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In vitro **cultivation of the trematode species** ***Trichobilharzia regenti***

In vitro kultivace motolice *Trichobilharzia regenti*

Master's thesis

Supervisor: RNDr. Martin Kašný, Ph.D.

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Prohlášení:

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ABSTRACT

The class Trematoda includes many pathogenic representatives. Main subject of this thesis, avian schistosome *Trichobilharzia regenti*, is a close relative to the important human pathogen *Schistosoma mansoni* (family Schistosomatidae).

In vitro cultivation of trematodes enables closer understanding of their biology and parasite-host interactions; however, no trematode species has been successfully kept *in vitro* from the egg stage to the adults producing eggs. Many studies are focused on the problematic of *S. mansoni* cultivation, but data concerning *T. regenti* cultivation remain scarce. Only the ability of *T. regenti* cercariae to transform into schistosomula *in vitro* was documented, with following survival in a culture medium for a few days.

Comparison of eight transformation methods was performed with *T. regenti* cercariae. Based on the number of tailless cercarial bodies obtained, five transformation methods were selected for further evaluation of the early schistosomula characteristics (glycocalyx shedding, penetration glands emptying and survival *in vitro*). It was observed that the largest quantity of cercarial bodies can be obtained by using a syringe needle or the BeadBeater cell disrupter. The largest quantity of schistosomula meeting the criteria of early schistosomulum was recorded after transformation of cercariae by penetration of the duck skin.

Viability of schistosomula in four different cultivation media was evaluated to find the optimal one for long-term cultivation. Also, the media supplements, such as red blood cells and duck serum were tested. The complete media SCM and ASCM were experimentally selected for long term cultivation. The addition of duck serum, in contrast to red blood cells, stimulated faster growth of schistosomula, gut development and prolonged *in vitro* survival, but the progress in these parameters was still delayed compared to *in vivo* developing schistosomula.

This thesis brings new information about *T. regenti* cercaria/schistosomulum transformation methods and subsequent long-term *in vitro* cultivation of schistosomula.

Keywords: *Trichobilharzia regenti*, *Schistosoma mansoni*, cercaria, schistosomulum, transformation, cultivation, development, penetration glands, glycocalyx, gut, growth, viability

ABSTRAKT

Třída Trematoda zahrnuje mnoho patogenních zástupců, mezi nimi významného lidského parazita druhu *Schistosoma mansoni* (čeleď Schistosomatidae). Její blízký příbuzný, ptačí schistosoma *Trichobilharzia regenti*, je hlavním předmětem této práce.

In vitro kultivace motolic může být vhodným krokem pro bližší pochopení biologie těchto organizmů a jejich vztahů s hostitelem. Žádný druh motolice však dosud nebyl v *in vitro* podmínkách kultivován od vajíčka po dospělce produkujícího opět vajíčka. Kultivací *S. mansoni* se do dnešní doby zabývalo mnoho studií, ale informace v literatuře týkající se kultivace *T. regenti* jsou prozatím strohé. V tomto ohledu bylo dosud pouze dosaženo *in vitro* transformace cerkárií *T. regenti* ve schistosomula s následným přežitím několik dní v kultivačním médiu.

S cílem získat co nejvyšší množství cerkárií s odlomeným ocáskem (jakožto stimulem pro transformaci cerkárií *T. regenti* ve schistosomula) bylo porovnáno osm transformačních metod. Vybraných pět metod bylo dále zhodnoceno z hlediska základních charakteristik časného schistosomula (svlečený glykokalyx, vyprázdněné penetrační žlázy, přežití v *in vitro* podmínkách). Bylo zjištěno, že transformace cerkárií s použitím injekční jehly či tkáňového homogenizátoru BeadBeateru poskytuje nejvyšší procento cerkárií s odlomeným ocáskem. Schistosomula nejlépe odpovídající kritériím časného schistosomula lze získat transformací cerkárií pomocí metody penetrace kachní kůží.

Dále byla zhodnocena životaschopnost schistosomulí ve čtyřech kultivačních médiích s cílem nalézt vhodné médium pro dlouhodobou kultivaci. Také byly testovány doplňky média, zahrnující kachní erytrocyty a kachní sérum. Na základě výsledků byla pro dlouhodobou kultivaci schistosomulí vybrána kompletní média, SCM a ASCM. Bylo zjištěno, že přídavek kachního séra stimuluje růst schistosomulí, vývoj střeva a prodlužuje přežití v *in vitro* podmínkách lépe než přídavek erytrocytů. Na druhou stanu bylo pozorováno, že je v tomto případě vývoj opožděný ve srovnání se schistosomuly vyvíjejícími se v definitivním hostiteli.

Tato práce přináší nové informace o *in vitro* transformacích cerkárií *T. regenti* ve schistosomula, včetně jejich následné *in vitro* kultivace.

Klíčová slova: *Trichobilharzia regenti*, *Schistosoma mansoni*, cerkárie, schistosomulum, transformace, kultivace, vývoj, penetrační žlázy, glykokalyx, střevo, růst, životaschopnost

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ABBREVIATIONS

ACD	Acid-Citrate-Dextrose solution
ADMEM	Advanced Dulbecco's Modified Eagle's medium
ANOVA	Analysis of Variance
BME	Basal Medium Eagle
BSA	Bovine Serum Albumin
c/s transformation	Cercaria/schistosomulum transformation
CB	Cercarial bodies
cDNA	Complementary DNA
ConA	Concanavalin A
dH_2O	Distilled water
DIC	Differential Interference Contrast
DMSO	Dimethyl Sulfoxide
EBSS	Earl's Balanced Salt Solution
ELAC	EBSS with Lactalbumin hydrolysate and other supplements (Clegg, 1965)
ELISE	Enzyme-Linked ImmunoSorbent Assay
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal bovine serum
FDA	Fluorescein Diacetate
FITC-phalloidin	Fluorescein Isothiocyanate-labeled phalloidin
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HBSS	Hank's Balanced Salt Solution
HEPES	Hydroxyethyl Piperazineethanesulfonic acid
HNRA2	Heterogeneous Nuclear Ribonucleoprotein A2 homolog 1
IHC	Immunohistochemistry
ITLR	Isoleucine-tRNA ligase
KEGG	Kyoto Encyclopedia of Genes and Genomes database
LAH	Lactalbumin Hydrolysate
LCA	*Lens culinaris* agglutinin
p. i.	Post infection
p. t.	Post transformation
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline Tween-20
PFA	Paraformaldehyde

PNA	Peanut agglutinin
qPCR	Quantitative Polymerase Chain Reaction
RBCs	Red Blood Cells
RNA	Ribonucleic Acid
RPMI-1640	Roswell Park Memorial Institute medium
RT	Room Temperature
SCIP-1	Intrinsic Schistosome C Inhibitory Protein; paramyosin-like protein
SCM	Schistosoma Culture Medium
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SGTP 1 and 4	Schistosoma Glucose Transporter 1 and 4
TAE	Tris-acetate-EDTA buffer
TEM	Transmission Electron Microscopy
TGS	Tris-Glycine-SDS buffer
TMB	3,3',5,5'-Tetramethylbenzidine
UEA I	*Ulex europaeus* agglutinin I
NCTC 109	Medium developed by the National Cancer Institute

1 INTRODUCTION

Members of the class Trematoda often represent important human or animal pathogens. Family Schistosomatidae that includes important human pathogens of the genus *Schistosoma* is a good example of this fact. Their close relatives, avian schistosomes of the genus *Trichobilharzia* have similar life cycles in their avian definitive hosts. Human can become an accidental host of these flukes and develop a skin reaction (Horák et al., 2008; Kouřilová et al., 2004). However, *Trichobilharzia regenti*, a subject of this study, has a unique life cycle due to the neurotropic behavior of the schistosomula stages (Horák et al., 1998a).

The *in vitro* cultivation of trematodes is often a prerequisite for successful realization of various experiments aimed at, for example, a discovery of important biological processes associated with life of the flukes and at possible interactions of flukes with the host body.

Even after decades of optimizations of the *in vitro* cultivations of trematodes, no record was reported on ability of any fluke representative to develop *in vitro* from egg stage to a fully developed adult able to reproduce. However, partial success has been achieved. For example, *S. mansoni* can be *in vitro* developed from cercarial stages to the mating adults laying infertile eggs (Basch, 1981a, 1981b). Current state of knowledge about cultivation of *T. regenti* schistosomula is insufficient. From the single study focused on this topic (Chanová et al., 2009), it is only known that cercariae are capable of *in vitro* transformation into the schistosomula after mechanical tail separation. It is then possible to keep such schistosomula alive in SCM 169 culture medium for maximum of 11 days (Chanová et al., 2009).

T. regenti is a model, that has been studied in the Laboratory of Helminthology (Faculty of Science, Charles University in Prague) since 1998. In order to extend current state of knowledge about possibilities of *in vitro* cultivations of *T. regenti* schistosomula, this thesis is focused on finding the optimal method for cercaria/schistosomulum transformation with subsequent cultivation of these stages *in vitro.*

2 AIMS OF THE THESIS

Main aim of this thesis was to verify the current knowledge related to the transformation of cercariae into the schistosomula followed by cultivation. The specific aims were following:

- to find the optimal method of cercaria/schistosomulum transformation for *T. regenti*, based on the number of obtained schistosomula and on defined criteria for transformation of early schistosomulum (emptying of penetration glands, shedding of glycocalyx, short-term viability)

- to find the optimal medium for long-term cultivations that would support growth and development of *T. regenti* based on evaluation of body growth and gut development

- to search for other markers of completed cercaria/schistosomulum transformation

3 LITERATURE REVIEW

3.1 *In vitro* cultivations

In vitro cultivations of parasitic organisms represent an essential basis of many experiments where standardized conditions are needed. *In vitro* experiments enable e.g., realization of antiparasitic drugs testing and experiments focusing on the parasite-host relationship, transcriptomic studies or uptake of macromolecules by the parasite (Gobert et al., 2010; Holtfreter et al., 2010; Thornhill et al., 2010). Moreover, many definitive host laboratory animals can be spared due to the introduction of *in vitro* experiments. Nevertheless, cultivation of multicellular organisms, such as helminths, is often difficult because of their complex body structure and complicated life cycles. Even after decades of research in the field of trematode cultivations, only partial success has been achieved. Up to now, no trematode species has been kept *in vitro* through the whole life cycle. This topic was summarized in my bachelor thesis called "Cultivation of Trematodes" (Vrbová, 2014).

3.2 Model organisms

This thesis focuses problematics of cercaria/schistosomulum transformation and cultivation of *Trichobilharzia regenti*, a trematode from the family Schistosomatidae. However, only limited information is available about *in vitro* development of *T. regenti* in contrast to the best-researched model of significant medical importance, *Schistosoma mansoni*. For this reason, the characteristics of *in vitro* and *in vivo* development of *S. mansoni* definitive host stages are also included in this review.

3.2.1 *Schistosoma mansoni*

Life cycle of *S. mansoni* includes two hosts, human as a definitive host and a pulmonate snail *Biomphalaria* spp. as intermediate host. Adults live in pairs in human mesenteric veins and females lay eggs after copulation. The eggs move into the intestine and are released into the environment. After stimulation by water, miracidia are released. Miracidia find and penetrate the intermediate host and after further development and asexual reproduction, cercariae are released from the snail host in high numbers. Cercariae then actively search for the definitive host and enter the host body through the skin (Roberts and Janovy, 2009).

Penetration of the definitive host skin and migration in the host body

Cercariae attached to the host skin are stimulated by the skin lipids, mostly fatty acids, to release proteolytic glands secretions and start to penetrate the skin (Haas et al., 1997). A high proportion (about 60%) of cercariae do not release the tail during the penetration. The cercariae

potentially may accept many molecules on their way through the skin, for example insulin, free amino acids and growth factors (Haas and Haeberlein, 2009; Kusel et al., 2009; Thornhill et al., 2010). The whole process of skin penetration is completed within 30 min and cercariae complete their transformation to schistosomula and enter the peripheral circulation in the time range of 24 h (Roberts and Janovy, 2009). Migration of schistosomula through the definitive host body was described in a laboratory definitive host, mouse (Clegg, 1959). On day 4 post infection (p. i.), schistosomula enter lungs and stay there till day 7 p. i., without body growth. From day 8 p. i. they enter portal vessels of the liver and begin to feed, grow and develop into adults that pair together on day 28 p. i. The paired adults migrate to the mesenteric vessels from day 35 p. i. and females start to lay eggs (Clegg, 1959).

3.2.2 *Trichobilharzia regenti*

T. regenti is a bird schistosome whose life cycle includes two hosts; waterfowl from the Anatidae family serves as definitive host and pulmonate snails *Radix peregra* as intermediate host (Horák et al., 1998a). However, mammals can serve as accidental hosts and infected humans can develop skin reaction against *T. regenti* cercariae, so-called cercarial dermatitis (Horák et al., 2008; Kouřilová et al., 2004). On the nasal tissue of definitive host females lay eggs from which miracidia are hatched after contact of eggs with water. Miracidia actively locate and penetrate the intermediate host where asexual reproduction results in production of large amounts of cercariae. Released cercariae search for the definitive host, attach to the host skin and penetrate into the host body (Horák et al., 1998a; Hrádková and Horák, 2002).

Penetration of the definitive host skin and migration in the host body

Fatty acids contained in the host skin, especially linoleic and linolenic acid, stimulate change of cercarial movements and emptying of circumacetabular and postacetabular glands of cercariae (Mikeš et al., 2005). The compounds of excretory/secretory products of *T. regenti* cercariae enable the skin penetration, because the contained proteolytic enzymes (such as cysteine peptidase cathepsin B2) are able to degrade skin proteins, e.g. elastin, collagen and keratin (Dolečková et al., 2009; Kašný et al., 2007). After skin penetration, cercariae complete the transformation into schistosomula and enter the peripheral nerves of the host where they can be found after 1.5 – 3 days p. i. Then the schistosomula continue migration through the spinal cord up and reach the brain 12 - 18 days p. i. Then they invade nasal cavity through the olfactory nerve. During the migration schistosomula grow and adults develop approximately 13-25 days p. i. The nasal cavity is a final destination where adults mate and first fertile eggs are laid 15 days p. i. (Chanová and Horák, 2007; Hrádková and Horák, 2002).

3.3 Comparison of *in vitro* and *in vivo* cercarial transformation to schistosomula

It is often problematic to obtain sufficient amount of schistosomula from the definitive host, because the worms migrate through the host body and many of them die during the process. Moreover, worms clean of the host tissue are often needed. *In vitro* cercaria to schistosomulum (c/s) transformation enables to produce and collect the schistosomula in standardized conditions and large numbers. Proper characterization of *in vitro* obtained schistosomula and their comparison to the schistosomula obtained *ex vivo* is crucial for realization of further *in vitro* experiments.

More about characterization of *S. mansoni* schistosomula is described in following chapters, including mostly morphologic characteristics. Morphologic characteristics were selected, because they have been used for a long time and are relatively easy to apply to the *T. regenti* model.

3.3.1 Characterization of transformed schistosomula – *S. mansoni*

Historically, many investigators addressed the problematics of characterization of *in vitro* produced schistosomula of *S. mansoni* and comparison to the *in vivo* transformed schistosomula. The knowledge about *S. mansoni* c/s transformation is broader than about bird schistosomes.

Removal of cercarial tail and motility changes

The most obvious morphological difference between cercaria and schistosomulum is presence of a tail in cercaria and its missing in schistosomulum; this is considered one of schistosomulum characteristics (Gilbert et al., 1972; Stirewalt and Uy, 1969; Wang et al., 2006; Yasuraoka et al., 1978). The separation of the cercarial tail is considered an important trigger in further development of schistosomula (Clegg, 1965; Yasuraoka et al., 1978) but purpose of the tail loss in the cercarial transformation remains unclear.

Some authors considered changes in movement to worm-like peristaltic constrictions to be a characteristic of transformed schistosomulum, even if the cercarial tail remained intact. *S. mansoni* cercariae move by inching movements (Gilbert et al., 1972; James and Taylor, 1976; Yasuraoka et al., 1978).

Cercarial penetration glands emptying

During the host skin penetration, cercariae empty their preacetabular and postacetabular gland contents, therefore acetabular glands emptying is considered important in cercaria/schistosomulum conversion (Brink et al., 1977; Cousin et al., 1981; Melo and Pereira, 1985; Ramalho-Pinto et al., 1974). However, evaluation of acetabular glands content alone is an insufficient characteristic of schistosomulum transformation as the tegument formation occurs even without acetabular glands emptying during cercarial transformation of *S. mansoni* (Eveland and Morse, 1975;

Michalick et al., 1979; Skelly and Shoemaker, 2000). *S. mansoni* cercariae incubated for 3 h in Earl's Balanced Salt Solution (EBSS) with 50% fresh rat serum or schistosomula obtained by passage through the syringe needle with subsequent incubation in RPMI-1640 emptied acetabular glands content in 24-48 h p. t. (Brink et al., 1977; Colley and Wikel, 1974). After incubation of cercariae with rat skin lipids or crude egg lecithin, the content of acetabular glands is depleted in 1 hour (Gilbert et al., 1972). James and Taylor (1976) demonstrated that *S. mansoni* cercariae emptied their acetabular glands during *in vitro* penetration of excised mouse skin as they were incapable of percutaneous infection of mice after the transformation. However, this method was not used for this purpose in later studies as staining the penetration glands content is quicker and cheaper. Preacetabular glands of *S. mansoni* can be stained by purpurin or alizarin red and postacetabular glands by Acid-Schiff reaction (Colley and Wikel, 1974; Eveland and Morse, 1975; Gazzinelli et al., 1973).

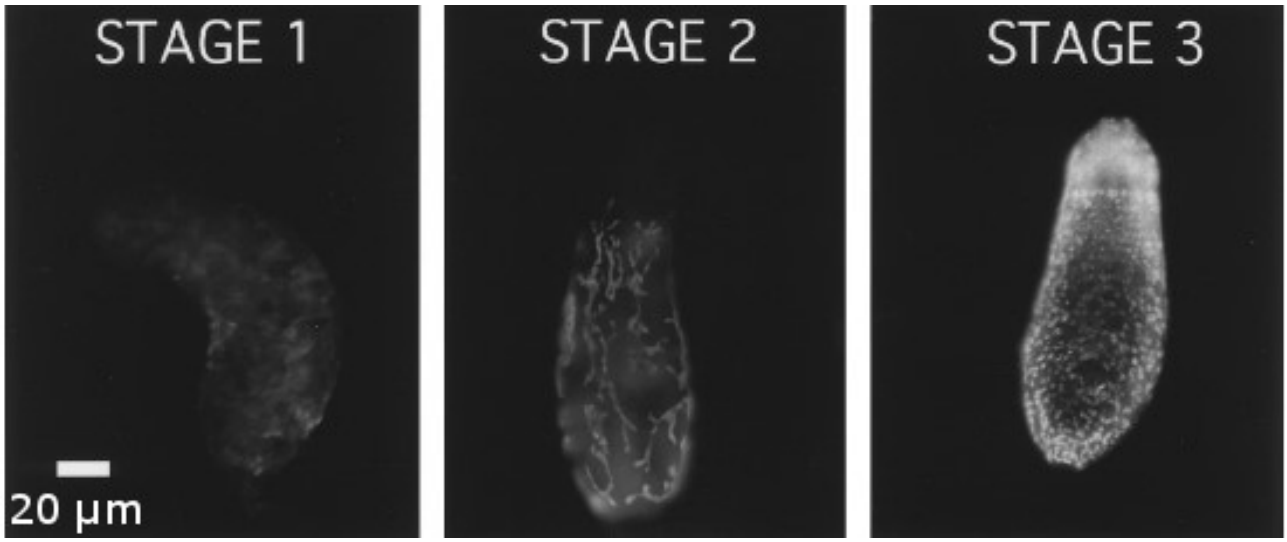
Surface development

Another property reported for transformed schistosomulum is water intolerance – schistosomulum can survive in a saline solution for days but dies in fresh water within 10-15 minutes. Worms become water intolerant within 20-60 min after c/s transformation by incubating cercariae in water with isolated rat skin lipids (Gilbert et al., 1972). Similar situation occurs *in vivo*, 90% of *S. mansoni* cercariae inoculated into mouse peritoneum become water intolerant after 30 min (Melo and Pereira, 1985). As an opposite to water intolerance, the saline tolerance was used as a criterion of transformed schistosomulum in early cercarial transformation experiments. High mortality of cercariae in culture medium NCTC-109 or in the phosphate buffer saline were reported, oppositely to schistosomula (Eveland and Morse, 1975; Gilbert et al., 1972; Stirewalt and Uy, 1969; Yasuraoka et al., 1978). However, cercariae survived in Earl's Balanced Salt Solution (EBSS) more than 5 h (Eveland and Morse, 1975). Both water intolerance and saline tolerance could be related to the development of the surface membranes of tegument. The tegument development is started after the worms is exposed to osmotic conditions 120-300 mOsm and for this reason evaluation of the tegument development would be more precise characteristic of schistosomulum (Skelly and Shoemaker, 2000; Stirewalt et al., 1983).

The tegument of *in vivo* produced schistosomula differs from that of cercariae. Cercarial subtegumental cells (cytons) are not connected with tegument by cytoplasmic bridges and cercariae have trilaminar tegumental surface membrane as opposite to the heptalaminar membrane of schistosomulum. Schistosomula evaluated 1 h after penetration of mice ear skin create a network of connected cytons and tegumental cells and their surface is covered by heptalaminar membrane. Cercarial cytons start to connect with the tegumental cells when cercariae are exposed to osmotic condition of 120-300 mOsm. When osmotic conditions of 300 mOsm combine with glucose addition

and temperature of 37°C (conditions met in the host blood), the cyton network is created in 1 h (Skelly and Shoemaker, 2000). The vesicle transport and cyton network can be observed using transmission electron microscopy (TEM) (Brink et al., 1977; Colley and Wikel, 1974; Cousin et al., 1981; Stirewalt et al., 1983). The specific antibody labeling of SGTP4 (type of *S.mansoni* glucose transporter presented in the outer membrane of developed tegument and in multilamellar and discoid bodies synthesized in cytons) can be used as a proof of on tegument formation (**Figure 1**; Hockley and McLaren, 1973; Skelly and Shoemaker, 2000, 1996)**.**

Figure 1 *S. mansoni* tegumental transformation stained, labeled by anti-SGTP4 antibody; Stage 1: diffuse pattern; Stage 2: discrete internal staining, cyton network; Stage 3: patchy surface staining (adapted from Skelly and Shoemaker, 2000)



The heptalaminar surface membrane is formed in ca 2-4 h post transformation (p. t.) on *S. mansoni* schistosomula transformed by syringe needle, penetration of dried rat skin or incubation in EBSS medium with 50% rat serum. Heptalaminar membrane formation can be documented by TEM (Brink et al., 1977; Cousin et al., 1981; Stirewalt et al., 1983).

The surface development of *S. mansoni* definitive host includes loss of cercarial glycocalyx to avoid immune reaction of host (Hockley and McLaren, 1973). The glycocalyx layer on the surface of cercariae is completely shed from the surface of schistosomula obtained 1.5 h after penetration of the mice ear skin (Cousin et al., 1981; Stirewalt et al., 1983). Schistosomula of *S. mansoni* obtained by incubation of cercariae in EBSS with 50% fresh rat serum or vortexing with subsequent incubation in ELAC (medium based on EBSS) shed their cercarial glycocalyx within 120 min p. t. (Brink et al., 1977). The glycocalyx shedding can be observed for example by using TEM, alcian blue staining (Brink et al., 1977; Colley and Wikel, 1974; Cousin et al., 1981; Gilbert et al., 1972; Stirewalt et al., 1983). For many years, so-called cercarienhüllen reaction (CHR) was used to determine that change of cercarial surface was completed. CHR is probably a reaction of immunoglobulins to the cercarial glycocalyx

and a gel-like “coat” containing glycoproteins is created around the cercarial body. Usually, serum from the mouse or human chronically infected by *S. mansoni* was added to the worms to induce CHR. If amorphous gel-like “coat” was formed around the worm, it was considered a cercaria (Colley and Wikel, 1974; Gazzinelli et al., 1973; Gilbert et al., 1972; Kemp, 1970; Kemp et al., 1973; Ramalho-Pinto et al., 1974; Stirewalt et al., 1983; Stirewalt and Uy, 1969). Ramalho-Pinto et al. (1974) reported to obtain CHR-negative schistosomula 12-48 h after transformation by vortexing combined with change of temperature (from 0°C to 25°C) followed by incubation in HBSS. Surface changes during cercaria/schistosomulum transformation are also connected with reaction of *S. mansoni* cercariae with fresh human serum which was also used as schistosomulum characteristic by Ramalho-Pinto et al. (1974). Cercariae are killed by the complement in 24 hours as the human complement is activated by *S. mansoni* glycocalyx (Ramalho-Pinto et al., 1974; Samuelson and Caulfield, 1986). However, the transformed schistosomula remain alive after incubation with fresh serum because they lack the glycocalyx layer (Ramalho-Pinto et al., 1974; Wang et al., 2006). Moreover, *S. mansoni* schistosomula inhibit complement by reaction of surface-exposed SCIP-1 paramyosin-like protein with C8 and C9 components of human complement cascade (Deng et al., 2003).

Organ systems development

The transformation of cercariae into schistosomula initiates development of organ systems. However, only nuclear condition of cells of germinal, muscle and nervous tissues and morphological changes of digestive and excretory systems were described for *in vitro* transformed schistosomula *S. mansoni* (Cousin et al., 1981; Stirewalt et al., 1983). Above mentioned cell nuclei of *in vivo* transformed schistosomula are euchromatic 1 h after infection, opposite to cercarial heterochromatic nuclei. But, cell nuclei of *in vitro* transformed schistosomula remain heterochromatic after 1 h of incubation in ELAC following transformation using dried rat skin penetration, passage through syringe needle and incubation of cercariae in medium with 50% rat serum (Cousin et al., 1981; Stirewalt et al., 1983).

Oral sucker of the *in vivo* transformed schistosomula is after 1 h p. t. described as everted, esophageal lumen as intermittently constricted and esophageal glands granules are located near the constricted lumen areas. In contrast, cercarial oral sucker of *S. mansoni* is alternately everted and inverted and esophageal lumen is dilated with granules located in the esophageal glands. *In vitro* transformed schistosomula by the syringe needle with incubation in ELAC still had cercarial characteristics of esophageal morphology after 1 h p. t. (Cousin et al., 1981). Evaluation of organ systems development in transformed schistosomula can be done by TEM (Cousin et al., 1981; Stirewalt et al., 1983).

Other characteristics of transformed schistosomulum

The completion of cercaria/schistosomulum transformation and the invasiveness of the transformation method can be evaluated by the viability assay supported by the growth measurements and/or development description (Clegg, 1965; Clegg and Smithers, 1972; Gold and Flescher, 2000). These characteristics are further elaborated in chapter 3.4. Independently on the transformation method tested (mechanical, incubation with rat serum, penetration the host skin) the schistosomula of *S. mansoni* develop into adults when injected into the definitive host (mouse) blood stream (Brink et al., 1977; Dorsey and Cousin, 1982; Gold and Flescher, 2000; Stirewalt et al., 1983; Stirewalt and Uy, 1969). Stirewalt et al. (1983) used cryopreservation of schistosomula in liquid nitrogen to determine if the development into schistosomulum was completed; cercariae were killed in the process of freezing, unlike schistosomula. According to this characteristic, the conversion into schistosomulum is completed after using different transformation methods, including mechanical transformation, penetration of dried rat skin and incubation of cercariae in ELAC medium with rat serum or ELAC medium alone for 3 h in 37°C.

3.3.2 Characterization of transformed schistosomula – *Trichobilharzia regenti*

Only little information is available about *in vitro* cultivated schistosomula of the genus *Trichobilharzia* (Chanová et al., 2009; Howell and Bourns, 1974). Following text summarizes markers of transformed *T. regenti* and *Trichobilharzia szidati* schistosomula. If available, comparison with the *in vivo* transformed schistosomula is included.

Cercarial tail

Cercarial tail is separated in the process of infecting the definitive host and the absence of tail was one of criteria for *T. regenti* and *T. szidati* schistosomulum set by Chanová et al. (2009).

Cercarial penetration glands emptying

T. regenti and *T. szidati* cercariae use contents of their acetabular glands during penetration of the host skin. But schistosomula transformed by syringe needle empty the contents of both postacetabular and preacetabular penetration glands in 48 h of cultivation p. t. (Chanová et al., 2009). Emptying of the cercarial acetabular glands content is considered to participate in shedding of *T. szidati* and *T. regenti* cercarial glycocalyx, however, the mechanism remains unknown (Horák et al., 1998a; Řimnáčová et al., 2017). A content of circumacetabular and postacetabular glands can be selectively stained by histological stains – lithium carmine and alizarin, respectively (Mikeš et al., 2005).

Surface development

Unlike the cercarial trilaminar tegumental membrane, the surface tegumental membrane of both *T. regenti* and *T. szidati* transformed schistosomula is heptalaminar after both *in vitro* and *in vivo* transformation. The tegument development is a quick process as it is completed in 24 h post transformation (p. t.) *in vitro* for *T. regenti* and *T. szidati* and in 12 h after *T. szidati* cercariae infect the definitive host (Chanová et al., 2009; Horák et al., 1998a). Initiation of tegument development of *T. szidati* can be quickly achieved by placing the cercarial bodies (CB) gained by passage through a syringe needle to osmotic conditions of 300 mOsm at temperature 39°C for 5 h. Then the structure of double membrane can be confirmed by TEM (Horák et al., 1998b).

Surface development relates to loss of glycocalyx which is documented after 24 h for both *in vivo* and *in vitro* transformed schistosomula of *T. regenti* and *T. szidati*. This change of the cercarial surface can be documented using FITC-lectin probes that bind saccharides from cercarial glycocalyx, e.g. *Ulex europaeus* agglutinin I (UEA I) for *T. regenti* and *Lens culinaris* agglutinin (LCA) for *T. szidati.* Schistosomula of *T. szidati* bind lectins Concanavalin A (ConA) and Peanut agglutinin (PNA) after their tegument is fully developed (Chanová et al., 2009; Horák et al., 1998a; Řimnáčová et al., 2017).

Organ systems development

Soon after the surface transformation of schistosomulum, functional oral sucker develops from cercarial oral opening and muscular cone of the cercarial head organ. *In vitro* transformed *T. regenti* schistosomula can achieve an advanced state of oral sucker development in 3 days p. t. when the cercarial muscular cone flattens (**Figure 2**; Bulantová, 2012). Fully developed oral sucker was observed on *in vivo* transformed *T. regenti* schistosomulum 5 days p. i. In developed oral sucker, many strengthened fibers of radial muscles connect the internal and external walls of oral sucker and basis of the oral sucker is formed from the muscular cone, as showed at **Figure 2** (Bulantová et al., 2011).

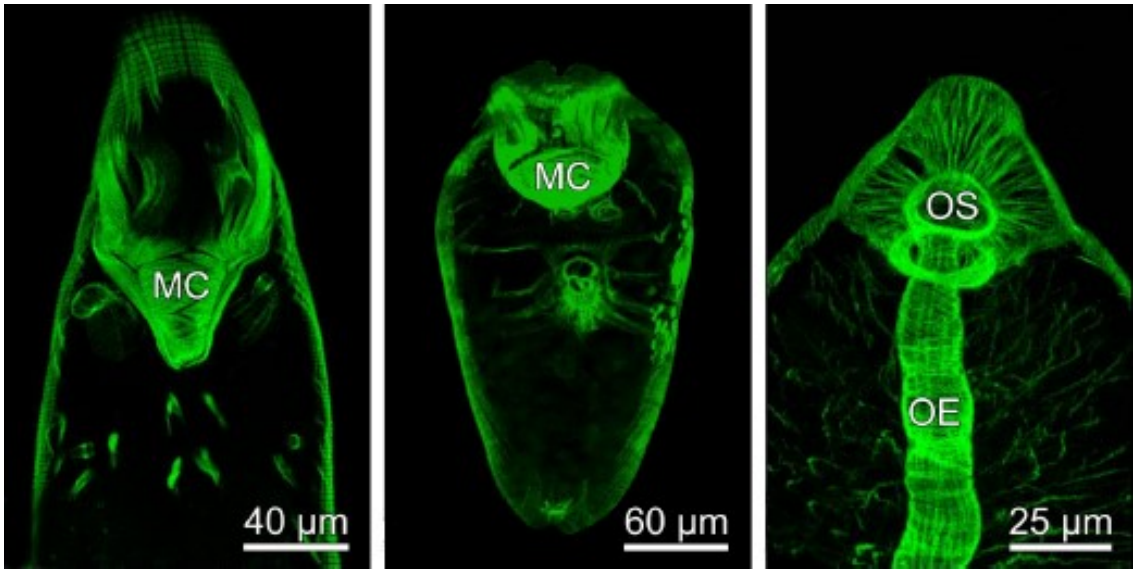
Only *T. regenti* schistosomula in early stages of development of digestive system were observed *in vitro*. In 24 h p. t., schistosomula transformed by syringe needle develop a double membrane of esophagus and in 3 days of cultivation when they start to uptake red blood cells (RBCs). The RBCs consumption is probably connected with the oral sucker development (Bulantová, 2012; Chanová et al., 2009).

Other characteristics of transformed schistosomulum

Ability to grow and develop *in vitro* is mostly a consequence of long-term cultivation and will be further summarized in chapter 3.4. Mean length (±SD) of *T. regenti* early schistosomula

transformed by the syringe needle and cultivated in Schistosoma culture medium (SCM)s changed from 269.6 ± 19.7 µm after 24 h p. t. to 341.1 ± 27.8 µm after 5 days p. t. (Chanová et al., 2009).

Figure 2 Development of oral sucker. Left – cercarial muscular conus (MC); Middle – Schistosomulum transformed *in vitro*, 3 days p. t.; Right – schistosomulum transformed *in vivo,* 5 days p. i.; OS – oral sucker, OE – esophagus (adapted from Bulantová, 2012)



3.3.3 Methods of cercaria/schistosomulum (c/s) transformation

C/s transformations of *S. mansoni*

Various methods of c/s transformation were tested on *S. mansoni* cercariae, most of them have been summarized in my bachelor thesis focusing on cultivation of trematodes (Vrbová, 2014). Brief description of the c/s transformation methods used for *S. mansoni* follows. **Table 1** summarizes gain of cercarial bodies after using different c/s transformation techniques, which are described in the text below.

Change of osmotic conditions: Short-term incubation of cercariae in osmotic condition of 120 mOsm PBS results in development of heptalaminar tegumental membrane but glycocalyx is still present even after 3 h incubation in 300 mOsm cultivation medium (Skelly and Shoemaker, 2000; Stirewalt et al., 1983). No additional development was tested as viability of cercariae rapidly drops after 5-8 days *in vitro* to 20-26% and after 14 days to 8-12% as opposite to cercariae transformed by incubation in 50% rat serum cultivated in the same conditions (Eveland and Morse, 1975).

Linoleic acid: Cercariae release their tails and empty their penetration glands after incubation with linoleic acid dissolved in water (Hara et al., 1993; Shiff et al., 1972). Worms are reported to die quickly after incubation as they probably become water-insensitive and glycocalyx

shedding is stimulated (Haas and Schmitt, 1982; Salafsky et al., 1988). But, when linoleic acid is added to the cultivation medium, acetabular glands remain full (Michalick et al., 1979). Moreover, when linoleic acid was added to the 5 mM phosphate buffer, no tegumental development of *S. mansoni* cercariae was observed (Skelly and Shoemaker, 2000).

Serum: Incubation of cercariae in medium with inactivated serum from definitive host stimulates tail shedding more than serum free medium. Fresh serum stimulates the tail shedding in much shorter time than inactivated serum. However, most of the cercariae are quickly killed by complement when fresh serum is used (Wang et al., 2006). Schistosomula transformed by 30 min incubation with fresh serum and subsequent cultivation in ELAC medium emptied their acetabular glands in 48 h p. t. and shed the glycocalyx in 3 h (Brink et al., 1977; Eveland and Morse, 1975). These schistosomula survived in ELAC medium in long-term cultivation experiments, the viability after 14 days p. t. was 95% (Eveland and Morse, 1975).

Mechanical tail removal: Best method for obtaining high numbers of mechanically transformed schistosomula is a passage of cercariae through the syringe needle gauge 22, 20× (Coultas and Zhang, 2012; Milligan and Jolly, 2011). High number of CB can be gained by using omnimixer, a laboratory homogenizer, according to the Dorsey and Cousin (1982). Mechanically transformed schistosomula develop heptalaminar tegument membrane in 3 h p. t., but the glycocalyx is completely shed after 96 h p. t. and schistosomula empty the content of their acetabular glands in 24-48 h p. t. (Colley and Wikel, 1974; Stirewalt et al., 1983).

Definite host skin: Schistosomula transformed by penetration of the skin isolated from the definitive host empty their penetration glands; shed the glycocalyx and develop heptalaminar structure of tegumental membrane in 3 h p. t. (Brink et al., 1977; Stirewalt et al., 1983).

Table 1 *S. mansoni* cercarial bodies (CB) gain depending on c/s transformation method

Method of c/s transformation *S. mansoni*	CB gain %	Reference
***in vivo* transformation**		
penetration of abdominal skin of mice (recovery of adults)	42-44	James and Taylor, 1976
***in vitro* transformation**		
24 h incubation in ELAC	0-10	Eveland and Morse, 1975
24 h incubation in DMEM	35,8	Wang et al., 2006
12 min incubation with 3 mM linoleic acid in water	90	Hara et al., 1993
24 h incubation in DMEM + 5% iFBS	59,5	Wang et al., 2006
24 h incubation in RPMI-1640 + 5% iFBS	92	Coultas and Zhang, 2012
3 h incubation in EBSS + 50% fresh rat serum	< 79	Brink et al., 1977
30 min incubation in DMEM + 50% fresh human serum	43	Wang et al., 2006
vortex, 2 min at 2700 RPM	50-70	Brink et al., 1977
centrifuge 15 min 6000g 0°C + 30-60 min in HBSS 7000g RT	N/A	Gazzinelli et al., 1973
passage through Pasteur pipette 20× + temperature change 0°C to 25°C	60	Wang et al., 2006
passage through syringe needle gauge 22 (0,72 mm diameter) 20×	99-100%	Coultas and Zhang, 2012
omnimixer	> 95	Dorsey and Cousin, 1982
penetration of mouse abdominal skin	20-30	Brink et al., 1977

C/s transformations of *T. regenti*

Currently used methods for *T. regenti* c/s transformation are repeated (20×) passage through the syringe needle of gauge 23 (diameter 0,72 or 0,63 mm; Chanová et al., 2009) and passage through the duck skin (according to RNDr. Jana Bulantová, Ph.D., unpublished). Number of tailless cercariae (cercarial bodies, CB) obtained by these methods has not been quantified yet. Syringe needle-transformed schistosomula cultivated in SCM for 24 hours shed the cercarial glycocalyx, developed heptalaminar structure of tegument and two-membrane structure of esophagus. Acetabular glands are completely emptied after 48 h p. t. (Chanová et al., 2009).

3.4 Long-term *in vitro* cultivations from schistosomula to adults

Many publications focus on *S. mansoni* definitive host stages and great progress has been achieved in *S. mansoni in vitro* development. *S. mansoni* cercariae can develop *in vitro* into adults laying infertile eggs after mechanical transformation (Basch, 1981a, 1981b).

No information is available about long-term *in vitro* cultivation of *T. regenti* and one study focuses on cultivation of *T. szidati* after *in vivo* transformation (Howell and Bourns, 1974).

3.4.1 Media for *in vitro* cultivations of schistosomula

Following text summarizes appropriate media for long-term cultivations and comparison of development *in vitro* and *in vivo*.

About 50 years ago, ELAC medium was a best choice for cultivation of *S. mansoni* schistosomula that reached sexual maturation and pairing of males and females (Clegg, 1965; Tiba et al., 1974). This medium was developed by Clegg (1965) and composes of EBSS enriched by 0,5% Lactalbumin hydrolysate (LAH), 1% rabbit RBCs and 50% heat-inactivated rabbit serum. ELAC medium with duck or chicken serum and complementary RBCs is also reported to support *in vitro* growth and development of reproductive system of *T. szidati* schistosomula transformed *in vivo*. When placed to ELAC with duck or chicken serum, schistosomula of *T. szidati* form a "gel-like coat" in 2 h. This "coat" encloses whole body of schistosomula in 2-3 days and about 5-6 days p. t. schistosomula get free of this material. The "coat" material is stained positively by Sudan IV which indicates lipid content. Nevertheless, it should be noted that the Sudan IV stain also reacts positively in presence of serum albumin (Howell and Bourns, 1974; Lu et al., 2011).

However, medium currently used for many *in vitro* experiments on *S. mansoni* schistosomula is SCM. It is a medium based on BME, in original composition enriched by a set of hormones, vitamins, LAH, 1% human RBCs and 10% inactivated human serum (Basch, 1981a). The main advantage of this medium is significantly lowered amount of added serum. Clemens and Basch (1989) further tried to formulate a completely serum-free medium and discovered that, during first 2 weeks, effect of human transferrin on growth and development of the intestine was the same as with the presence of human serum. The serum-free SCM with addition of bovine serum albumin (BSA) also supports survival of *T. regenti* and *T. szidati* schistosomula transformed *in vitro* without further growth or development (Chanová et al., 2009)

Medium RPMI-1640 enriched by 5% inactivated fetal bovine serum is sometimes used for short-term cultivations of adult *S. mansoni* as it is commercially available (Holtfreter et al., 2010; Howe et al., 2015). However, *in vitro* transformed schistosomula survive only a few days and do not develop in this medium (Coultas and Zhang, 2012).

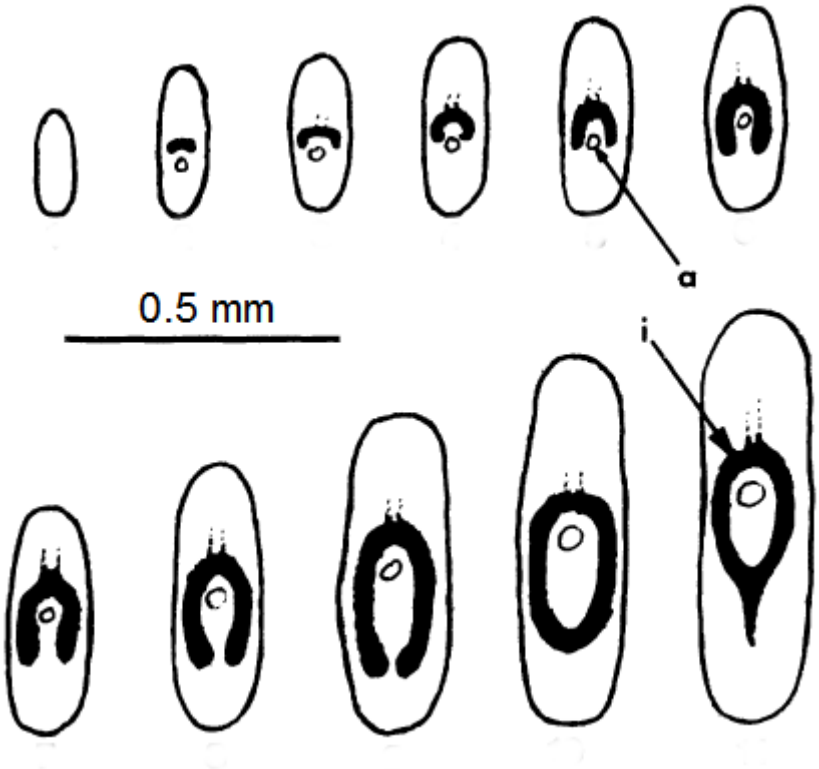
3.4.2 Comparison of *in vitro* and *in vivo* development of gut and reproductive system

After *S. mansoni* infection of mice, schistosomula begin to feed on RBCs on the day 8 p. i. and begin to grow (summarized in **Table 2**). On day 15 p. i. the gut caeca connect posterior to the ventral sucker (development of *S. mansoni* early schistosomula gut shown at **Figure 3**). After 21 days from infection, *testes* start to form in male and uterus in female juvenile stages and 28 days old worms start to form pairs and copulate. On day 30 after infection egg-shell proteins starts to form and first eggs are laid on day 35 p. i. The stated time is a day when most worms fulfilled the criteria (feeding, gut caeca connecting, formation of reproductive system). Variations of a few days in reaching a development stage is common in both *in vivo* and *in vitro* conditions (Clegg, 1965).

Table 2 Growth of *S. mansoni* in the definitive host (data adapted from Clegg, 1965)

Stage of *S. mansoni*	Length	Comment
Cercarial body	157 µm	Without tail
Schistosomulum 7 days p. i.	252 µm	No cell division = no growth
Adults 34 days p. i. – males	5.0 – 8.8 mm	-
Adults 34 days p. i. – females	5.1 – 9.4 mm	-

Figure 3 Development stages of early schistosomula gut; a – acetabulum; i – intestinal caeca (adapted from Clemens and Basch 1989)



S. mansoni schistosomula transformed *in vitro* start to digest RBCs from day 4 – 7 p. t. and the schistosomula reach gut-closed stage 11-15 days p. t., therefore the development of early

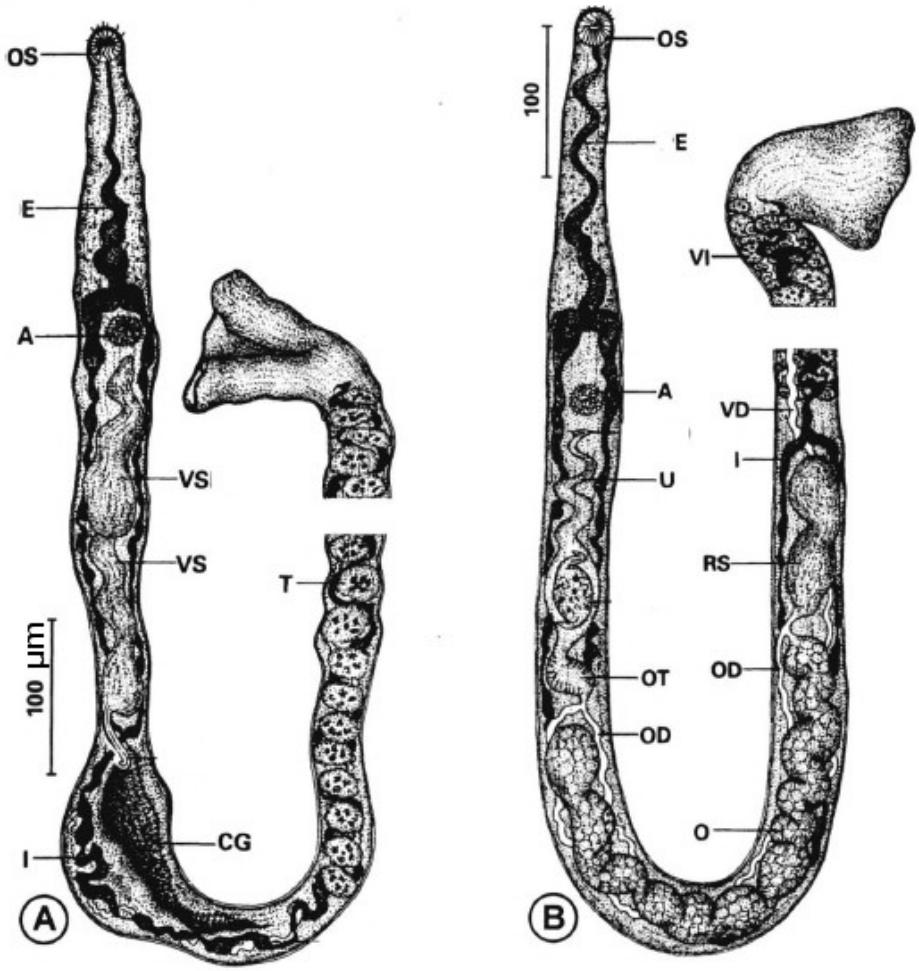
schistosomula is similar to the development in the definitive host (Basch, 1981a; Bogitsh and Carter, 1977; Tiba et al., 1974; Yasuraoka et al., 1978). However, then the organogenesis slows down and the reproductive system is formed a week later than in mice (Clegg, 1965). Mating of the adult worms *in vitro* occurs 30-49 days p. t. and only few of paired adults lay deformed eggs lacking the germinal disc (Basch, 1981b; Clegg, 1965; Yasuraoka et al., 1978). *In vitro* cultivated schistosomula grow more slowly than those in the definitive host and average length of adult *in vitro* cultivated worms is 3.5 mm for females and 4.5 mm for males approximately 40 days p. t. (Clegg, 1965).

T. regenti starts to uptake food 3 days after infecting the definitive host, probably parts of host nervous tissue (Blažová and Horák, 2005). Schistosomulum has bifurcated caecum on day 3 p. i. and the caeca connect to form a single caecum leading to the posterior end of the body on day 6 p. i. After that, intestine length increases. Reproductive system is partially formed on day 9 p. i. when male and female worms can be distinguished. *Vesicula seminalis*, basis of *canalis gynaecophorus* and tiny *testes* form in males on day 9 p. i. and *ovary* and *vitellaria* in females. Around 12-17 days p. i. males have about 160 *testes* and females form *uterus* and *receptaculum seminis*. First immature eggs are produced from 14 days p. i. (Blažová and Horák, 2005). The description of *T. regenti* adults is shown at **Figure 4**. Significant growth was noted from day 6 p. i., as shown **Table 3** (Blažová and Horák, 2005). Growth of early *T. regenti* schistosomula *in vitro* was included in the table based on findings of Chanová et al. (2009).

Table 3 Growth of *T. regenti* in the definitive host (data adapted from Blažová and Horák, 2005; Horák et al., 1998; Chanová et al., 2009)

	Length ± SD (μm)		
Stage of *T. regenti*	***In vivo***	***In vitro***	**Comment**
Cercarial body	225 ± 32		Without tail
Schistosomulum 3 days p. i.	278 ± 33	265.2 ± 18.4	×
Schistosomulum 5 days p. i.	×	341.1 ± 27.8	×
Schistosomulum 6 days p. i.	578 ± 221	×	×
Schistosomulum 9 days p. i.	1300 ± 545	×	×
Schistosomulum 12 days p. i.	2086 ± 958	×	×
Schistosomulum 15 days p. i.	1793 ± 1137	×	×
Adult male	11 000	×	Estimated length

Figure 4 *T. regenti* adults. A: anterior and posterior of a male; B: anterior and posterior of a female; OS – oral sucker, E – esophagus, A – acetabulum, VS – vesicula seminalis, I – intestine, CG – canalis gynaecophorus, T – testes, U – uterus, OT – ootype, OD – oviduct, O – ovary, RS – receptaculum seminis, VD – viteloduct, VI vitellaria (adapted from Horák et al. 1998)



Literature resources concerning *T. regenti* cultivation remain scarce, therefor *in vitro* development of *T. szidati* is described as the only study focused on long-term cultivation of the genus *Trichobilharzia* (Howell and Bourns, 1974). *T. szidati* schistosomula obtained *ex vivo* 1 h p. i. start to feed after 2-3 days of cultivation. On day 4 p. t. males and females could be distinguished. Starting from day 7, the gut of these worms became pale and inflated and no ingestion of RBCs were observed after day 10. Maximum growth and development occurs before day 12 p. t., when females had *ovaria*, *receptaculum seminis* free of spermatic cells and *uterus* and their body size reaches 1.4 mm. Males had rudimentary *testes* and seminal vesicles free of spermatic cells and reached the size of 2.1 mm (Howell and Bourns, 1974).

4 MATERIALS AND METHODS

4.1 Model organism – *Trichobilharzia regenti*, *Schistosoma mansoni*

The Laboratory of Helminthology (Charles University in Prague, Parasitology department) routinely maintains a life cycle of *Trichobilharzia regenti* by using *Radix peregra* as an intermediate host and *Anas platyrhynchos* f. *domestica* as a definitive host.

Cercarial stages of *Trichobilharzia regenti* were collected for further experiments. The *R. lagotis* snails previously infected with *T. regenti* were collected into the glass beakers filled with dechlorinated water. Then the beakers were placed under the artificial light source for approximately 60 minutes to let the cercariae shed. After that, the water containing cercariae was poured into the Erlenmeyer flask. The flask was darkened by the aluminum foil so that the water was illuminated by the artificial light only from above. Utilizing their positive phototaxis, the cercariae concentrated in the upper water layer. Then they were transferred to the sterile 50 ml centrifuge tubes using Pasteur pipette and immobilized on ice for consequent reducing the water amount and concentration of cercariae. The cercariae were always used the day they were collected.

The number of cercariae was established by counting the cercariae in twenty 50 µl aliquots, then extrapolating the average count value to the actual volume of cercariae-containing water collected before.

The *Schistosoma mansoni* cercariae were used for testing of anti-SGTP1 and anti-SGTP 4 antibodies (chapter 4.8.2). They were obtained from Mgr. Marta Chanová, Ph.D. (Institute of Immunology and Microbiology, First Faculty of Medicine, Charles University) in a 500-ml glass flask. First, the cercariae were immobilized by cooling the flask on ice for 1 h and after they dropped to the flask bottom, they were concentrated by discharging of the water and transferred to the cultivation media SCM.

4.2 Media, buffers and other solutions used for c/s transformation and cultivations

4.2.1 Schistosoma culture medium (SCM) and advanced culture medium (ASCM)

The Schistosoma culture medium (SCM) was used for short-term cultivation of *T. regenti* cercarial bodies (or *S. mansoni* cercariae, chapter 4.8.2) and both SCM and advanced SCM (ASCM) were used for long-term cultivation experiments (see 4.7).

Medium SCM is a complete medium based on Basal Medium Eagle (BME; Life Technologies, 41010). Medium ASCM is a complete medium based on Advanced Dulbecco's Modified Eagle's medium (ADMEM; Life Technologies, 12491). ADMEM is a medium originally based on BME. It contains about 3-5× higher amino acid concentration, 4× higher vitamins concentration and some

other components, including human transferrin and ferric nitrate, ascorbic acid, sodium pyruvate, ethanolamine and trace elements (vanadium, copper, manganese, selenium).

Basal media were completed by components presented in **Table 4**. More detailed procedure of complete media preparation is described in following text.

Table 4 Composition of the SCM and ASCM (adapted from Basch, 1981a) with bovine serum albumin (BSA) instead of human serum (Chanová et al., 2009)

Component	Vendor and catalog number	Final concentration in the SCM	Final concentration in the ASCM
Lactalbumin hydrolysate (LAH)	Fluka, 61302	1 g/l	1 g/l
Glucose	Sigma-Aldrich, G7528	111×10^{-4} M	25×10^{-3} M
Hypoxanthine	Sigma-Aldrich, H9636	5×10^{-7} M	5×10^{-7} M
Serotonin	Sigma-Aldrich, S9523	1×10^{-6} M	1×10^{-6} M
Insulin	Sigma-Aldrich, I5500	8 µg/ml	18 µg/ml
Hydrocortisone	Sigma-Aldrich, H0888	1×10^{-6} M	1×10^{-6} M
Triiodothyronine	Sigma-Aldrich, T6397	2×10^{-7} M	2×10^{-7} M
MEM Vitamins 100×	Life Technologies, 11120	0,5×	0,5×
Schneider's Insect Medium	Sigma-Aldrich, S9895	5% (v/v)	5% (v/v)
HEPES	Sigma-Aldrich, H4034	1×10^{-3} M	1×10^{-3} M
BSA	Sigma-Aldrich, A9418	1% (w/v)	1% (w/v)

Complete media preparation: First, it was necessary to prepare stock solutions of all the hormones mentioned in **Table 4** (summarized in **Table 5**). The stock solutions of hormones were stored in -20°C. Second, the components listed in **Table 4** were added to the basic medium BME or ADMEM. The procedure followed a protocol from the Dr. James McKerrow (UC San Diego, Skaggs School of Pharmacy and Pharmaceutical Sciences; unpublished). HEPES and glucose were dissolved in basal medium and the lactalbumin hydrolysate was added. Then all the hormones were added according to **Table 5**. After adjusting the pH to 7.4 with pH meter (inoLab, WTW), the Schneider Insect medium and MEM Vitamins solution aliquots were added. The medium was supplemented with BSA (Chanová et al., 2009) instead of human serum (Basch, 1981). After this, the pH was readjusted to 7.4 and osmolarity was measured using osmometer (Os3000, Marcel) and adjusted to

275 mOsm. The medium was sterile filtered and used for cultivation experiments. Before the use, antibiotics solution Penicillin-Streptomycin-Amphotericin B (Sigma-Aldrich A5955) was added.

Table 5 Stock solutions of hormones added to the SCM or ASCM (adapted from Basch, 1981a)

Hormone	Solvent	Stock solution concentration	Volume / 1 l of media
Hypoxanthine	1M NaOH solution	1×10^{-3} M	0,5 ml
Serotonin	10^{-4} M HCl solution	1×10^{-3} M	1 ml
Insulin	10^{-3} M HCl solution	1 g/ml	8 ml
Hydrocortisone	96% (v/v) Ethanol, then add dH_2O to make final concentration	1×10^{-3} M	1 ml
Triiodothyronine	1M NaOH solution	2×10^{-4} M	1 ml

4.2.2 Other solutions used in c/s transformation and cultivation experiments

Phosphate buffered saline (PBS)

PBS of osmolarity 300 mOsm was used for washing the red blood cells (RBCs) and for washing the schistosomula prior to the cercarial penetration glands staining and viability assay. It was prepared following the protocol from Food and Agriculture Organization of the United Nations (FAO; Grimes, 2002). All the components listed in **Table 6** were dissolved in dH_2O and pH was adjusted to 7.4

Table 6 Composition of 300 mOsm PBS, pH 7.4 (Grimes, 2002)

Component	Final concentration in PBS (mM)
NaCl	136
KCl	2.68
KH_2PO_4	1.47
Na_2HPO_4	6.48

Acid-citrate-dextrose solution (ACD)

The ACD was used during collection of duck blood to prevent it from clotting. It was prepared following the protocol from FAO (Grimes, 2002). All the components listed in **Table 7** were dissolved in dH_2O.

Table 7 Composition of ACD (Grimes, 2002)

Component	Final concentration in PBS (mM)
Citric acid	17.54
Sodium citrate	38.4
Glucose	61

Hank's Balanced Salt Solution (HBSS) for washing of the CB

HBSS (Life Technologies, 14185045) with addition of 1% antibiotics (Penicillin-Streptomycin-Amphotericin B solution) was used for washing the cercarial bodies (CB) before placing the worms in the cultivation medium.

Linoleic acid solution

Linoleic acid (Sigma-Aldrich L1012) was used for *T. regenti* c/s transformation. Stock solution of concentration 1 mg/ml linoleic acid in dimethyl sulfoxide (DMSO) was prepared and stored in -20°C.

4.3 Media supplements preparation

4.3.1 Duck serum processing

Duck serum was used to initiate c/s transformation of *T. regenti* and in long-term cultivation experiments. The serum was obtained from ducks kept in our animal husbandry as follows.

A duck was decapitated and the whole blood was collected into 50 ml centrifuge tubes. Then the blood was clotted in room temperature (RT) for an hour and after it was centrifuged at 6000 g for 4 mins in RT to remove the blood clot. The supernatant was transferred to the clean tube and sterile filtered. It was heat-inactivated in 56°C for 90 min and stored in -20°C.

4.3.2 Collection of duck blood and preparation of washed RBCs

Duck erythrocytes (RBCs) were used for long-term cultivation experiments as a medium supplement. It was collected from ducks kept in our animal husbandry.

The collection of blood and washing of RBCs followed the protocol from FAO (Grimes, 2002). First, the ACD buffer was added to the microtube to the ratio of 1:3 to the blood to be added. No anesthesia was used and the blood was drawn from the *aa. metatarsales dorsales* with the insulin syringe needle after disinfecting the area around the bleeding site with 70% (v/v) ethanol. The blood was quickly transferred to the microtube with ACD buffer and mixed gently with the buffer.

The collected anticoagulated blood was transferred into centrifuge tubes with sterile-filtered PBS and centrifuged 10 mins at 500 g in RT. The washing steps were repeated 2× with PBS and 1× with the culture medium. The washed RBC suspension was stored in 4°C for maximum of 2 days.

4.4 Methods of *T. regenti* c/s transformation

The *T. regenti* cercariae were let for 1 h in room temperature (RT) to accommodate to the temperature difference after immobilization on ice. Then the c/s transformation methods were applied as follows.

4.4.1 Incubation of cercariae in SCM

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- HBSS containing 1% (v/v) Penicillin-Streptomycin-Amphotericin B
- SCM

The cercariae were rinsed with HBSS and placed to 35 mm tissue culture dishes into SCM. The cercariae were incubated according to the cultivation conditions described below (chapter 4.5).

4.4.2 Incubation of cercariae with linoleic acid

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- HBSS containing 1% (v/v) Penicillin-Streptomycin-Amphotericin B
- 1 mg/ml linoleic acid stock solution (chapter 4.2.2)

The cercariae were incubated for 60 min in water solution containing 100 µg/ml of linoleic acid, then the CB were washed in HBSS and cultivated as described in chapter 4.5.

4.4.3 Incubation of cercariae in SCM with 50 % inactivated duck serum

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- HBSS containing 1% (v/v) Penicillin-Streptomycin-Amphotericin B
- SCM with 50% inactivated duck serum (v/v)

The cercariae were rinsed with HBSS and placed to the SCM with 50% (v/v) of sterile heat-inactivated duck serum for 2 h.

4.4.4 Mechanical transformation – vortex

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- 15ml centrifuge flask
- Vortex mixer (TTS2 IKA yellowline test tube shaker)

The Cercariae were concentrated in 15ml centrifuge flask and vortexed for 4 min at 2500 RPM (adapted from Brink et al., 1977).

4.4.5 Mechanical transformation – centrifuge

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- 1.5 ml Eppendorf tubes
- Centrifuge (Eppendorf Minispin)

Water with concentrated cercariae was pipetted into the 1.5 ml Eppendorf tubes and centrifuged for 2 min at 13 000 g in RT. Then the CB were placed to the 35 mm culture dishes.

4.4.6 Mechanical transformation – syringe needle

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- 5 ml Eppendorf tube
- Syringe needle gauge 23 (0.6 mm in diameter, 60 mm long)
- 5 ml plastic syringe (Braun)
- HBSS containing 1% (v/v) Penicillin-Streptomycin-Amphotericin B

The cercariae were aspirated into the syringe needle from the 5 ml Eppendorf tube and pushed back, this was repeated 20× with maximal pressure (adapted from Chanová et al., 2009). Then the CB were placed to the 35 mm culture dishes and washed in HBSS.

4.4.7 Mechanical transformation – BeadBeater

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- 1.5 ml Bead tubes
- Mini-Beadbeater-1 (BioSpec) cell disrupter
- HBSS containing 1% (v/v) Penicillin-Streptomycin-Amphotericin B

The BeadBeating tubes were filled by 1.5 ml of cercarial suspension and the BeadBeater was set to 2500 RPM and 12 s. After breaking the cercarial tails, CB were transferred to the 35 mm culture dishes and washed in HBSS.

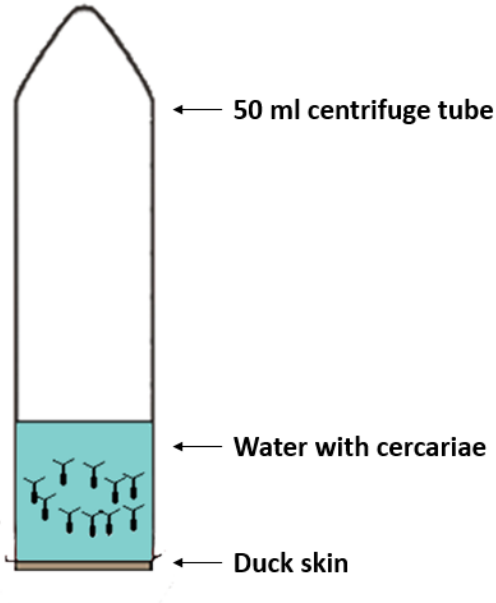
4.4.8 Transformation through the duck skin

Materials:

- 35 mm BioLite Tissue Culture Dishes (Thermo Scientific)
- Interdigital webbing skin from ducks, approximately 14 days old, stored in -20°C
- 50 ml centrifuge tube
- SCM preheated at 37°C

The cercariae were incubated for one hour in water in contact with the outer side of the duck skin that was covering the top of centrifuge tube (**Figure 5**). The cercariae were in contact with 660.5 mm^2 of duck skin in RT. Then the skin was cut into stripes afterward and the stripes were incubated in the SCM at 37°C for 40 min to let the CB leave the skin stripes. In the end the CB were separated from the duck skin and transferred to the 35 mm culture dish with fresh medium (adapted from protocol of RNDr. Jana Bulantová, Ph.D., unpublished).

Figure 5 Method of c/s transformation – penetration of the duck skin



4.5 Establishment of the *in vitro* cultures of *T. regenti*

4.5.1 Washing the CB

The cercarial bodies (without tails) were concentrated in the middle of the tissue plate by circular movements and washed three times by the HBSS with 1% (v/v) Penicillin-Streptomycin-

Amphotericin B. In some cases, the CB were attached tightly to the plate surface, therefore there was no need to concentrate them in the middle of the plate during washing. After the procedure, washing medium was replaced by the culture medium and the plates with CB were transferred into the incubator.

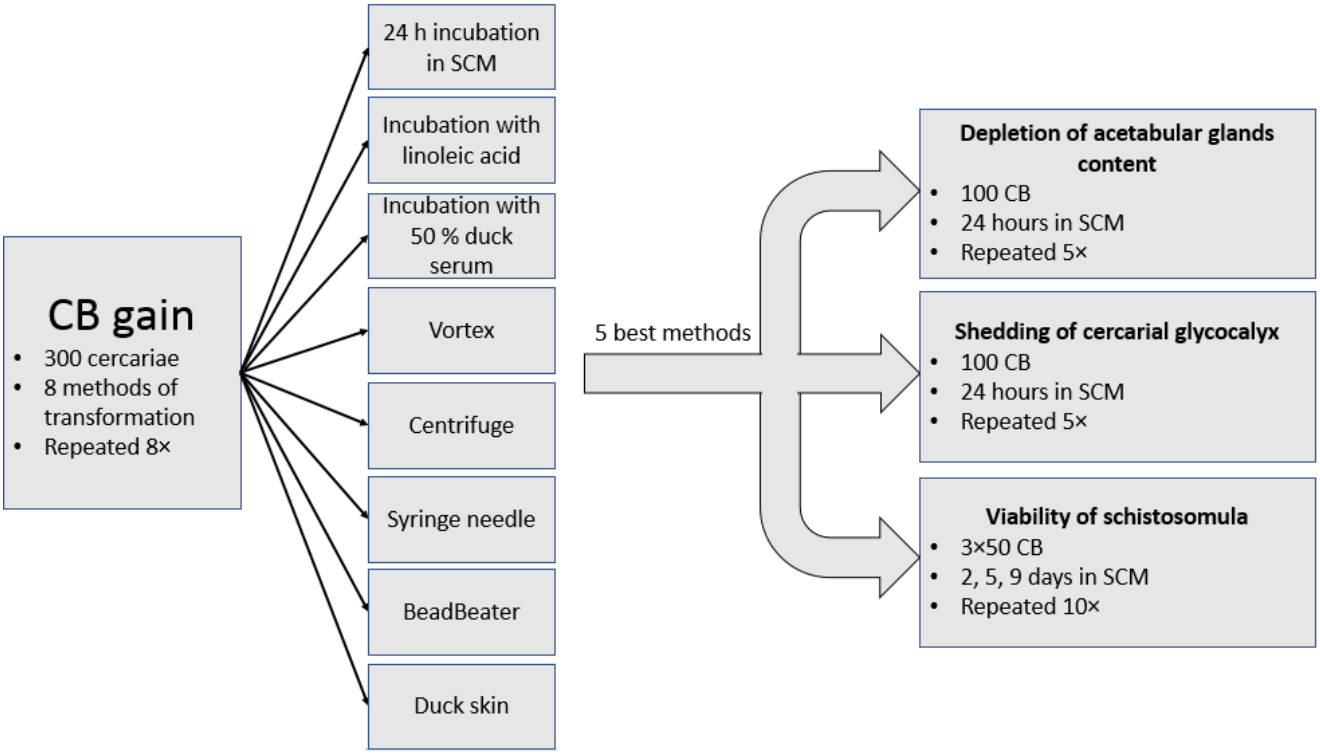
4.5.2 Cultivation conditions

The worms were incubated in 35mm BioLite Tissue culture dishes (Thermo scientific), in 3 ml of cultivation medium, at 37°C and 5% CO_2 atmosphere according to Chanová et al. (2009). The cultivation medium was changed every three days.

4.6 Evaluation of transformation methods suitability

The number of obtained *T. regenti* CB was recorded for each transformation method and the accomplishment of c/s transformation and subsequent viability in the culture medium was evaluated. To evaluate the ratio of worms with schistosomulum characteristics, cercarial penetration glands emptying and cercarial glycocalyx shedding was observed. The scheme of experiments related to the methods of c/s transformation is shown at **Figure 6**.

Figure 6 Experimental scheme of the c/s methods comparison

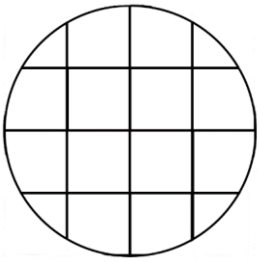


4.6.1 Counting the CB gained by transformation methods

Assuming that tail removal is an important trigger for further *in vitro* development, the CB gain was evaluated for each c/s transformation method. Cercariae were concentrated to the count of 300 ± 26 (mean ± SD) per ml of water to ease the counting and the c/s transformation method was applied. The transformation was either performed directly in the 35-mm plastic culture dish (incubation in SCM, with linoleic acid, with duck serum) or the CB were transferred to the dish after the cercarial tail removal (mechanical methods of transformation, duck skin penetration).

The dish was filled with 2 ml of water to ease the manipulation with the CB and to keep the CB wet. The 4×4 grid was drawn on the bottom of the dish so it was divided into fields small enough to be visible in one field of view in the stereomicroscope (**Figure 7**). When counting, the system was employed as follows on inner squares: only the worms set within the counting field or the right-hand or top boundary line were counted. Based on CB gain, 5 methods were selected for evaluation of glands emptying, shedding of glycocalyx and viability. Also, the influence of tail breakage on glands emptying and glycocalyx shedding was evaluated.

Figure 7 The 4×4 grid drawn on the bottom of the 35 mm plastic dish for CB counting



4.6.2 Evaluation of penetration glands emptying

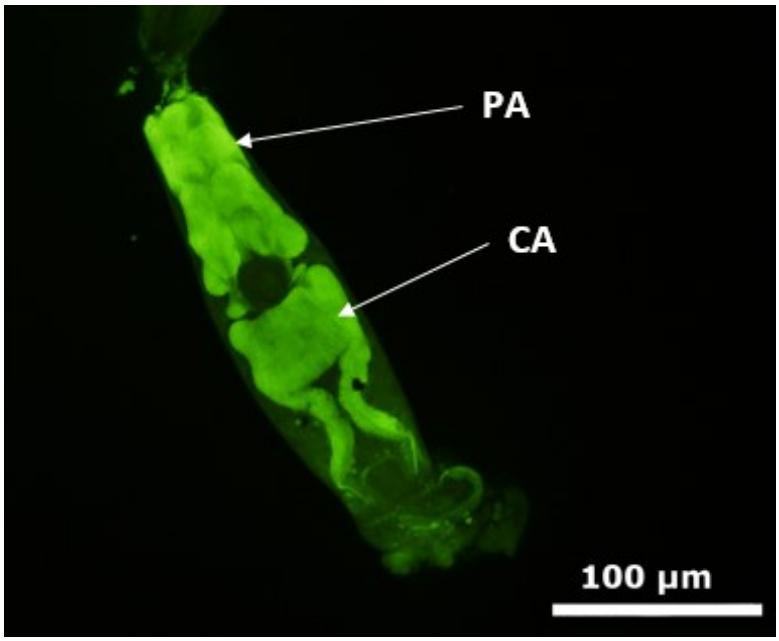
Solutions and reagents:

- PBS (chapter 4.2.2)
- 2 mg/ml fluorescein diacetate (FDA; Sigma-Aldrich F7378) stock solution in acetone
- Procedure:

Five methods of c/s transformation were selected based on CB gain and applied at concentrated cercariae (about 1000 cercariae/ml). The CB were incubated in SCM for 24 h according to the conditions stated above (chapter 4.5.2). Then, about 100 CB were randomly selected for evaluation of penetration glands emptying. The experiment was repeated 5× in total.

The cercarial post- and preacetabular glands were stained with FDA to visualize if the glandular content was emptied. In every experiment, 10 freshly shed cercariae were used as a positive control (**Figure 8**).

Figure 8 *T. regenti* cercaria stained with FDA, acetabular glands not emptied; PA – postacetabular gland; CA – circumacetabular gland



After the 24 h incubation, the CB were washed in PBS. and stained with the FDA in final concentration 0.5 µg per ml of PBS, for 30 min at 37°C, darkened by the aluminum foil (Peak et al., 2010). The stained worms were immediately observed under a fluorescence microscope Olympus BX51 using appropriate excitation/emission filters. Photos were taken by Olympus DP70 camera and acquired with QuickPHOTO MICRO 3.0. Results were noted as two categories of worms – that with acetabular glands emptied completely and the rest – with acetabular glands not completely emptied.

4.6.3 Evaluation of cercarial glycocalyx shedding

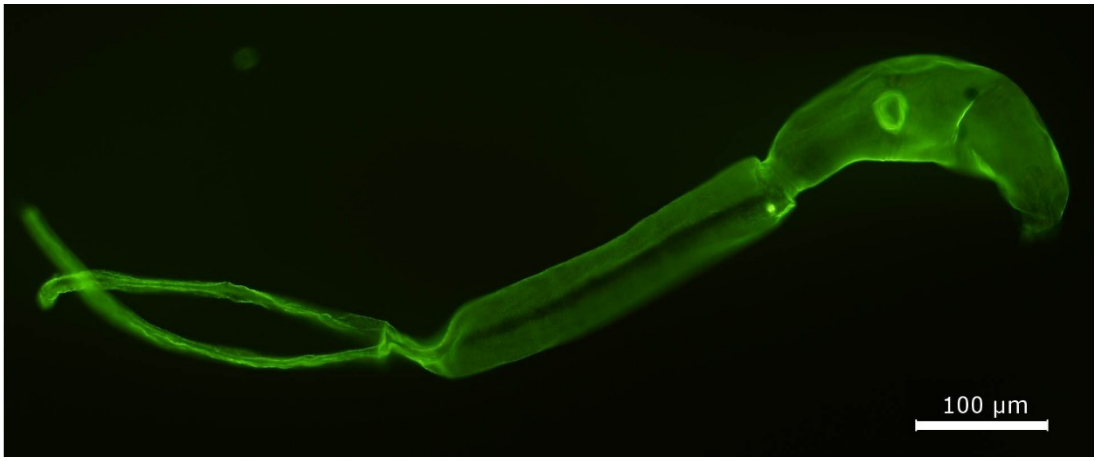
Solutions and reagents:

- PBS (chapter 4.2.2)
- 4% (w/v) paraformaldehyde solution in PBS
- Fluorescein-labeled *Ulex Europaeus* Agglutinin I (UEA I; Vector FL-1061)
- VECTASHIELD® Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; Vector)

T. regenti cercarial glycocalyx binds lectin UEA I, unlike schistosomula with glycocalyx shed (Řimnáčová et al., 2017). Therefore, UEA I was used for evaluation of glycocalyx shedding in my experiments. The procedure starts the same as in evaluation of penetration glands emptying experiment (chapter 4.6.2). Five methods of c/s transformation selected based on CB gain were applied on cercariae concentrated on about 1000 cercariae/ml. The CB were incubated in SCM for 24 h according to the conditions stated above (chapter 4.5.2). Then, about 100 CB were randomly

selected for evaluation of glycocalyx shedding. The experiment was repeated 5× in total. Every time, 10 freshly shed cercariae were used as positive control (**Figure 9**).

Figure 9 *T. regenti* cercaria with glycocalyx labeled by fluorescent-conjugated UEA I, glycocalyx not shed



After 24 h incubation in SCM, the worms were fixated in 4% (w/v) paraformaldehyde solution for one hour in RT. Then they were washed 3× in PBS and Fluorescein-labeled UEA I was added in the final concentration 20 µg/ml. Then the sample was incubated in dark for 30 min, mounted in VECTASHIELD® Mounting Medium and examined under the fluorescent microscope Olympus BX51 using appropriate excitation/emission filters. Photos were taken by Olympus DP70 camera and acquired with QuickPHOTO MICRO 3.0. The worms were evaluated in two categories: first, worms with glycocalyx shed completely with no positive reaction with UEA I and second, all the rest of worms showing positive reaction with UEA I.

4.6.4 Viability assays

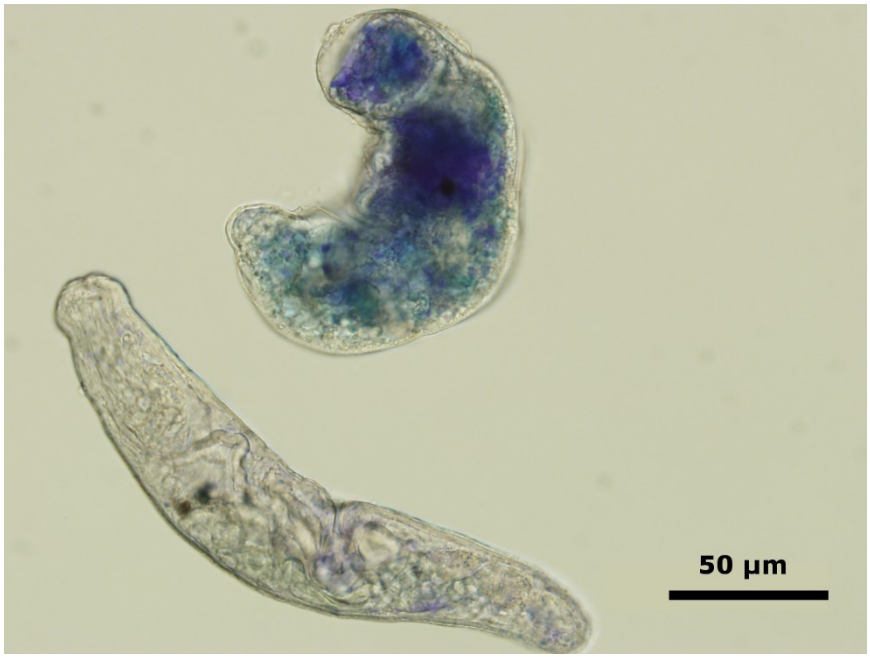
Solutions and reagents:

- PBS (chapter 4.2.2)
- 0.15% (w/v) methylene blue solution in PBS
- 2 mg/ml FDA (Sigma-Aldrich F7378) stock solution in acetone

Viability of schistosomula transformed by 5 selected c/s transformation methods was assessed after 2, 5 and 9 days of incubation in SCM to evaluate the damaging effect of used transformation method. Cercarial bodies were placed into cultivation dish, 50 worms per dish and one dish was prepared for each time of cultivation and the worms were cultivated as described in chapter 4.5.2. The experiment was repeated 10× in total.

FDA and methylene blue assays were used to evaluate viability of worms as FDA crosses the membranes of living cells and is converted to fluorescein and methylene blue stains only dead cells (Gold, 1997; Peak et al., 2010). Both FDA and methylene blue assay were used simultaneously: First, the schistosomula were washed in PBS and methylene blue solution was added in ratio 1:1 to the PBS and let to stain the worms for 15 min in RT. Second, the methylene blue was washed away and FDA was added to the final concentration 0.5 µg/ml of PBS. The worms incubated with FDA for 30 min in 37°C while darkened by the aluminum foil. After the last step, the worms were immediately observed under inverted microscope Olympus IX71 with the use of proper emission/excitation filter for FDA staining, then the methylene blue staining was evaluated using light microscopy. The photos were taken by Olympus DP70 camera and processed by QuickPHOTO MICRO 3.0. Only worms viable according to both FDA and methylene blue assays were counted as being alive, which means worms positive in FDA staining and negative in methylene blue staining. If more than half of worm body was stained by methylene blue, the worm was considered stained. **Figure 10** illustrates a schistosomulum alive (bottom) and a dead schistosomulum (top) by the methylene blue staining.

Figure 10 Schistosomulum transformed by BeadBeater, stained by methylene blue; top: dead schistosomulum; bottom: schistosomulum alive



4.7 Long-term cultivation experiments

In long-term cultivation experiments, selected characteristics were evaluated after 9, 14 and 21 days p. t. First, viability in two basic commercially available media (BME, ADMEM) and two complete media (SCM, ASCM) was determined for BeadBeater-produced schistosomula. This

experiment was only performed on BeadBeater-schistosomula, because BeadBeater transformation was the main method intended for following experiments based on the production of CB.

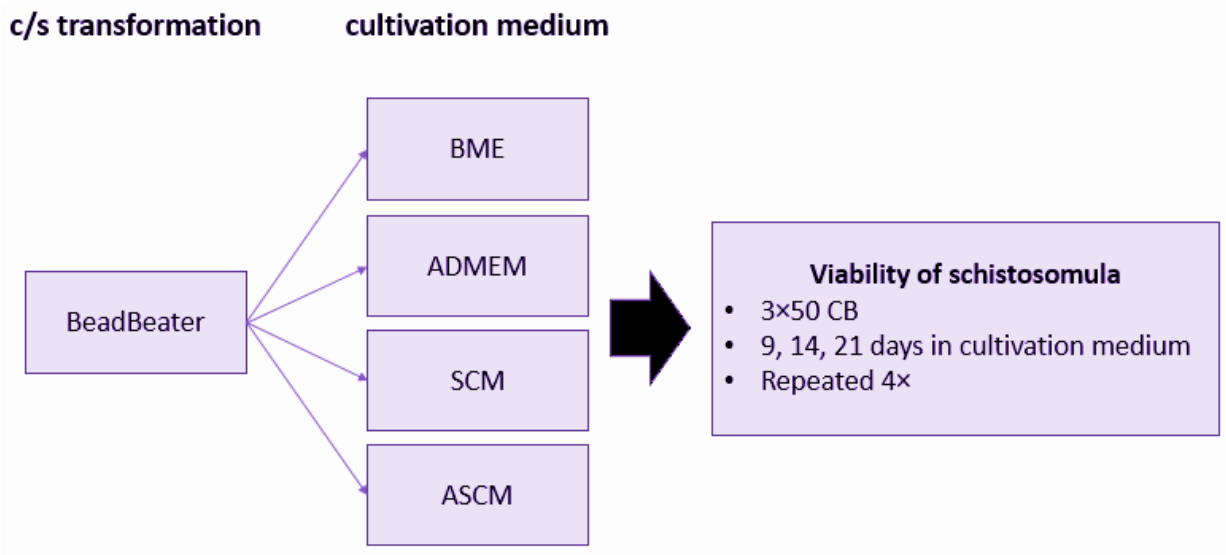
Then, effect of RBCs and duck serum was tested on body growth and gut development of BeadBeater-schistosomula and duck skin-schistosomula. Also, effect of SCM and ASCM as cultivation media was compared. The worms were cultivated as described in chapter 4.5.2.

4.7.1 Medium for long-term cultivation

The scheme of this experiment is shown at **Figure 11**. Schistosomula transformed by BeadBeater were placed to media BME, ADMEM, SCM and ASCM, 50 worms in a culture dish. One dish was used for each time of cultivation and the experiment was repeated in total 4×.

Viability after long-term cultivation in tested media was tested as described in chapter 4.6.4. using FDA and methylene blue.

Figure 11 The scheme of experiment evaluating viability in 4 cultivation media



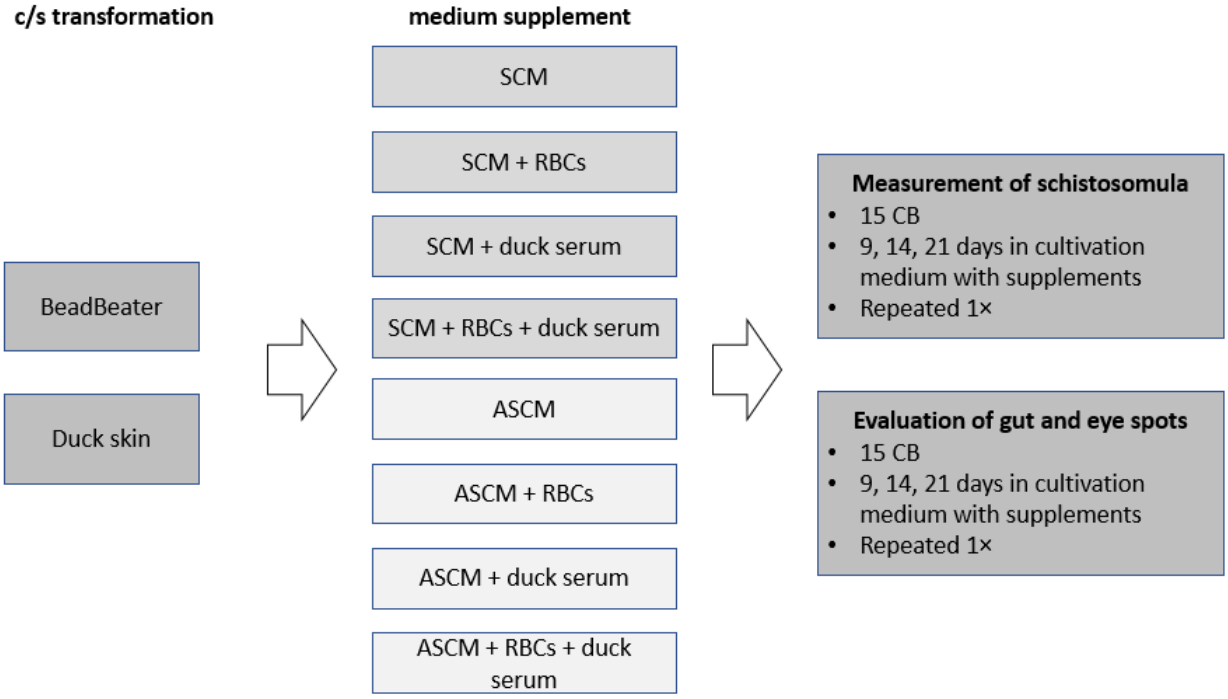
4.7.2 Culture media supplements

First, survival of BeadBeater-schistosomula was tested in SCM with 10% (v/v) heat-inactivated duck serum and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco 10270) for 14 days as preliminary experiment. On day 14 p. t., viability and growth were evaluated. Based on the result, FBS was omitted from further testing.

Effect of duck RBCs and heat-inactivated duck serum in SCM and ASCM media was tested as pictured in **Figure 12**. Schistosomula transformed by BeadBeater or duck skin penetration were placed into SCM, SCM with 1% RBCs, SCM with 10% (v/v) duck serum, SCM with 1% (v/v) RBCs and

10 % (v/v) duck serum, ASCM, ASCM with 1% (v/v) RBCs, ASCM with 10% duck serum or ASCM with 1% (v/v) RBCs and 10 % (v/v) duck serum. Duck RBCs and serum was obtained and processed as mentioned above (chapter 4.3). Duck skin schistosomula were included in this experiment because of their better optical state at the end of short-term cultivations. After 9, 14 and 21 days of cultivation, 15 randomly selected worms from each group were evaluated as follows.

Figure 12 Experiment schema - effect of medium supplements on the growth and development of *T. regenti* schistosomula



Evaluation of media supplements – length of the schistosomula body

Solutions and reagents:

- PBS (chapter 4.2.2)
- 7.14 % $MgCl_2$ solution in PBS (w/v)
- 4% (w/v) paraformaldehyde solution in PBS

Schistosomula cultivated as mentioned above (chapter 4.5) were measured to evaluate the growth of long-term cultivated schistosomula depending on medium supplement added.

The worms were washed in PBS and placed for 10 min to the $MgCl_2$ solution in RT to relax their musculature. After that they were fixated in paraformaldehyde solution and photographed using Olympus BX51 microscope, Olympus DP70 camera and QuickPHOTO MICRO 3.0. All the measurement values were acquired using ImageJ 1.50i.

Evaluation of media supplements – developmental stage of the gut and eye spots state

The schistosomula fixed in paraformaldehyde were further analyzed after taking the measurement. The state of gut development was analyzed for caeca connection and continuation as single gut as opposite to cercarial two caeca (Horák et al., 1998a). Additionally, the state of pigments spots was evaluated, as it was noticed that cercariae have 2 pigment eye spots and adult worms obtained from the definitive host (25 days p. i.) were noted to have many smaller pigment dots instead (personal observation).

Evaluation of media supplements – development of reproductive system

Addition of duck serum resulted in prolonged viability and significant body growth, therefore two of the duck skin transformed schistosomula were examined by differential interference contrast microscopy to evaluate state of reproductive system using Olympus BX51 microscope. As a control, adult male and female worms 25 days p. i. (available from the laboratory cycle of *T. regenti*) were analyzed. The photographs were acquired by the Olympus DP70 camera and QuickPHOTO MICRO 3.0.

4.8 Other potential markers of completed transformation into schistosomulum

Following potential markers of successful c/s transformation were selected and evaluated: development of oral sucker, appearance of glucose transporters (GTP1 and 4) and expression of selected genes.

4.8.1 Development of oral sucker – musculature labeling

Solutions and reagents:

- PBS (chapter 4.2.2)
- 4% (w/v) paraformaldehyde solution in PBS
- Incubation solution: 1% (w/v) sodium dodecyl sulfate (SDS); 0.3% (v/v) Triton X-100; 1% (w/v) BSA; 10% (w/v) sucrose; 0.1% (w/v) NaN_3 in PBS
- FITC-phalloidin (Sigma-Aldrich P5282)
- VECTASHIELD® Mounting Medium with DAPI (Vector)

The musculature was labeled by Fluorescein Isothiocyanate-labeled phalloidin (FITC-phalloidin) to evaluate the development state of schistosomular oral sucker.

First, cercariae were transformed by syringe needle and incubated 36 h in SCM following the procedure mentioned above (chapter 4.5). Second, 40 schistosomula were fixed in paraformaldehyde solution for 4 hours in RT. Then the worms were washed 4× in PBS and

transferred into the incubation solution. FITC-phalloidin was added to the final concentration 200 ng/ml and the specimens were incubated at 4°C in dark for 48 h (Mair et al., 2000). After that, the worms were mounted in VECTASHIELD® Mounting Medium and observed under the fluorescent microscope Olympus BX51 with the use of proper emission/excitation filter. The photographs were taken by Olympus DP70 camera and QuickPHOTO MICRO 3.0

4.8.2 Development of tegument – glucose transporters GTP1 and 4 labeling

T. regenti cercariae and schistosomula were tested for expression and presence of glucose receptors GTP1 and GTP4 that were proved to form on the *S. mansoni* cercarial surface after incubation in appropriate medium (Skelly and Shoemaker, 2000).

To determine if the glucose receptors GTP 1 and 4 are present in *T. regenti* genome and transcriptome, the alignment of *S. mansoni* DNA sequences coding the GTP1 and 4 and *T. regenti* genomic DNA sequence was performed (blastn). Then, Mgr. Roman Leontovyč compared *T. regenti* DNA sequences matching to the *S. mansoni* GTP1 and 4 coding genes to the *T. regenti* local transcriptome sequence database.

The results showed that *S. mansoni* glucose transport protein 1 and 4 (SGTP1 and 4) could be labeled by primary antibodies rabbit anti-SGTP1 and anti-SGTP4 (Skelly and Shoemaker, 1996; Zhong et al., 1995). These antibodies were kindly donated by Patrick J. Skelly, Ph.D., The Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine, Tufts University. These antibodies were used for checking the cross-reactivity with *T. regenti* schistosomula GTPs and thus their localization in whole mount immunohistochemistry (IHC), ELISA and Western blot experiments. *S. mansoni* cercariae serving as positive control were incubated for 24 h in SCM (according to conditions mention in chapter 4.5.2.), whereas *S. mansoni* cercariae without incubation were used as negative control.

Whole mount IHC

Solutions and reagents:

- 4% (w/v) paraformaldehyde in PBS solution
- PBS (chapter 4.2.2)
- Permeabilization solution A: 0.3% (v/v) Triton X-100; 0.5% (w/v) SDS; 2 µg/ml Proteinase K (Sigma-Aldrich P2308) in PBS
- Permeabilization solution B: 0.3% (v/v) Triton X-100; 0.5% (w/v) SDS in PBS
- Blocking solution: 1% (w/v) BSA; 0.3% (v/v) Triton X-100; 0.05% (v/v) Tween 20 (BIO–RAD) in PBS

- Primary antibodies: rabbit anti-SGTP1 and anti-SGTP4; mouse anti-tubulin antibody (Sigma-Aldrich T7451)
- Secondary antibodies: goat anti-rabbit Alexa Fluor 488; goat anti-mouse Alexa 568
- VECTASHIELD® Mounting Medium with DAPI (Vector)

Tested *T. regenti* stages: cercariae, cercariae incubated for 24 h in SCM, schistosomula produced by BeadBeater incubated in SCM for 24 h and 5 days

T. regenti cercariae and schistosomula without addition of antibodies were used to check for autofluorescence and *T. regenti* cercariae and schistosomula with only secondary antibodies to check for nonspecific binding of the antibody. *T. regenti* schistosomula incubated for 24 h were labeled by anti-tubulin antibody (1:200) to verify that the protocol leads to successful IHC labeling.

The worms were fixed in 4% (w/v) paraformaldehyde solution for 3 hours in RT and then washed in PBS 3×. Then they were permeabilized in a permeabilization solution A for 5 min in 37°C and then in permeabilization solution B for 15 min, in RT. After washing 2× in PBS, the specimens were transferred into blocking solution and incubated for 2 h in RT. Then the primary antibodies were added (anti-SGTP4 1:100; anti-SGTP1 1:200), worms were incubated in 4°C for 12 h. Then the antibodies were washed away in blocking solution for 2 h in RT and the secondary antibodies were added (dilution 1:400). After incubation in 4°C for 12 h, the worms washed in PBS and mounted in VECTASHIELD® Mounting Medium and evaluated under the with appropriate emission/excitation filter on microscope Olympus BX51. The photographs were taken by Olympus DP70 camera and QuickPHOTO MICRO 3.0.

ELISA

Solutions and reagents:

- Quant-iT Protein Assay Kit (Thermo Fisher Scientific)
- 0.1 M PBS (pH 7.2): 360 ml 0.2 M Na_2HPO_4; 140 ml 0.2 M NaH_2PO_4; 120 mM NaCl
- PBS - T: 0.05% (v/v) Tween-20 in 0.1 M PBS (pH 7.2)
- Bicarbonate/carbonate coating buffer (pH 9.6): 100 mM $NaHCO_3$; 33.5 mM Na_2CO_3 in dH_2O
- Blocking solution: 1% (w/v) Blotting Grade Blocker Non-fat dry milk (BIO-RAD) solution in PBS-T
- Primary antibodies: rabbit anti-SGTP1 and anti-SGTP4
- Secondary antibody: anti-Rabbit IgG, whole molecule – peroxidase antibody produced in goat (Sigma-Aldrich A0545)
- 3,3’,5,5’-tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich)

Tested *T. regenti* stages: cercariae, cercariae incubated for 24 h in SCM, schistosomula produced by BeadBeater incubated in SCM for 24 h

To detect SGTP 1 and 4 transporters, an indirect antigen-down assay was performed on homogenized *T. regenti* stages mentioned above. All homogenates in coating buffer alone were used as a blank and homogenates with secondary antibody and homogenates with negative rabbit serum and secondary antibody were used to detect nonspecific binding of the antibody.

Samples of 600 worms were stored in 200 µl of water (cercariae) or 0.1 M PBS (schistosomula). Cercariae and incubated worms were washed in water or PBS, respectively. The material was stored at -20°C and homogenized by sonication on Vibracell 72405 for 9 × 10s in 200 µl of water/PBS. The homogenate was centrifuged at 13 000 g, 4°C for 20 min. For the detection, both suspensions and supernatants from centrifuged homogenates were used.

The microplate (NUNC MaxiSorp) was coated with 5 µl per well of antigen solved in bicarbonate/carbonate coating buffer at 4°C for 12 h (proteins were quantified using the Quant-iT Protein Assay Kit, Thermo Fisher Scientific). Then the plate was washed 3× in PBS-T and blocked in RT for 1 h with blocking solution. After that, primary antibodies (1:100) were added and the plate was incubated for 3 h in RT and washed 3× with PBS-T afterward. Then the plate was incubated in RT for 1 h with secondary antibody (1:5000). After incubation with secondary antibodies, the plate was washed in PBS-T 3× and incubated with TMB substrate for 15 min in RT. After reaction developed, the absorbance was measured using 450 nm filter at Infinite M200 (Tecan). The samples were always measured in doublets.

Western blot

Solutions and reagents:

- Quant-iT Protein Assay Kit (Thermo Fisher Scientific)
- Laemmli sample buffer
- 0.1 M PBS (pH 7.2): 360 ml 0.2 M Na_2HPO_4; 140 ml 0.2 M NaH_2PO_4; 120 mM NaCl
- TGS buffer: 30 g Tris, 144 g glycine, 10 g SDS
- Marker BIO-RAD Precision Plus Protein Dual Xtra Standards
- Blotting buffer: 140 ml dH_2O, 20 ml TGS, 40 ml methanol
- Blocking solution: PBS + 5% (w/v) Blotting Grade Blocker Non-fat dry milk (BIO-RAD) + 2,5% BSA + 0,05% Tween
- PBS-T: 0.05% (v/v) Tween-20 in 0.1 M PBS (pH 7.2)
- Opti-4CN Kit (BIO-RAD)

Tested *T. regenti* stages: cercariae, schistosomula produced by BeadBeater incubated in SCM for 24 h and 5 days

Negative controls to detect non-specific binding were as follows: negative rabbit serum instead of primary antibodies, homogenate and only secondary antibodies.

Detection of SGTP 4 transporter was also tested using western blot technique. A sample of 600 worms was stored in 200 µl of water (cercariae) or 0.1 M PBS (schistosomula) in -20°C for each tested variant. The samples were homogenized by sonication on Vibracell 72405 for 9 × 10s in 200 µl of water (cercariae) or PBS (schistosomula). For the detection, both suspensions and supernatants from centrifuged homogenates (concentrated using 3000 kDa filter Microcon YM3) were used. Protein concentration in samples was quantified using the Quant-iT Protein Assay Kit (Thermo Fisher Scientific) and homogenate was diluted in Laemmli sample buffer. Samples (13 µg) were loaded onto the 12% polyacrylamide gel. Proteins were separated by the SDS electrophoresis (sodium dodecyl sulfate) in reducing conditions of TGS buffer at 150 V (BIO-RAD Power Pac Universal) on BIO-RAD Mini Protean 3. After separation, the control gels were stained by Coomassie Brilliant Blue R-250 (Serva Electrophoresis) to visualize the protein profile. Proteins from other gels were transferred onto Immun-BlotR polyvinylidene difluoride (PVDF) membrane (BIO–RAD) by Trans-Blot Turbo Transfer system (BIO–RAD) with the use of blotting solution. The transfer time was 15 min at constant current of 1,5 A. After blotting, the residual proteins in gels were stained by Coomassie Brilliant Blue to document their successful transfer to the membrane. Then the membrane was incubated for 12 h in blocking solution in RT – while shaking all the time – to prevent non-specific binding of the antibodies. Blocked membrane was incubated with primary antibody (anti-SGTP 4, 1:200) dissolved in PBS-T with 2,5% BSA for 1,5 h RT. The membrane was then 3× washed in PBS-T for 5 min and incubated with secondary antibody (goat-anti-rabbit IgG; HRP conjugate, Sigma A0545, 1:1000) in PBS-T with 2,5% BSA for 1 h in RT. After washing 3× washed in PBS-T for 5 min, the membrane was developed for 30 min using Opti-4CN Kit (BIO-RAD). The membranes were scanned by GS-800 Calibrated Densitometer (BIO–RAD) after the development by the kit.

4.8.3 Gene expression of schistosomula

To evaluate if changes in gene expression of selected genes could serve as a marker of c/s transformation, expression of selected genes was tested on *T. regenti* stages. The stage tested were cercariae, BeadBeater-schistosomula 2, 5 and 9 days p. t. incubated in SCM and BeadBeater-schistosomula 9 days p. t. incubated in SCM with 10% duck serum.

Selection of genes for expression evaluation

The genes for evaluation were selected based on transcriptomic data provided by Mgr. Roman Leontovyč who generated a list of 148 genes with the highest expression level in

T. regenti schistosomula 7 days p. i., in contrast to cercariae – significantly less active stage in expression of selected genes.

Three genes that could be associated with development or digestive processes of the worm were selected based on the gene onthology (KEGG and Swiss-Prot database were used).

Selection of reference genes for expression evaluation

The reference genes were selected based on transcriptomic data provided by Mgr. Roman Leontovyč who generated a list of 7 genes with stable expression level in *T. regenti* cercariae and 7 days old schistosomula. Three of these genes were selected based on gene onthology. List of selected genes is shown at **Table 8**.

Table 8 Selected genes for evaluation of the *T. regenti* expression in cercariae and cultivated schistosomula; GAPDH - Glyceraldehyde 3-Phosphate Dehydrogenase; HNRA - Heterogeneous Nuclear Ribonucleoprotein A2 homolog 1, ITLR - Isoleucine-tRNA ligase

Selected gene product	Sequence ID	Primer
Axon guidance protein	Treg_008144	TrAxGP
Hemoglobinase	Treg_015036	TrHem
Hexosaminidase	Treg_015036	TrHex
Reference gene – GAPDH	Treg_000122	TrGAPDHref
Reference gene – HNRA2	Treg_004035	TrHNRAref
Reference gene - ITLR	Treg_015058	TrITLref

Designing of primers

Primers for all the selected six genes were designed according to their cDNA sequences for amplification of the gene fragment of size 107-208 base pairs (**Table** 9). The fragments were designed short because of the intended usage in qPCR. All the primers were designed using Clone Manager 9 program to have similar parameters of CG content and melting temperature.

Table 9 Table of primers for evaluation of the *T. regenti* expression in cercariae and cultivated schistosomula

Primers	5'-3'	Amplicon size (bp)
TrAxGPFw	GGGGAAACGGATAATGCCAGCATACTTGG	152
TrAxGPRe	GAAACAGGGAAATATCCTGGATGGCGAC	
TrHemFw	GCCCAACTAGATTCTCGTGGATTTGCAG	208
TrHemRe	CTCCTGGAAGCGTTGAATTCCCTGAAG	
TrHexFw	GGATTCCAACCACGACTGAGCATTCG	107
TrHexRe	GCGGTAGCGGAACGACTATGGAC	
TrGAPDHrefFw	GAAAATGTGGCGAGACGGTCGTG	159
TrGAPDHrefRe	GTAAGATCGACGACTGAAACATCCGCTG	
TrHNRArefFw	GTATCCTGGAAGGAAACAGGCGAGTC	118
TrHNRArefRe	CTGCTCTGCTGGAGCCTATGTGATG	
TrITLrefFw	GTTGGTTCGAGTCTGGCTCTATGCC	125
TrITLrefRe	CCACCCTCGGGTCTGATCAATACC	

Material preparation

Solutions and reagents:

- PBS
- High Pure RNA Tissue Kit (Roche), version 09
- Transcriptor First Strand cDNA Synthesis Kit (Roche)

Material for all the *T. regenti* stages tested was prepared as follows: 500 of worms were washed in PBS (in water in case of cercariae), transferred to 100 µl of the Lysation buffer from the High Pure RNA Tissue Kit and stored in -80°C for maximum a month.

The worms were homogenized by the electric rotor-stator homogenizer. The RNA was isolated with the High Pure RNA Tissue Kit according to the kit instructions and eluted into 5 µl of elution buffer (part of the kit). The RNA concentration was measured using spectrometer Nanodrop ND 1000 (Thermo Scientific). The cDNA was synthesized from the same amount of RNA for each stage (20 ng) according to kit instructions, using the enclosed OligodT primers (Transcriptor First Strand cDNA Synthesis Kit) and stored in -20°C.

PCR performed on cDNA of *T. regenti* stages

Solutions and reagents:

- Specific primers for the 3 genes tested and 3 reference genes (**Table** 9)
- MasterMix EmeraldAmp GT PCR (Clontech)
- SYBR Green Gel Stain (Life Technologies)
- TAE buffer (Life Technologies)
- Gene Ruler Ladder Mix (Thermo Scientific)

The PCR detection was performed. The cDNA from all the tested *T. regenti* stages was diluted to 400 ng/µl and used as a template in the PCR performed on C 1000 Thermal Cycler (BIO–RAD) with specific primers. The particular selected fragments of the cDNA were amplified in PCR as showed in **Table 10**.

Results were evaluated by electrophoresis run on 2% agarose gel containing SYBR Green in TAE buffer, using Gene Ruler Ladder Mix as a standard. The electrophoresis was set to 120 V and 40 min.

Table 10 PCR reaction

Reaction mixture for PCR		PCR amplification cycle		
EmeraldAmp GT PCR Master Mix (Clontech)	12.5 µl	95°C	4 min	
Primer Fw (**Table** 9)	0.5 µl	95°C	30 s	35×
Primer Re (**Table** 9)	0.5 µl	55°C	30 s	
cDNA, 400 ng/µl	1 µl	72°C	30 s	
dH_2O	10.5 µl	72°C	5 min	

qPCR

Solutions and reagents:

- Specific primers for the 3 genes tested and 3 reference genes (**Table** 9)
- iQ SYBR Green Supermix (BIO–RAD)

All the cDNA samples diluted to 400 ng/µl and all 6 primers were used in a qPCR reaction with parameters set as shown in **Table 11**. The reaction was processed by iQ5 Multicolor Real-Time PCR Detection System (BIO–RAD), in doublets.

Table 11 qPCR reaction

Reaction mixture for qPCR		qPCR amplification cycle		
iQ SYBR Green Supermix (BIO–RAD)	6.25 µl	95°C	4 min	
Primer Fw (**Table** 9)	0.5 µl	95°C	15 s	50×
Primer Re (**Table** 9)	0.5 µl	55°C	15 s	
cDNA, 400 ng/µl	1 µl	72°C	15 s	
dH_20	6.25 µl	72°C	1 min	

4.9 Statistics

Proportional data (schistosomula viability after different transformation methods or in different cultivation media, gland emptying, glycocalyx shedding, tail detachment) were arcsine-transformed and data on schistosomula length were log-transformed to reach normality (Zar, 1999), which was verified by Shapiro-Wilk test. Differences among groups undergoing different transformation methods were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. As for the examination of the influence of two factors on the differences among groups, two-way ANOVA was applied. If the P-values were lower than 0.05, the

difference was considered significant. In the figures, data are presented as arithmetic mean ± standard deviation (SD). All analyses were performed using GraphPad Prism, version 6.

5 EXPERIMENTAL RESULTS

5.1 Cercariae/schistosomula transformation

Cercaria to schistosomulum transformation (c/s) methods were compared in the cercarial bodies (CB) gain, emptying of acetabular glands, cercarial glycocalyx shedding and viability in Schistosome culture medium (SCM) after 2, 5 and 9 days. The percentages of worms with schistosomulum-like characteristic (tail-loss, glands emptying, glycocalyx shedding, being alive) are summarized in **Table 12** and **Figures 13-16** and the results are further described in chapters 5.1.1-5.1.8 and complete data with p-values from the statistical analysis are available in the Supplement file.

The influence of tail breakage on the glands emptying an glycocalyx shedding was also evaluated. When the cercariae were placed into SCM without tail removal, only 4.60% worms had the glands emptied and 1.29% shed their glycocalyx 24 h p. t. (**Table 12**). When cercarial tails were removed by BeadBeater or syringe needle, 35.01% or 35.98% (respectively) of worms incubated for 24 h emptied their acetabular glands and 22.19% or 26.88% (respectively) shed their glycocalyx. However, no statistical difference was confirmed between incubated cercariae and mechanically transformed schistosomula after 24 h incubation in SCM (**Table 12, Figure 13**).

During the glycocalyx shedding evaluation, stages with glycocalyx present and glycocalyx shed were observed. However, stages with glycocalyx present only on the posterior part of body were observed as well. These stages were evaluated as still possessing glycocalyx layer. Viability of schistosomula transformed by all the methods was decreasing in time, but it seemed to decrease slower for skin schistosomula (**Figure 16**).

The worms transformed via different methods were indistinguishable under light microscope during first 2 days (except for incubated cercariae in SCM that mostly still had tails). But, after 5 days p. t. slight differences in appearance were noted (further described in chapters 5.1.1 to 5.1.8). The schistosomula often had “bubble-like” material on the surface (**Figure 21**) 5 and 9 days p. t. but the structures were only visible on live schistosomula; after fixation in 4% FDA the surface became smooth (**Figure 20**).

Figure 13 CB gain depending on various transformation methods (% from initial number of cercariae); mean ± SD; n = 8

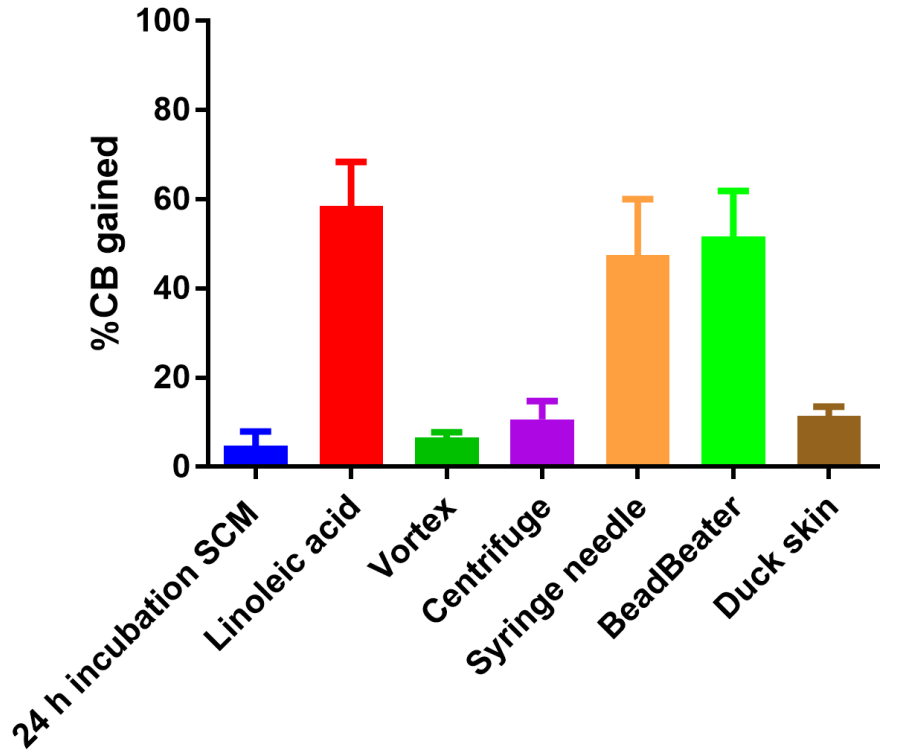


Figure 14 Percentage of schistosomula with emptied penetration glands depending on the transformation methods used, after 24 h incubation in SCM; mean ± SD; n = 5

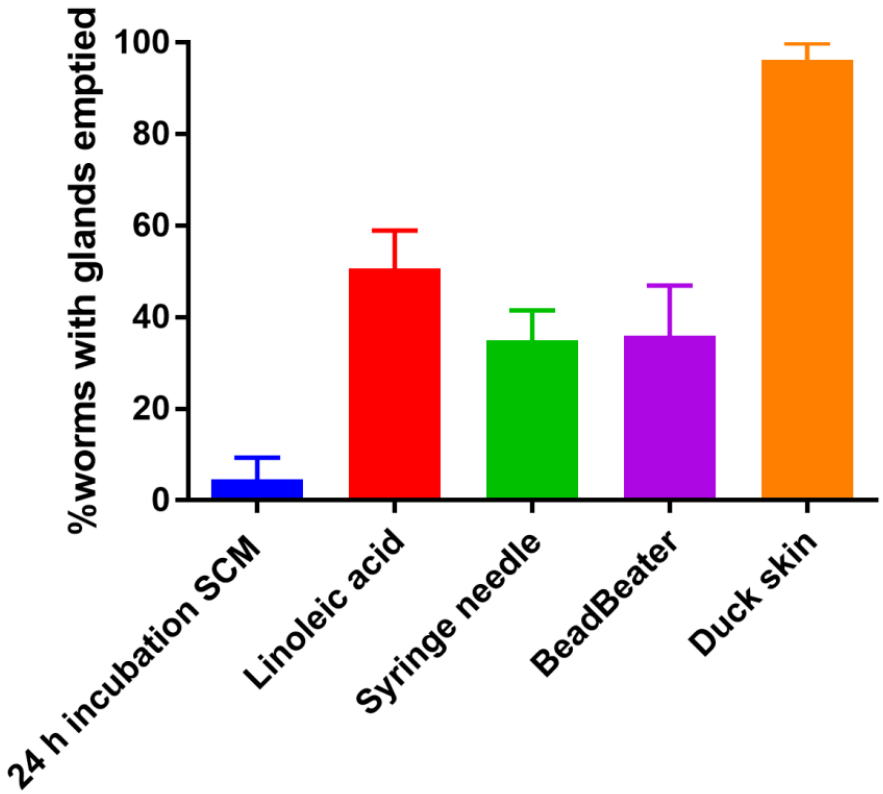


Figure 15 Percentage of schistosomula with glycocalyx shed depending on the transformation methods used, after 24 h incubation in SCM; mean ± SD; n = 5

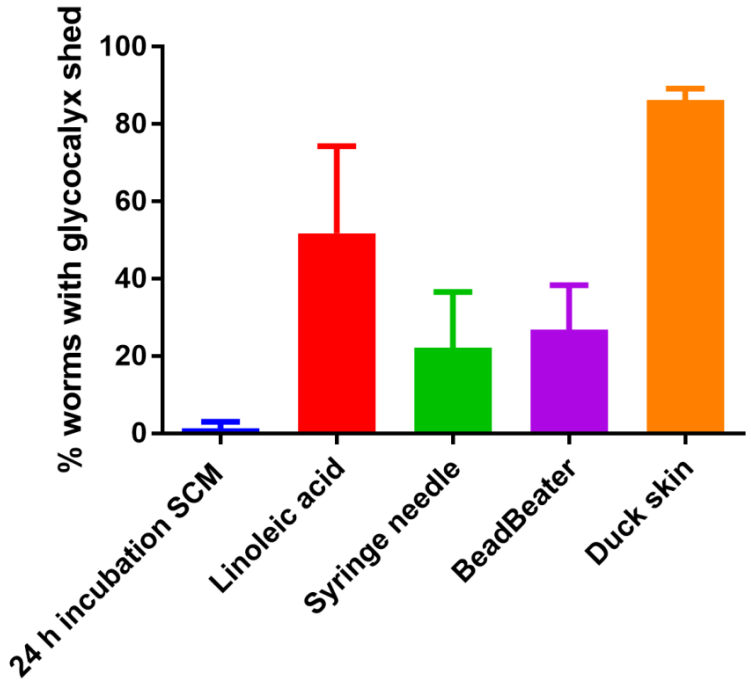


Figure 16 Viability of schistosomula depending on transformation methods, visualised in time; mean ± SD; n = 10; p < 0.05 = *; p < 0.01 = **; p < 0.001 = *

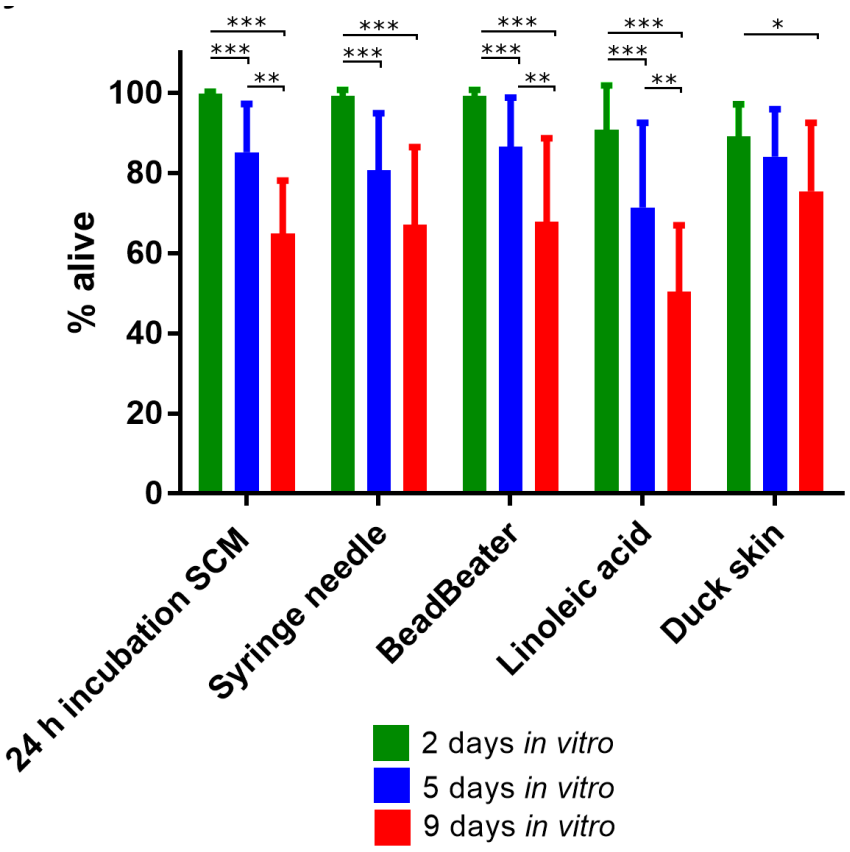


Figure 17 Viability of schistosomula depending on transformation methods, comparison of methods; mean ± SD; n = 10; p < 0.05 = *; p < 0.01 = **; p < 0.001 = ***

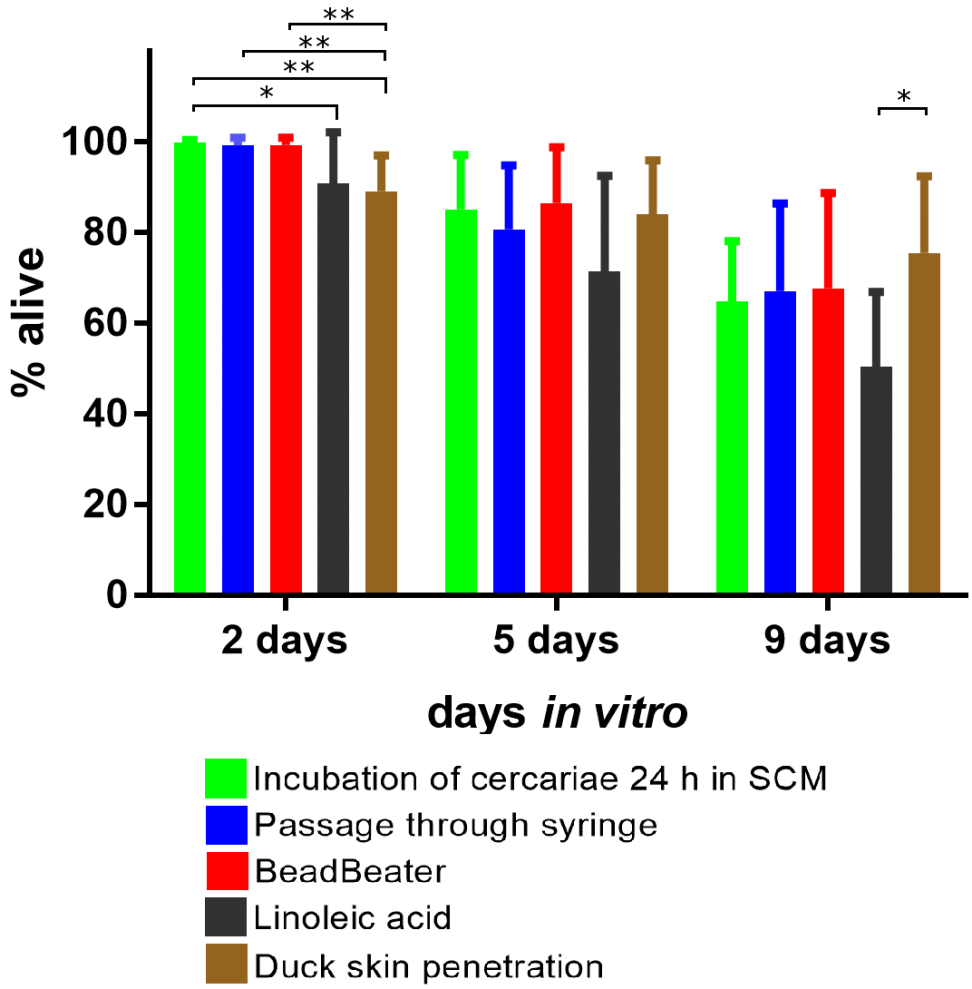


Table 12 Comparison of the c/s transformation methods – summary; mean % of CB gain, emptied penetration glands, glycocalyx shed and viability after 2, 5 and 9 days p. t.; grey = significantly best result

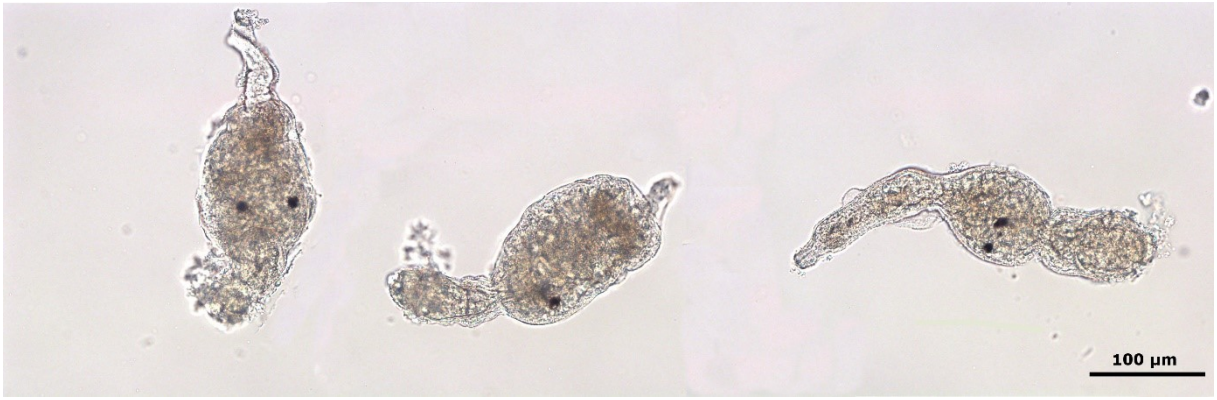
Transformation method	CB gain	Glands	Glycocalyx	Viability 2	Viability 5	Viability 9
24 h incubation SCM	4.83	4.60	1.29	99.81	85.18	64.85
Centrifuge	10.67	N/A	N/A	N/A	N/A	N/A
Vortex	6.60	N/A	N/A	N/A	N/A	N/A
Syringe needle	47.56	35.01	22.19	99.28	80.72	67.17
BeadBeater	51.58	35.98	26.88	99.32	86.54	67.77
Linoleic acid	58.44	50.65	51.79	90.84	71.47	50.48
Duck skin	11.45	96.22	86.28	90.30	84.08	75.51
Duck serum	N/A	N/A	N/A	N/A	N/A	N/A

5.1.1 24 h incubation of cercariae in SCM

The number of cercarial bodies gained by cultivation in SCM for 24 h is low (4.83%, **Table 12**) and the ratio of worms with acetabular glands emptied and glycocalyx shed is significantly lower than that of worms produced by other methods (**Figure 14 and Figure 15**). Although the viability of stages produced by the incubation in SCM is comparable with that of stages produced by BeadBeater or syringe needle in all tested time points (**Table 12, Figure 17**), high number of worms was obviously malformed and damaged after 9 days in *in vitro* conditions even though they still passed the viability test (**Figure 18**).

It should be noted that after incubation of cercariae for 4 days in SCM, the number of tailless organisms became similar to BeadBeater-schistosomula, needle-schistosomula and LA-schistosomula (about 38.83%). Most of the worms had their penetration glands emptied (88.20%) and glycocalyx shed (52.30%). Data concerning cercariae incubated for 4 days remained statistically unprocessed as all the transformation methods were evaluated in the same time frame of 24 hours.

Figure 18 *T. regenti* cercariae incubated in SCM for 9 days, fixed in 4% PFA; visible body deformations



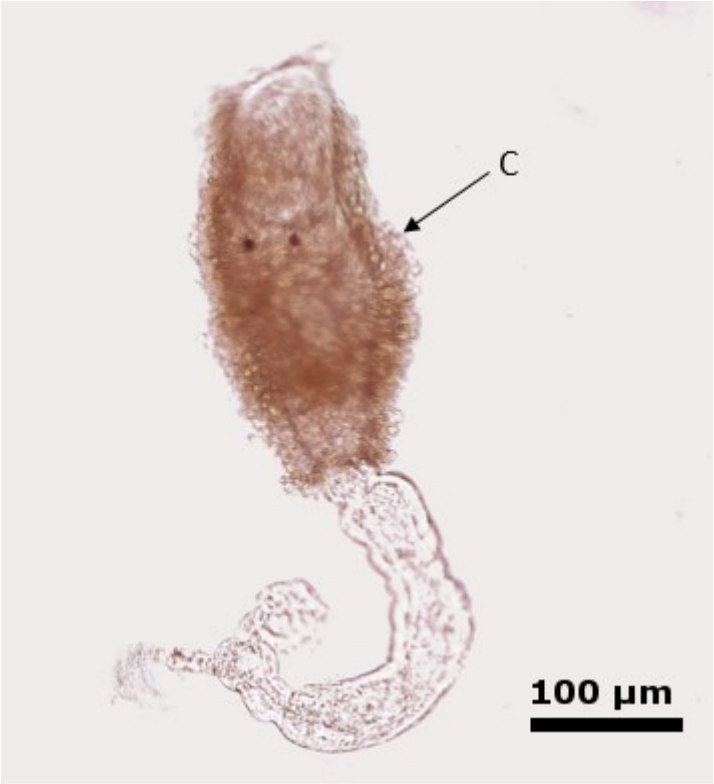
5.1.2 Incubation of cercariae in SCM with linoleic acid

After incubation of cercariae with 100 µg/ml linoleic acid in water for one hour, similar number of CB (58.44%) with similar ratio of acetabular glands emptied and glycocalyx shed can be gained as is for mechanically by BeadBeater or syringe needle passage schistosomula (**Table 12, Figure 13, Figure 14, Figure 15).** Significant decrease in viability was noted compared to cercariae incubated in SCM after 2 days p. t. After 9 days p. t., viability decreases in comparison to duck skin schistosomula. The schistosomula were visibly damaged from day 5 p. t. on, even though the organisms passed the viability assay. On day 9 p. t. the schistosomula looked similar to the cercariae incubated in SCM for 9 days.

5.1.3 Incubation of cercariae in SCM with 50 % duck serum

After 1 h incubation of cercariae with 50% heat-inactivated duck serum, only small amount of CB (about 6%) can be harvested. Cercariae react with unknown factor from serum and form a gel-like "coat" around their body (**Figure 19**) which remains intact even after 24 h incubation in 37°C. The "coats" tend to clog together and it is impossible to count the CB precisely, therefore the data couldn't be included in the statistical analysis and this method was not further tested.

Figure 19 *T. regenti* cercaria incubated 1 h with 50% heat-inactivated duck serum, with formed "coat" on the surface (C)



5.1.4 Mechanical transformation – vortex

Only small number of cercariae lost tails during vortexing, similarly as during incubation of cercariae in SCM for 24 h (**Table 12, Figure 13**). For this reason, this method was omitted from further testing.

5.1.5 Mechanical transformation – centrifuge

Only small number of cercariae lost tails during centrifuging, similarly as during incubation of cercariae in SCM for 24 h (**Table 12, Figure 13**). For this reason, this method was omitted from further testing.

5.1.6 Mechanical transformation – syringe needle

Ratio of tailless organisms obtained by repeated passage through the syringe needle is comparable with linoleic acid- or BeadBeater-produced schistosomula (**Table 12, Figure 13**), however the percentages of schistosomula with acetabular glands depleted and glycocalyx shed are not significantly different from that of incubated cercariae (**Figure 14, Figure 15**). The worms are similarly viable after 2, 5 and 9 days as BeadBeater-schistosomula and the cercariae incubated in SCM (**Figure 17**). Less schistosomula appear damaged than after linoleic acid incubation or incubation of cercariae in SCM at day 9.

5.1.7 Mechanical transformation – BeadBeater

Results of all the evaluations are highly similar to the ones for needle-transformed schistosomula (**Table 12, Figure 13, Figure 14, Figure 15, Figure 17**) and even appearance of schistosomula at day 9 is the same for the needle-schistosomula as for BeadBeater-schistosomula (**Figure 20**).

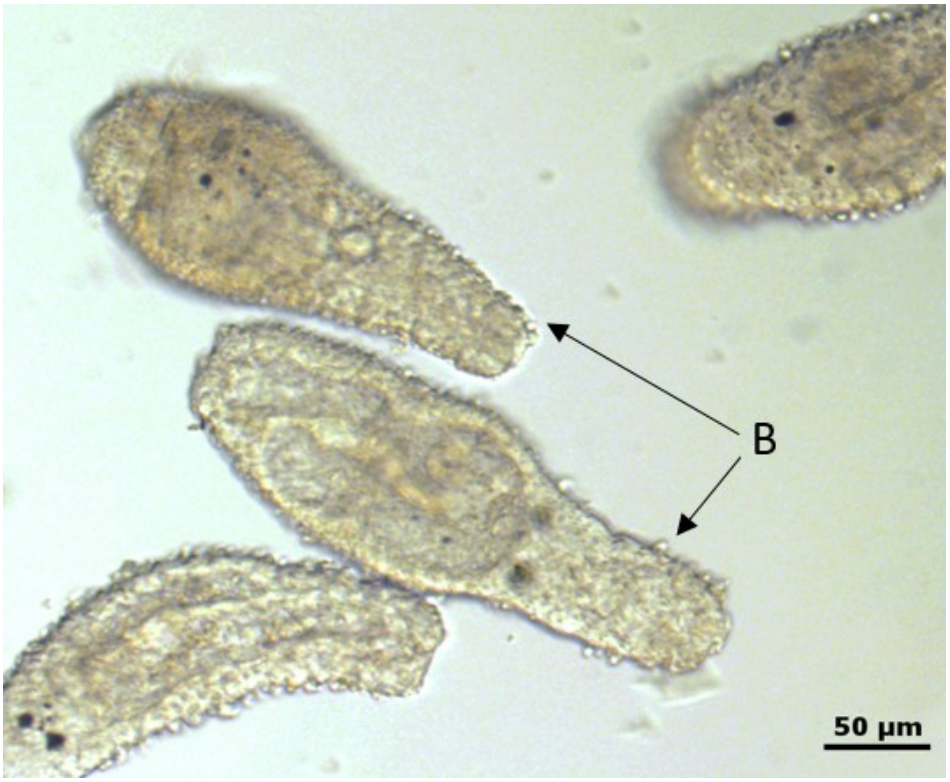
Figure 20 *T. regenti* BeadBeater-schistosomula, 9 days p. t., fixed in 4% PFA

5.1.8 Transformation through the duck skin

The gain of CB by the penetration of cercariae through the isolated duck skin is low, similarly to the percentage of CB gained by centrifuging or vortexing (**Table 12, Figure 13**). However, most of the skin-transformed worms released the content of their acetabular glands and shed the cercarial glycocalyx (**Figure 14, Figure 15**). After initial drop in viability on day 2, the percentage of viable schistosomula is comparable to the other transformation methods in day 5 and 9 (**Figure 17**). On day

5 p. t. only minimum of duck-skin transformed schistosomula showed body deformations unlike to the rest of the methods tested (BeadBeater, syringe needle, linoleic acid stimulation and cercariae incubated in SCM), shown at **Figure 21**.

Figure 21 Duck skin schistosomula, 9 days p. t.; live photo from the culture dish; B – bubbles on surface



5.2 Long-term cultivations

Viability of schistosomula in 4 cultivation media was tested long-term (up to 21 days) and the best media supporting survival were used for subsequent cultivations where growth and gut development was tested.

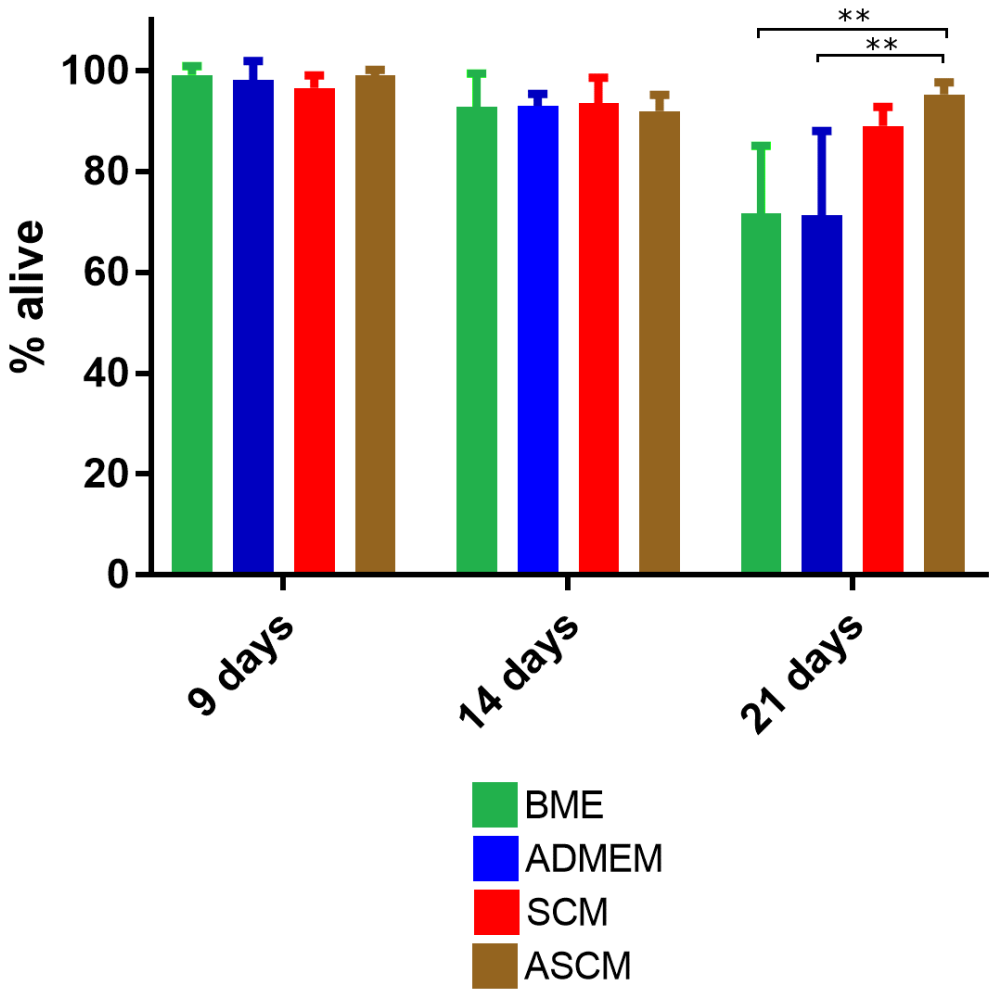
5.2.1 Medium for long-term cultivation – viability assay

Viability of BeadBeater-schistosomula was tested after 9, 14 and 21 days of cultivation in media BME, DMEM, SCM and ASCM. Complete data for the long-term viability of schistosomula with p-values from the statistical analysis are available in the Supplement file.

During 14 days of *in vitro* cultivation, no differences in schistosomula viability were noted among media. However, after 21 days of cultivation significantly higher viability was noted in complete medium ASCM as opposite to incomplete basic media BME and ADMEM (**Figure 22**).

Viability in complete medium SCM was also higher than that incomplete media, but remained without statistical confirmation.

Figure 22 Long-term viability of schistosomula in 4 different cultivation media; mean ± SD; n = 4; p < 0.01 = **



5.2.2 Cultivation supplements

First, the preliminary 14-day incubation of BeadBeater-schistosomula in SCM, SCM with 10% heat-inactivated fetal bovine serum (FBS) or 10% heat-inactivated duck serum was tested. Schistosomula didn't survive 14 days of cultivation with FBS, unlike when cultivated with duck serum or in serum-free medium (data not shown). Therefore, only duck serum was used in further experiments.

BeadBeater-schistosomula were tested in the main experiment, but the data is not complete due to the time requirements of the experiment. The body length of BeadBeater-schistosomula is summarized in **Table 13** and when possible, gut development state and state of the eye spots was evaluated. Duck skin schistosomula were tested because their appearance after 9 days *in vitro* was

superior to other methods used as most worms remained undeformed. Complete results were obtained only for duck skin schistosomula and only these results were statistically examined. Data of body growth measurements are available in the Supplement file.

Developmental stage of the gut and eye spots state: Addition of duck red blood cells (RBCs) and 10% heat-inactivated duck serum to the complete media SCM and ASCM was tested in comparison to serum free media and control cercariae. After 9, 14 and 21 days *in vitro*, the schistosomula were measured (**Table 13**) and gut development and state of eye spots was evaluated. At least one from the measured schistosomula in each tested group had the gut in the state of fused caeca continuing with single gut to the posterior end at day 9 p. t. when duck serum or RBC was added (**Figure 24**) or on day 14 when SCM or ASCM alone was used. Schistosomula with one dominant caecum not yet connected to the second one were often observed from day 9 p. t. (**Figure 23**). Gut was usually the most noticeable structure of the worm body as it often became somewhat dilated (**Figure 23**). At day 21 p. t., all the schistosomula cultivated with duck serum or RBCs had adult-like gut.

Figure 23 *T. regenti* duck skin schistosomulum 14 days p. t.; incubated in SCM + 10% duck serum; fixed in 4% PFA; DC – one gut caecum dominant; D – dilated gut

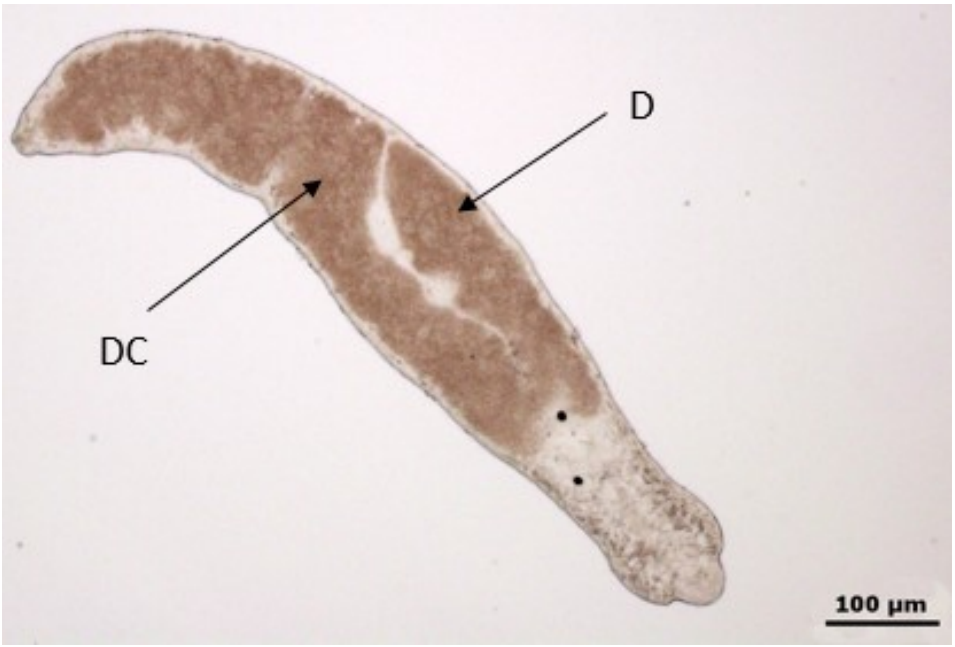
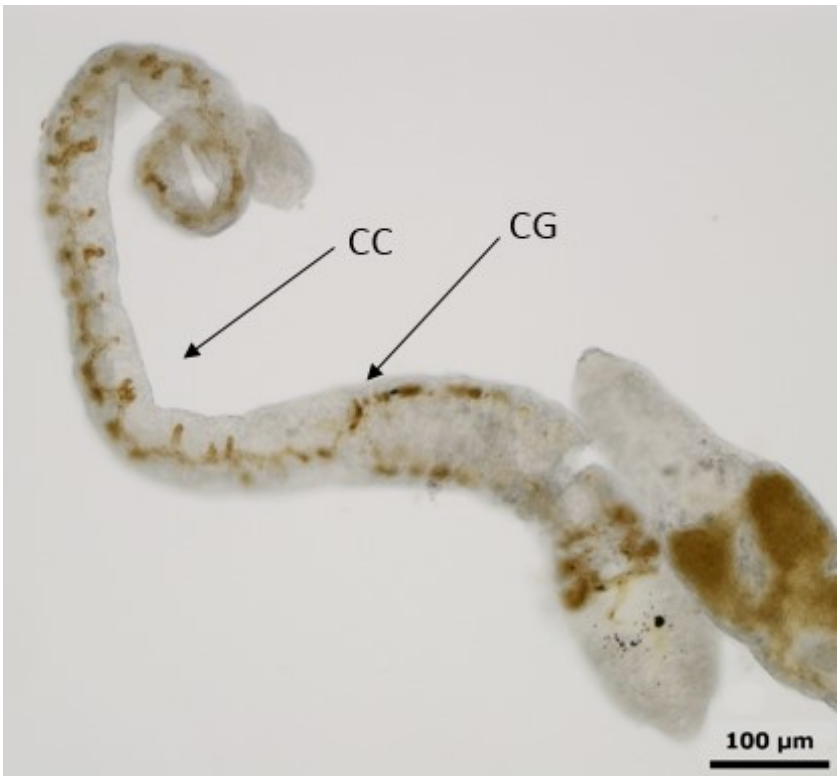


Figure 24 *T. regenti* duck skin schistosomulum 14 days p. t.; incubated in SCM + 10% duck serum + RBCs; fixed in 4% PFA; connected gut (CG) caeca with common caecum (CC) continuing to the posterior part of body



Cercarial eye spots scattered in many pigment spots (**Figure 25**) were observed in all the tested groups, independently on the body growth or gut development of the schistosomula. Some schistosomula still had eye spots intact even when growth occurred (**Figure 23**).

Figure 25 *T. regenti* duck skin schistosomulum 9 days p. t.; incubated in ASCM; fixed in 4% PFA; E – scattered eye spots

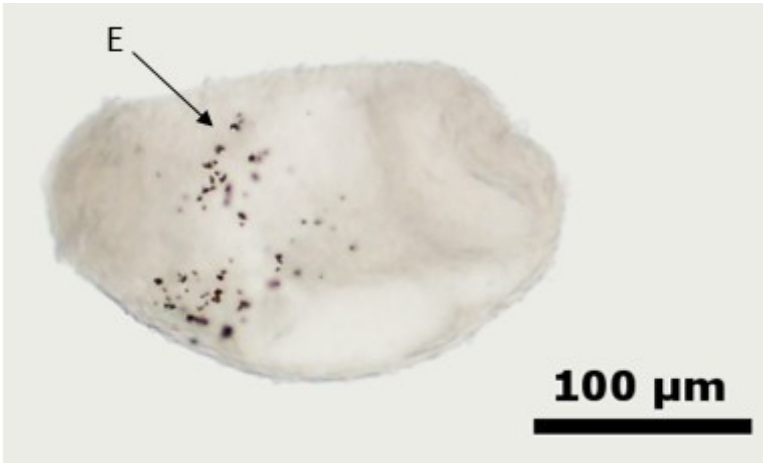


Table 13 Length of *T. regenti* schistosomula (µm) cultivated 9, 14 and 21 days p. t with different media supplements; mean ± SD; n = 15 (mean ± SD)

C/s transformation method →	BeadBeater			Duck skin		
Medium supplement ↓	9 days	14 days	21 days	9 days	14 days	21 days
SCM	**228.34** ± 35.75	×	×	**198.25** ± 41.66	**286.00** ± 87.99	**297.97** ± 63.79
SCM + RBCs	**269.68** ± 23.57	×	×	**313.73** ± 68.01	**369.98** ± 87.40	**480.10** ± 194.21
SCM + duck serum	**395.66** ± 93.26	**454.51** ± 191.02	**592.26** ± 266.09	**562.36** ± 155.05	**784.79** ± 418.70	**837.98** ± 420.97
SCM + RBC + duck serum	**377.18** ± 68.82	×	×	**497.85** ± 212.92	**913.75** ± 386.28	**1234.59** ± 589.95
ASCM	**279.89** ± 40.46	×	×	**245.54** ± 41.21	**278.09** ± 78.56	**272.56** ± 55.79
ASCM + RBCs	**305.89** ± 20.13	×	×	**291.99** ± 64.89	**340.75** ± 84.93	**462.99** ± 191.34
ASCM + duck serum	**300.82** ± 55.80	**425.76** ± 120.14	**739.43** ± 408.29	**423.25** ± 106.56	**516.09** ± 151.31	**1134.57** ± 477.79
ASCM + RBCs + duck serum	**343.93** ± 71.55	×	×	**503.65** ± 149.57	**469.43** ± 152.57	**1422.79** ± 438.33
***T. regenti* cercariae**	**342.65** ± 38.49					

Addition of RBCs

After addition of RBCs to the complete culture medium, a few worms reached around 900 µm on day 21 p. t.; however, any significant growth wasn't proved statistically compared to fixed cercariae that served as control. When compared to the medium without RBCs, after 21 days p. t. significant growth occurred in presence of RBCs (**Table 13**). Ingestion of RBCs was observed on day 5 p. t. Schistosomula cultivated with RBCs attached these cells to their surface from day 2 p. t. but when placed under the cover slip to document this, the RBCs were loosened.

Addition of serum

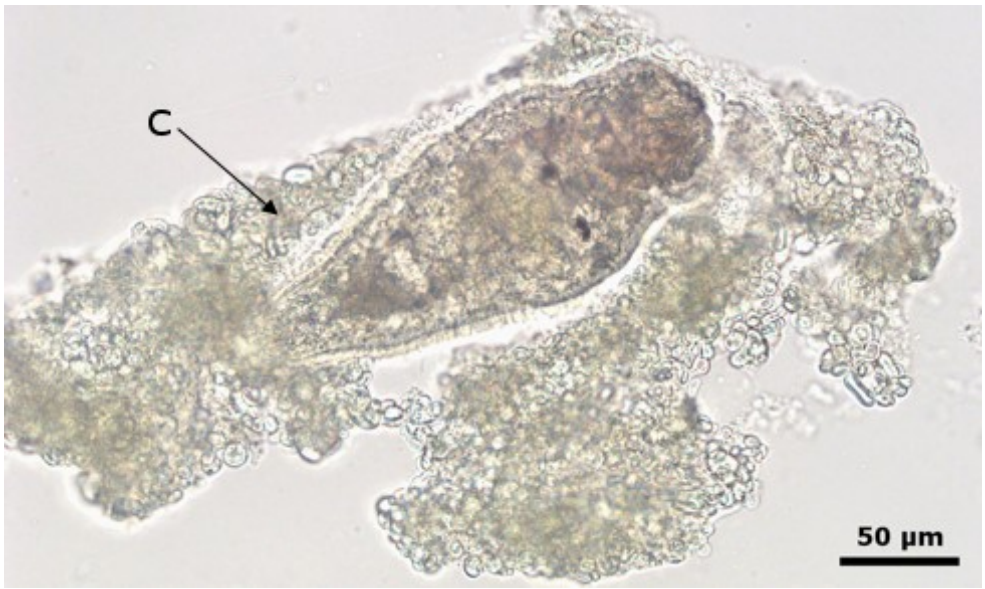
Heat-inactivated duck serum stimulated growth of schistosomula in comparison to control cercariae and to worms cultivated in serum free medium in all time points (**Table 13**). Gel-like "coat" surrounding the schistosomula was observed after 24 h of cultivation in medium with duck serum (**Figure 26**), starting to form at the both anterior and posterior end of the worm, continuing to the

middle of the body. After 5 to 7 days, majority of worms leave the “coat” and continue living freely in medium. No gel-like structure was observed when using FBS during the preliminary experiment.

Even if no RBCs were added, light brown pigment was observed in the intestine of schistosomula cultivated with duck serum starting from day 9 p. t. (**Figure 23)**.

Addition of duck serum to the SCM prolonged the ability of schistosomula to survive *in vitro* for extended time of at least 71 days for duck-skin schistosomula and at least 52 days for BeadBeater-schistosomula. The schistosomula may have lived longer but both cultures had to be terminated due to contamination. In contrast to this finding, most duck-skin schistosomula die after day 28 days p. t. when cultivated in serum-free SCM and BeadBeater-schistosomula die on day 25 p. t.

Figure 26 “coat” (C) formed around *T. regenti* duck skin schistosomulum; 5 days p. t., incubated in SCM + 10% duck serum; specimen pressured under a cover slip



After 58 days p. t. in SCM with 10 % duck serum, duck-skin schistosomula were observed by differential interference contrast (DIC) microscopy to evaluate state of reproductive system. In place where adult *T. regenti* have developed reproductive system (**Figure 32** and **Figure 33**), tissue different from the rest of somatic cells was observed at *in vitro* cultivated schistosomula (**Figure 27, Figure 28, Figure 29**). The end of the body of adult *T. regenti* is filled with *testes* (**Figure 31**) or *vitellaria*. However, 58 days old *T. regenti* schistosomula cultivated with the duck serum have the body end filled with caecum (**Figure 30**).

Figure 27 Anterior of *T. regenti* schistosomulum, 58 days p. t., incubated in SCM with duck serum; DIC; inset A: magnification of the part between acetabulum and caeca; AC – acetabulum, C – caeca, PR – potential primordia of reproductive system

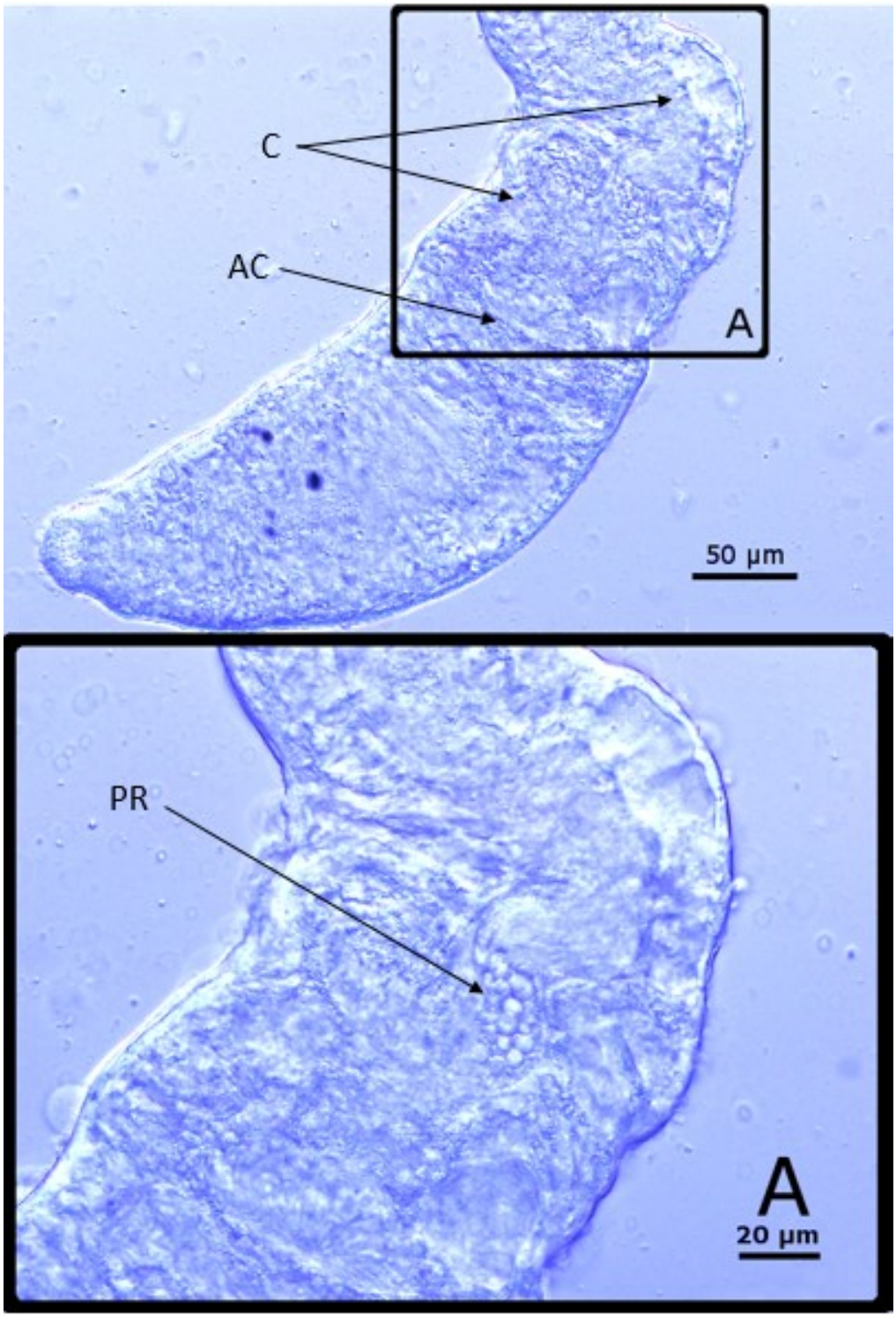


Figure 28 *T. regenti* schistosomulum, 58 days p. t., incubated in SCM with duck serum; DIC; inset A: magnification of the part between acetabulum and caeca (Figure 29); inset C: magnification of the body end (Figure 30)

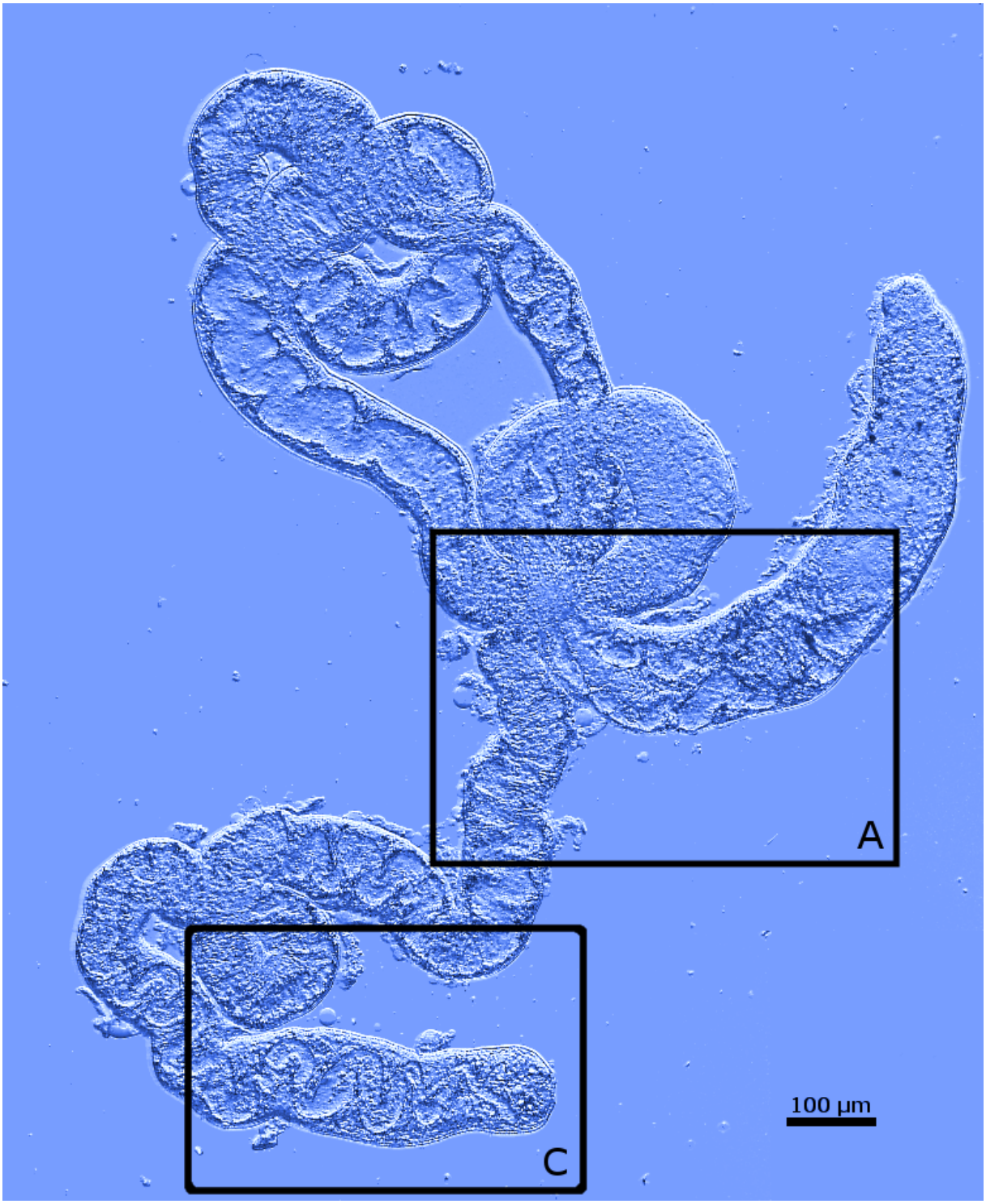


Figure 29 *T. regenti* schistosomulum, 58 days p. t., incubated in SCM with duck serum; DIC; Inset B: magnification of the part between caeca posterior to acetabulum; AC – acetabulum, CA – caeca, PR – potential primordia of reproductive system

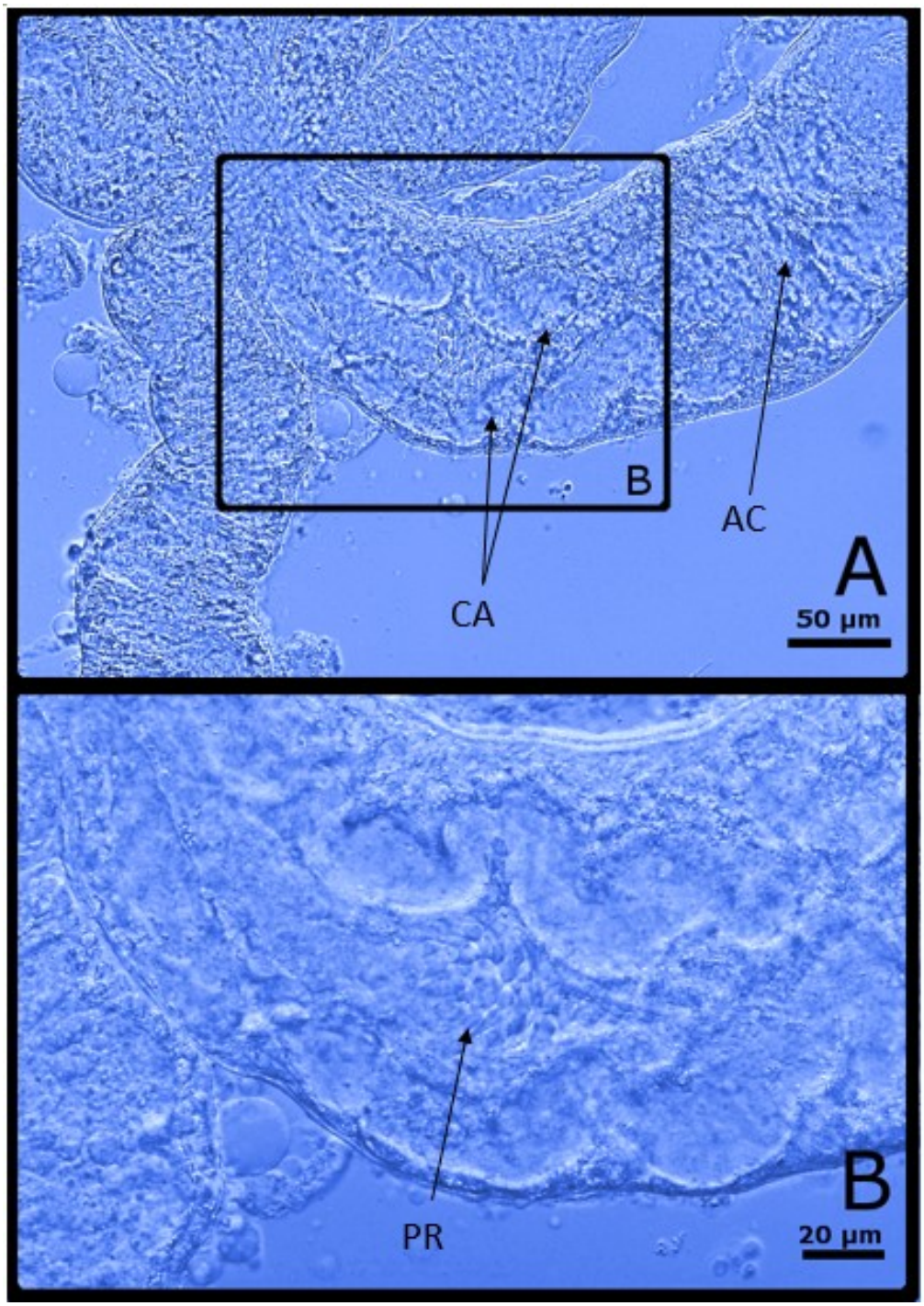


Figure 30 Posterior of *T. regenti* schistosomulum, 58 days p. t., incubated in SCM with duck serum; DIC; CA – caecum

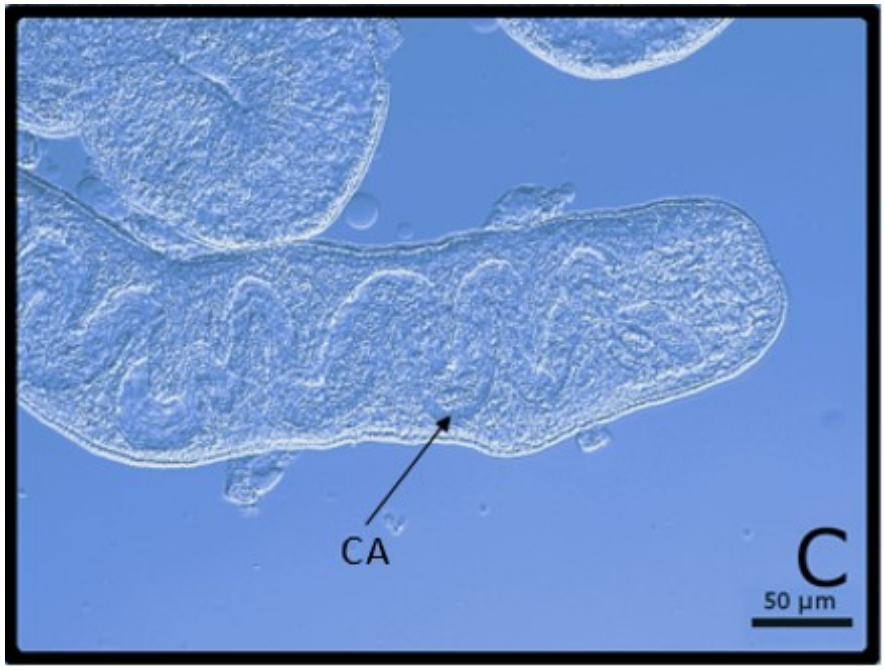


Figure 31 Posterior of *T. regenti* adult male, 25 days p. i.; DIC; T – *testes*

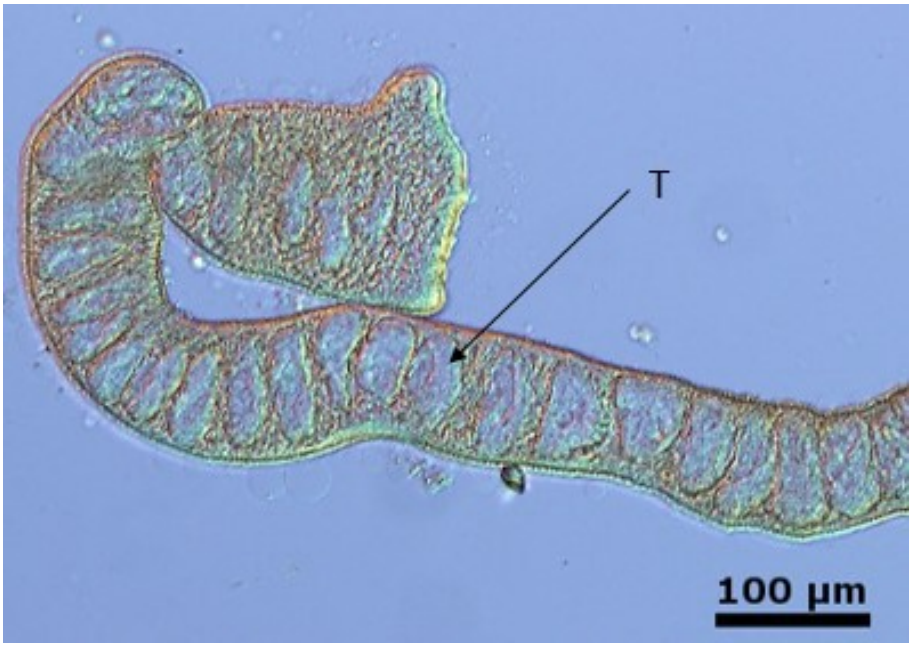


Figure 32 Anterior of *T. regenti* adult male, 25 days p. i.; DIC; AC – acetabulum; CA – caeca; - VS – *vesiculum seminalis*; CG – *canalis gynaecophorus*

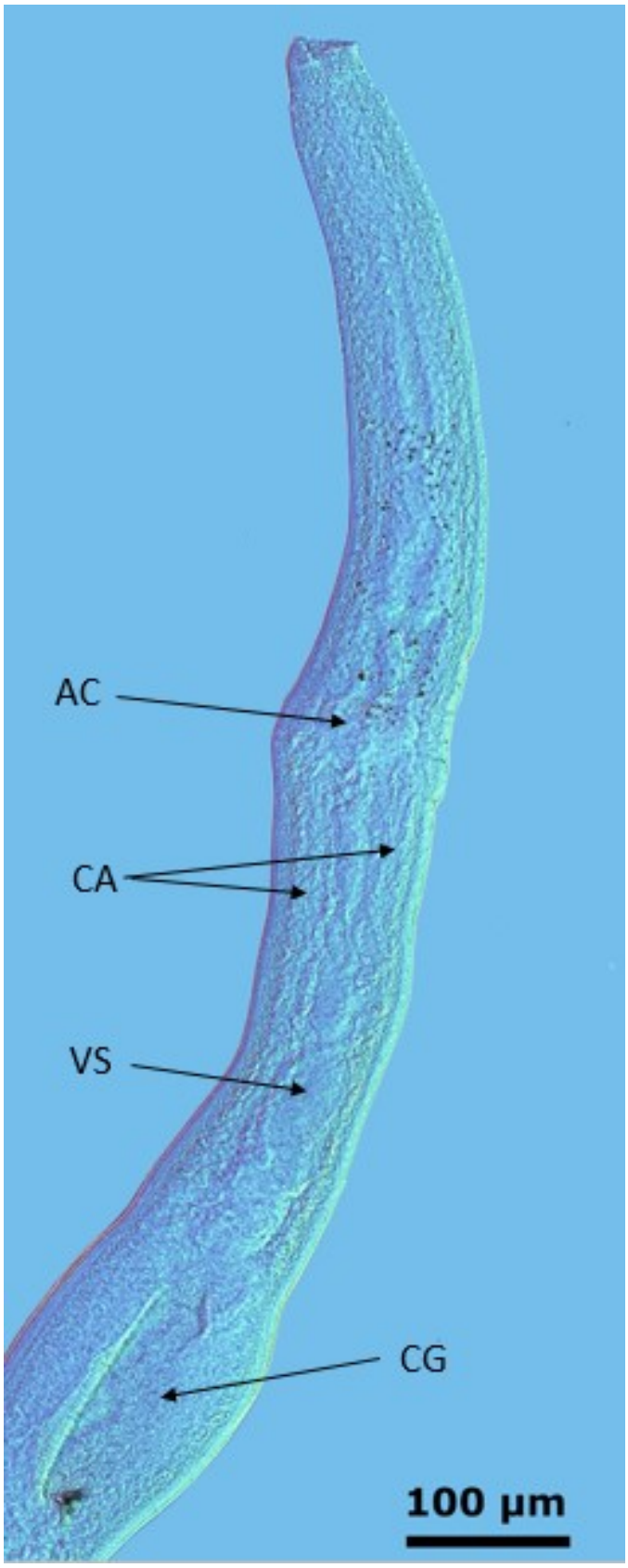
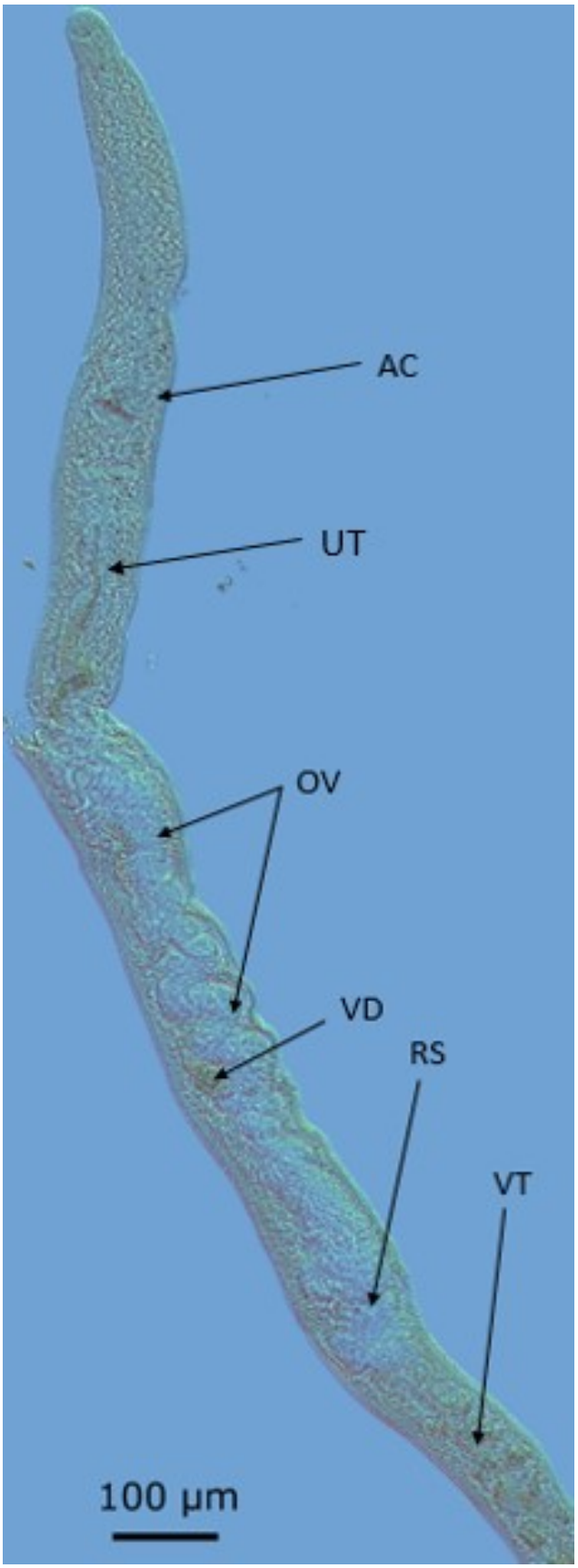


Figure 33 Anterior of *T. regenti* adult female; 25 days p. i.; DIC; AC – acetabulum; UT – *uterus*; OV – *ovaria;* VD – *vitelloduct*; RS – *receptaculum seminis*; VT - *vitellaria*



SCM vs ASCM

The medium ASCM supports viability and growth of schistosomula with similar effect as SCM (**Table 13**). When no medium supplement was added, the length of schistosomula measured was even shorter than that of control cercariae in all time points.

5.3 Other potential markers of completed transformation into schistosomulum

Following schistosomulum characteristics were tested as markers of development of *in vitro* cultivated schistosomula – oral sucker development, presence of glucose transporters GTP1 and 4 in the tegument membrane, documenting tegument development and elevated expression of hemoglobinase, hexosaminidase and axon guidance protein in schistosomulum.

5.3.1 Development of oral sucker – musculature labeling

Musculature of syringe-needle transformed schistosomula cultivated in SCM for 3 days was labeled by FITC-phalloidin. About 14 worms from a total of 40 labeled were evaluable under the fluorescent microscope as it was difficult to focus on the muscular fibers properly (**Figure 34, Figure 35, Figure 36**). Confocal microscopy would be a solution but a quick method with potential to evaluate a high number of samples in a short time was needed. For that reason, development of oral sucker wasn't used in the main experiment to compare methods of c/s transformation. One syringe needle schistosomulum developed the oral sucker almost completely 3 days p. t. (**Figure 36**).

Figure 34 *T. regenti* cercaria with FITC-phalloidin labeled musculature; MC – muscular cone of head organ

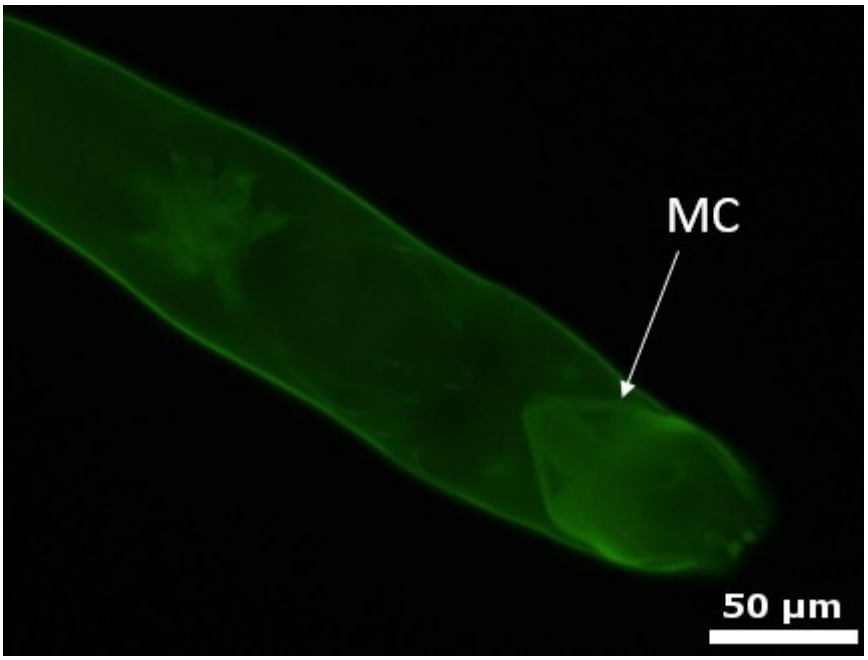


Figure 35 *T. regenti* syringe needle schistosomulum with FITC-phalloidin labeled musculature; 3 days p. t., incubated in SCM; MC – muscular cone of head organ

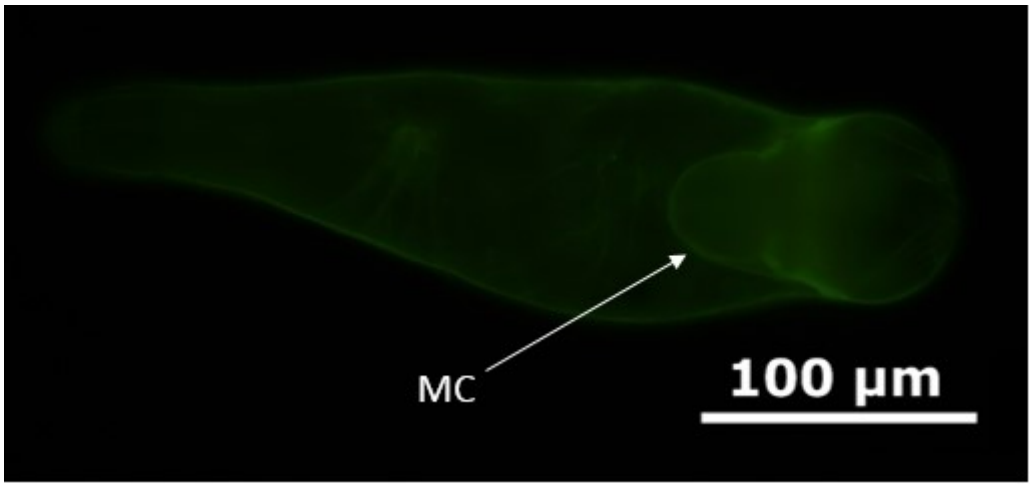
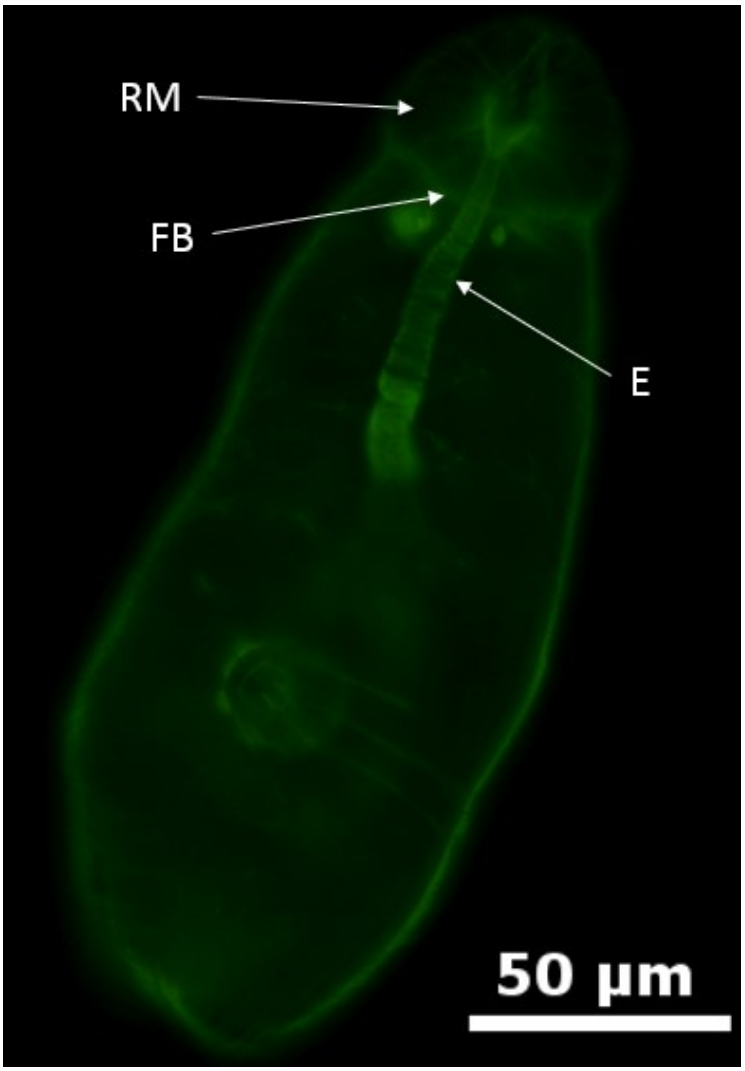


Figure 36 *T. regenti* syringe needle schistosomulum with FITC-phalloidin labeled musculature; 3 days p. t., incubated in SCM; flattened base of the oral sucker (FB), radical muscle fibers in the oral sucker (RM), esophagus (E)



5.3.2 Development of tegument – glucose transporters GTP1 and 4 labeling

Anti-SGTP1 and 4 (*S. mansoni* glucose transporter 1 and 4) antibodies were tested on *T. regenti* cercariae and schistosomula, with *S. mansoni* cercariae and cercariae incubated in SCM for 24 h as a control. Whole mount IHC, ELISA and western blot methods were used, as described below.

The sequence coding glucose transporters of *S. mansoni* showed 79% identity with a fragment of *T. regenti* genome which belongs to yet nonannotated gene (GenBank ID LL000240.1).

A corresponding sequence was also found in *T. regenti* transcriptome; however, it was impossible to distinguish to which GTP of *S. mansoni* the transcript corresponds to.

It should be noted that, unlike *T. regenti* cercariae, all the *S. mansoni* cercariae lost their tails during 24 h incubation in SCM.

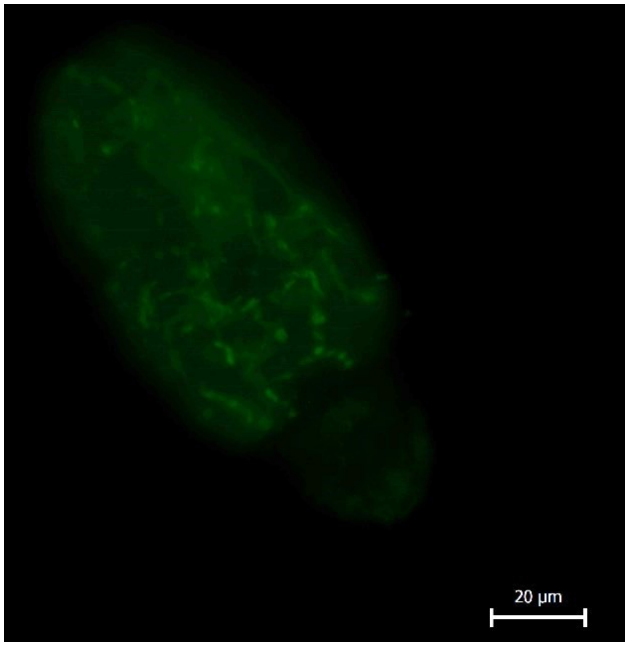
Whole mount IHC

T. regenti cercariae, cercariae incubated in SCM for 24 h, duck-skin-schistosomula with subsequent 24 h incubation, duck-skin-schistosomula incubated in SCM 5 days were incubated with both antibodies. None of the tested samples bound the antibodies, except for anti-SGTP 4 labeled *S. mansoni* cercariae incubated in SCM that served as positive control (**Figure 37**). Anti-SGTP 1 antibody bound neither samples nor positive control (**Table 14**).

Table 14 Whole mount IHC – Binding of anti-SGTP 1 and anti-SGTP 4 antibodies

Binding of antibodies anti-SGTP 1 and anti-SGTP 4		
Tested sample	**Anti-SGTP 1**	**Anti-SGTP4**
***T. regenti* cercariae**	×	×
***T. regenti* cercariae incubated 24 h in SCM**	×	×
***T. regenti* duck skin-schistosomula, 24 in SCM**	×	×
***T. regenti* duck skin-schistosomula, 5 days in SCM**	×	×
***S. mansoni* cercariae – negative control**	×	×
***S. mansoni* cercariae incubated 24 h in SCM – positive control**	×	✓

Figure 37 *S. mansoni* cercaria incubated 24 h in SCM, labeled by anti-SGTP4 antibody



ELISA

T. regenti cercariae, cercariae incubated in SCM for 24 h and BeadBeater-schistosomula incubated for 24 h in SCM, with *S. mansoni* cercariae and cercariae incubated in SCM for 24 h as negative and positive control were used, respectively. However, the results were unconvincing as the measured optical density values very similar for negative controls and positive control. Also, the optical density values were low (between 0.007 and 0.117), which means poor or low signal, due to insufficient color development in 96-well plate.

Western blot

T. regenti cercariae and cercariae incubated in SCM for 24 h were tested, with *S. mansoni* cercariae and cercariae incubated in SCM for 24 h as negative and positive control, respectively. Tested homogenates were successfully separated by SDS electrophoresis as confirmed by Coomassie staining and then transferred to the PVDF-membrane which was confirmed by Coomassie staining of the gel. However, no band appeared on the membrane after the color reaction was induced.

5.3.3 Gene expression of schistosomula

Selection of genes for the comparison of expression; selection of reference genes

Based on the transcriptomic data provided by Mgr. Roman Leontovyč, genes in **Table 15** were selected for the comparison of expression among *T. regenti* cercariae and *in vitro* cultivated schistosomula and as reference genes.

Table 15 Selected genes for the comparison of expression among *T. regenti* cercariae and *in vitro* cultivated schistosomula and reference genes

Selected gene product	Sequence ID
Axon guidance protein [*Schistosoma mansoni*]	Treg_008144
Hemoglobinase (C13 family) [*Schistosoma mansoni*]	Treg_015036
Beta-hexosaminidase B [*Schistosoma mansoni*]	Treg_015036
Reference gene – GAPDH – Glyceraldehyde 3-phosphate dehydrogenase	Treg_000122
Reference gene – HNRA2 – heterogeneous nuclear ribonucleoprotein A2 homolog 1 [*Schistosoma japonicum*]	Treg_004035
Reference gene – ITLR – Isoleucine--tRNA ligase [*Schistosoma mansoni*]	Treg_015058

RNA isolation

Amount of RNA isolated from all the stages was low as shown in **Table 16**.

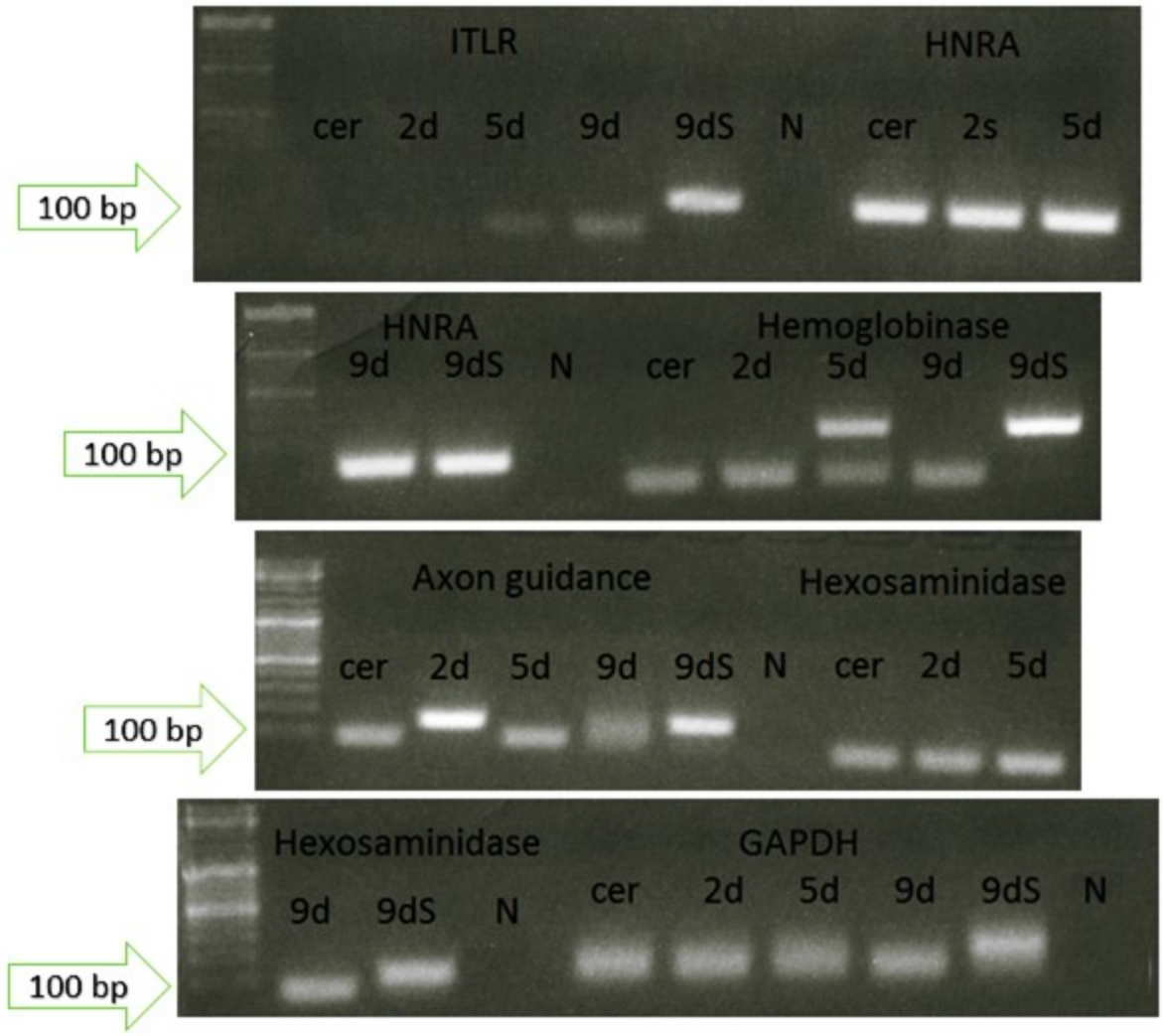
Table 16 Concentration of isolated RNA

T. regenti stage	RNA concentration (ng/µl)
Cercariae	5.4
BeadBeater-schistosomulum, 2 days p. t., SCM	7.3
BeadBeater-schistosomulum, 5 days p. t., SCM	5.0
BeadBeater-schistosomulum, 9 days p. t., SCM	4.8
BeadBeater-schistosomulum, 9 days p. t., SCM + 10% duck serum	10.3

PCR detection of tested genes in cDNA of *T. regenti* stages

Results of the PCR detection of the tested genes and reference genes in cDNA of *T. regenti* cercariae and schistosomula cultivated for different times *in vitro* are shown in **Figure 38**. A product of size comparable to the size of desired amplicon was shown by electrophoresis for HNRA, for cDNA produced by all tested stages. For the other primers, product was shown mostly for cDNA from schistosomula 9 days p. t. incubated with duck serum (ITLR, Hemoglobinase, Axon guidance protein, GAPDH). The amplicon sizes are summarized in **Table 9**. Sometimes, bands much lower than the one of product were shown, supposedly the primers. But, the products were not verified by sequencing.

Figure 38 Test of primer specificity; cer – cercariae; 2d – schistosomula incubated 2 days; 5 d – schistosomula incubated 5 days; 9d – schistosomula incubated 9 days; 9dS – schistosomula incubated 9 days with duck serum; N – negative control



qPCR

The qPCR reaction was performed but the results were inconclusive, beause the product sequencing was not accomplished and the C_t (threshold cycle) values mostly didn't differ from the negative control. The C_t value represents relative measure of concentrations of the PCR product in the reaction.

The only difference from the negative control value was observed when applying the TrHem or TrGAPDHref primer at cDNA of 9 days old schistosomula incubated with duck serum (**Table 17**).

Table 17 Results of qPCR. Only samples with C_t value different from negative control (N) were observed

cDNA + primer	C_t value	C_t value N
9dS + TrHem	25.8	30.9
9dS + TrGAPDHref	24.11	31.7

6 DISCUSSION

Production of schistosomula stages *in vitro* could be very useful prior to experiments focused on worm biology and development, reaction of schistosomula to potential anthelmintics and immunology studies.

Over the years, the problematics of *in vitro* production of *S. mansoni* schistosomula and observation of their *in vitro* development has been addressed by many researchers. In contrast to this, only little is known about c/s transformations of *T. regenti* and no long-term cultivation experiments of this bird schistosome were conducted. *T. regenti* can be used as a good model organism for studying interactions of host nervous system immunity and a helminth. Therefore, it is reasonable to focus on this parasite in further studies.

Current state of knowledge about *in vitro* incubations of *T. regenti* schistosomula could be summarized as follows. The schistosomula can be harvested *in vitro* using repeated passage through the syringe needle with subsequent incubation in SCM. These early stages of schistosomula are comparable to the schistosomula transformed *in vivo* in terms of tegument development, glycocalyx shedding, uptake of food and size and survive maximum of 11 days in SCM (Chanová et al., 2009). The results of this thesis significantly extend this kind of knowledge.

6.1 Cercariae/schistosomula transformation

The loss of cercarial tail occurs *in vivo* during the process of definitive host skin penetration and is probably synchronized with emptying of penetration glands (Hrádková and Horák, 2002; Mikeš et al., 2005). *In vivo* transformed schistosomula also shed the cercarial glycocalyx, probably as a consequence of penetration glands emptying (Řimnáčová et al., 2017). For these reasons, shedding of glycocalyx and emptying of acetabular glands was evaluated after applying of different c/s transformation methods.

6.1.1 Loss of cercarial tail

The removal of cercarial tail was reported to initiate transformation of *S. mansoni* (Clegg, 1965; Yasuraoka et al., 1978), therefore impact of cercarial tail loss on early schistosomula characteristics was evaluated in this study. When no tail breakage was initiated by *T. regenti* c/s transformation in my experiments (cercariae were directly placed into SCM), vast majority of worms (98.71%) remained with glycocalyx unshed and penetration glands content was present (95.45%) 24 h p. t. However, when cercarial tails were removed by mechanical c/s transformation using syringe needle or BeadBeater, 22.19% or 26.88% (respectively) of worms incubated 24 h p. t. shed

their glycocalyx. Only 35.01% or 35.98% of all syringe needle and BeadBeater-transformed schistosomula (respectively) emptied acetabular glands. Nevertheless, no statistical difference was confirmed between incubated cercariae and mechanically transformed schistosomula after 24 h incubation in SCM. This partly contradicts the results of Chanová et al. (2009) who characterized *T. regenti* syringe needle schistosomula as lacking glycocalyx after 24 of cultivation in SCM. But, number of evaluated schistosomula or a proportion of schistosomula with corresponding characteristics was not mentioned in the study (Chanová et al., 2009). On the other hand, 88.20% of the *T. regenti* cercariae incubated in SCM for 4 days emptied their acetabular glands and 52.30% shed their glycocalyx. These proportions are equivalent to schistosomula transformed by incubation with linoleic acid followed by 24 h incubation in SCM. To summarize influence of tail loss to the glycocalyx shedding and emptying of acetabular glands, the tail separation probably accelerates the transformation process of *T. regenti* cercariae although the details of the mechanism remain unclear.

CB production

The CB production was determined for each c/s transformation method to select the method enabling the production of the largest CB quantity. The best methods in terms of *T. regenti* CB numbers are incubation with linoleic acid (58.44%), mechanical transformation by passage through the syringe needle (47.56%) and usage of BeadBeater (51.58%). The rest of the tested methods – penetration of duck skin (11.45% CB), centrifuging (10.67 % CB) or vortexing (6.60% CB) and 24 h incubation of cercariae in SCM (4.60% CB) provide similarly low numbers of CB. Many of these c/s transformation techniques provide at least twice lower number of *T. regenti* CB compared to *S. mansoni*. This can be caused by the easier induction of tail loss on *S. mansoni* cercariae as they lose tail even after repeated passage through Pasteur pipette (Wang et al., 2006). Moreover, cultivation of *S. mansoni* cercariae in SCM for 24 h resulted in 100% tailless worms in my experiments. Another reason for obtaining a lower number of *T. regenti* CB could be the fact that immediately after losing the tail, *T. regenti* cercarial bodies attach to the surfaces (e.g., plastic syringe or Eppendorf tubes) by their acetabulum. The situation was not improved by the addition of 5% BSA into water prior to the transformation or by using lo-binding tubes. In addition, the syringe needle passage depends on the pressure applied by hand on the syringe plunger during *T. regenti* transformation, for this reason maximum pressure I could produce was applied during the experiments. CB number obtained after duck skin penetration (11.45%) is lower than the number of schistosomula retrieved after duck infection (22-95%; Blažová and Horák, 2005). Similar situation was observed in experiments with *S. mansoni* transformation trough the mouse abdominal skin where CB gain was 20-30% in contrast to 42-44% of worms retrieved after infecting mice (Brink et al., 1977; Stirewalt and Uy, 1969). But *in vitro* skin penetration can be further optimized in many ways, including changes in number of

cercariae incubated with the skin, volume of water containing cercariae or exclusion of light (Stirewalt and Uy, 1969).

6.1.2 Glycocalyx shedding and acetabular glands emptying

Early *T. regenti* schistosomula should have their surface free of cercarial glycocalyx and their acetabular glands should be emptied (Chanová et al., 2009; Řimnáčová et al., 2017). Therefore, these characteristics were tested to compare methods of c/s transformation.

Passage of *T. regenti* cercariae through the duck skin is the best method in terms of number of schistosomula with glycocalyx shed (86.28%) and penetration glands emptied (96.22%). This corresponds with glycocalyx and glands emptying of *S. mansoni* schistosomula transformed by the dried rat skin penetration in comparison to mechanical transformation, serum incubation and incubation of cercariae in cultivation medium (Stirewalt et al., 1983).

Incubation with linoleic acid results in 51.79% schistosomula without glycocalyx layer and 50.65% with acetabular glands emptied, but the linoleic acid seems to influence the worms survival as they have the lowest viability ratio from all the other tested methods. To achieve balance between CB gain and viability, linoleic acid was used in high concentration 100 µg/ml after preliminary experiments (data shown in Supplement file). Zídková (2003) described death of *T. regenti* cercariae after incubation with linoleic acid (100 µg/ml) in 40 min but in my experiments most of the CB were moving after 1 h incubation in the same conditions. In preliminary experiment, penetration glands emptying did not occur when linoleic acid was added to the cultivation medium instead of water. This corresponds to the results for *S. mansoni* cercariae (Michalick et al., 1979).

During evaluation of glycocalyx shedding of *T. regenti* schistosomula, worms with glycocalyx shed completely, with glycocalyx intact and those with glycocalyx partially shed were observed. This is in accordance with findings of Řimnáčová et al. (2017) who described the process of glycocalyx shedding in detail.

Penetration glands were stained by FDA instead of traditionally used alizarin and lithium carmine, because the method can be used on living worms and because no distinguishing between circumacetabular and postacetabular glands was needed for my experiments. FDA is used for viability assay of *S. mansoni* schistosomula (Peak et al., 2010) but the acetabular glands content activates the fluorescein from the FDA molecule more that the living body cells that results in stronger signal by acetabular glands.

6.1.3 Short-term viability of schistosomula

The viability of schistosomula transformed by different transformation methods was compared. Except for initial significant decrease of living duck skin schistosomula and linoleic acid

schistosomula on day 2 p. t., all the methods provide similarly viable schistosomula on day 5 p. t. On day 9 p. t., higher mortality was observed for schistosomula transformed by incubation with linoleic acid than when other methods were used. Viability of schistosomula produced by all the transformation methods decreased in time, but the duck skin-schistosomula underwent this process at slower pace. This study is the first to determine the proportion of *T. regenti* schistosomula-like organisms in dependence on the c/s transformation method. To conclude, short-term viability of schistosomula can be implemented on the early schistosomula to help with selection which c/s method to choose.

Viability of *T. regenti* cercariae incubated in SCM is similar for mechanically transformed schistosomula after 2, 5 and 9 days p. t. However, even though most of the incubated cercariae passed the viability assay 9 days p. t. (64.85%), the optical evaluation is also necessary as the incubated cercariae show more deformations than mechanically transformed schistosomula. This result is in contrast with that of Eveland and Morse (1975) who proved that viability of *S. mansoni* cercariae decreases significantly after 5-8 days of cultivation (20% viable), unlike schistosomula transformed by incubation with fresh rat serum (88% still viable). To conclude, removal of cercarial tail seems to be a necessary premise for completing the c/s transformation of *T. regenti*. Also, the viability assay of methylene blue and FDA should be accompanied by evaluation of visual appearance of the cultivated schistosomula.

6.2 Long-term cultivations

6.2.1 Medium for long-term cultivation – viability assay

First, four media were tested to find appropriate medium for long-term cultivations. The results suggest that complete medium (SCM or ASCM) is necessary for mechanical schistosomula survival in later stages of cultivation (between day 14 and 21 p. t.). Until day 14 p. t., no significant difference between viability of schistosomula in basic media (BME and ADMEM) and in complete media was observed. This is surprising, because Chanová et al. (2009) reported that *T. regenti* schistosomula did not survive in BME a single day and live maximum of 11 days in SCM. This could be due to the difference in a batch of *T. regenti* cercariae used in my experiments. Even during my experiments variability in outcoming results was observed: the proportion of living BeadBeater-produced schistosomula was 67.77% on day 9 p. t. in the experiment evaluating short-term viability. However, in the long-term cultivation experiment that was conducted a year later, 96.59% of the BeadBeater-schistosomula were alive in the SCM medium on day 9 p. t., even though the viability assay was performed and evaluated in the same way.

Another reason for different results in my experiments compared to experiments of Chanová et al. (2009) could be adoption of different methods used for viability assay. In this study, staining with FDA and methylene blue was used to decide if a worm is alive. No information about the method used for evaluation of worm viability was mentioned by Chanová et al. (2009). From my experience, evaluation of worm movement may not be a good viability marker as sometimes, for example after the medium change, schistosomula were motionless for a few hours but the next day they were moving vigorously.

6.2.2 Cultivation supplements

Addition of serum, RBCs or using advanced medium ASCM were tested to find cultivation supplements supporting growth and development of *T. regenti* schistosomula.

RBCs

Addition of duck RBCs causes significant growth of duck skin-schistosomula on day 21 p. t. in both SCM (480.10 ± 194.21 µm) and ASCM (462.99 ± 191.34 µm). The size of these corresponds to the size of duck skin schistosomula incubated with serum on day 9 p. t. However, no difference was noted in size of schistosomula cultivated with or without RBCs when duck serum was also present in the medium. To conclude, addition of RBCs stimulates growth of *T. regenti* schistosomula, but slower than duck serum. The studies focused on long-term cultivation of *S. mansoni* schistosomula used serum-rich media, because the presence of serum significantly enhances growth and gut development of worms (Clemens and Basch, 1989). The influence of RBCs on the growth of *S. mansoni* was not tested but RBCs are a necessary additive to reach complete reproduction system development of *S. mansoni* adults (Basch, 1984).

Ingestion of RBCs was first observed on day 5 p. t. regardless of the medium used or addition of serum, whereas Chanová et al. (2009) described feeding of mechanical schistosomula from day 3 p. t. and Blažová and Horák (2005) noted feeding of schistosomula *in vivo* also on day 3 p. t. From day 1 p. t., RBCs were observed to attach to the schistosomula surface. Similar phenomenon was observed on *S. mansoni* possibly as a part of molecular camouflage (Caulfield and Cianci, 1985).

Serum

Addition of 10% FBS from two different batches did not support growth or survival of *T. regenti* schistosomula in the preliminary experiment. Basch (1981a) described that FBS can support growth of *S. mansoni*, but it depends on the serum batch as some can even inhibit the

schistosomula growth. Therefore, another batch of FBS may be usable for *T. regenti* cultivation, but the use of FBS was omitted from further experiments.

When 10% duck serum was used, significant growth of *T. regenti* duck skin schistosomula was noted at all tested time points – day 9, 14 and 21 p. t. – in comparison with schistosomula incubated in serum-free medium. Duck skin schistosomula incubated in ASCM with duck serum for 21 days reached comparable size to the *in vivo* transformed schistosomulum 9 days p. i.: the *in vitro* produced schistosomula were 1134.57 ± 477.79 µm long on day 21 p. t. and the *in vivo* produced schistosomula reached 1300 ± 545 µm on day 9 p. i. (Hrádková and Horák, 2002). Even though duck skin schistosomula reached a longer mean length when cultivated in SCM with duck serum and RBCs (1234.59 ± 589.95 µm) or ASCM with duck serum and RBCs (1422.79 ± 438.33 µm, growth of *in vitro* transformed schistosomula is slower than in the definitive host after. Growth and development of *S. mansoni* schistosomula cultivated *in vitro* is also slowed down from ca day 15 p. t. (Clegg, 1965). In contrast to *S. mansoni* schistosomula, the slowing of growth and development happens sooner in cultivation of *T. regenti* but this bird schistosome has shorter life cycle than *S. mansoni* (Blažová and Horák, 2005; Clegg, 1959). BeadBeater-schistosomula seem to grow even slower than duck skin schistosomula. BeadBeater-schistosomula cultivation in SCM with duck serum reach mean length of 592.26 ± 266.09 µm on day 21 p. t., but the duck skin schistosomula cultivated in the same conditions reach mean length 562.36 ± 155.05 µm on day 9 p. t. To conclude, if schistosomula with development closer to the *in vivo* situation are needed, transformation by the duck skin penetration should be preferred method, even though the CB gain is low. If stages transformed at more defined conditions are needed, transformation using the BeadBeater method is appropriate.

Duck skin schistosomula had light-brown pigment in the intestine 9 days p. t. even when incubated without RBCs and can live at least 71 days without RBCs. Serum proteins combined with complete medium can probably serve as substitutional nutrient source.

Duck skin schistosomula incubated with duck serum for 58 days did not develop the reproductive system, only possible primordia were found. In contrast to this, *T. regenti* can lay eggs after 14 days p. i. in the duck host and destiny of the adult worms remains unknown after day 25 p. i. (Chanová and Horák, 2007). The life span of immature schistosomula kept *in vitro* can be for almost three times longer than that of the *in vivo* developed worms. This may be caused by the fact that energy was not invested into reproduction system of the *in vitro* cultivated schistosomula.

Both BeadBeater- and duck skin-schistosomula created “coat” from gel-like material when incubated with duck serum. This seems to be similar to the “coat” formed during CHR of *S. mansoni* cercariae in serum of immune host. The “coat” mostly contains glycoproteins (Kemp, 1970) as the CHR is a reaction of immunoglobulins with cercarial glycocalyx (Kemp et al., 1973). However, the “coat” formed even around *T. regenti* schistosomula produced by penetration of duck skin when

duck serum was added on day 2 p. t. At this time, most schistosomula should have shed their glycocalyx. Similar "coats" were observed to form around *in vivo* transformed *T. szidati* schistosomula cultivated with duck or chicken serum (Howell and Bourns, 1974). These coats were described as lipid particles based on Sudan IV staining (Howell and Bourns, 1974); however, recently an interaction of Sudan IV with serum albumin was discovered (Lu et al., 2011) and that could mean that at least a part of the "coat" is formed by serum proteins.

SCM vs ASCM

No difference was found in growth or gut development of schistosomula cultivated in SCM and ASCM. To observe significant difference, longer cultivation should probably be applied.

Development of gut and disintegration of the eye spots

Many duck skin schistosomula develop somewhat inflated gut that occupies most of the schistosomular body on day 14 p. t. Howell and Bourns (1974) observed pale and inflated caeca of a related species – *T. szidati* about 7 days p. t. and after that no schistosomula accepted RBCs. However, *T. regenti* schistosomula in my experiments had the light brown pigment in the not connected caeca and if in presence of RBCs, they continued feeding. It remains unclear if the caecum dilatation is a pathological process or not. First duck skin schistosomula with connected caeca and single gut continuing to the posterior body end were observed on day 9 p. t. when duck serum or RBCs were present. In SCM or ASCM alone, first stages with this state of gut development were observed on day 14 p. t. Schistosomula obtained from the definitive host have connected caeca with single caecum continuation on day 6 p. t. (Blažová and Horák, 2005), therefore the gut development probably occurs sooner *in vivo.* However, the state of gut development was not evaluated sooner than on day 9 p. t.

On day 21 p. t., most of the schistosomula had typical adult caeca, but up to the day 21, many worms had bifurcated, nonconnected gut with one caecum dominant and longer. There is a possibility that the dominant caecum continues as the single gut after the shorter caecum connects to it; even though the gut of *S. mansoni* is formed by connection of the caeca followed by single gut formation (Basch, 1981a).

The eye spots disintegrate independently on the process of the gut development or body growth; therefore, evaluation of the eye spots disintegration probably isn't a good marker of schistosomulum development.

6.3 Other potential markers of completed transformation into schistosomulum

6.3.1 Development of oral sucker

Oral sucker development could be used as a marker of completed c/s transformation, as one of the tested mechanical schistosomula had oral sucker almost completely differentiated. Flattened cercarial muscular conus and radical muscular fibers in the oral sucker were observed, as described by Bulantová et al. (2011). Nevertheless, it is difficult to focus the muscle fibers under the fluorescence microscope, therefore it was impossible to evaluate most of the FITC-phalloidin labeled schistosomula. The oral sucker should be evaluated using confocal microscopy, but that way only limited number of worms can be examined in short time.

6.3.2 Development of tegument

GTP1 and 4 were tested as potential markers of *T. regenti* tegument development because it serves as such on *S. mansoni* schistosomula (Skelly and Shoemaker, 2000; Zhong et al., 1995). The anti-SGTP4 antibody was functional, as confirmed on *S. mansoni* cercariae incubated in SCM. Anti-SGTP1 did not bind any target on *S. mansoni* controls but that could be caused by its location in the basal membrane of tegument, unlike SGTP4 that is located in apical membrane (Skelly and Shoemaker, 2000; Zhong et al., 1995). Both antibodies did not label any *T. regenti* stage. Although the observed DNA sequence similarity between *S. mansoni* and *T. regenti* GTP was high (79%), the peptides bound by the monoclonal antibodies could be different. The *T. regenti* GTP could also adopts different conformation. Testing of the two antibodies by ELISA resulted in unconvincing results as even positive control was not clearly positive. This could be caused by insufficient protein isolation during homogenization, because both transporters are intramembranous. No signal was detected on the PVDF membrane during the western blot experiment. In this case, a positive control in form of antibody functional against some *T. regenti* structure should be used to confirm the method was performed correctly, but this was unavailable. Both ELISA and Western blot need to be further optimized for SGTP1 or 4 detection but not enough antibody was available. To conclude, anti-SGTP1 and SGTP4 antibodies cannot be used on *T. regenti* on whole mount IHC to document tegument development.

6.3.3 Gene expression of schistosomula

Changes in the expression of selected genes could be potentially a good marker of c/s transformation. However, primers with verified specificity and efficiency would be needed for all selected genes. Moreover, stability of reference genes expression should be evaluated to enable assessment of qPCR (Bustin et al., 2009). Also, material optimization would be needed, 500 worms of

every stage used for RNA isolation provide low concentration of isolated RNA. Integrity of isolated RNA would need to be tested, but almost all the isolated amount of RNA (20 ng) was used for reverse transcription in my experiment. Even though the same amount of RNA was used as a reverse transcription template for all the *T. regenti* stages, the cDNA used in qPCR should have been diluted by the same volume of water in all the samples. And last, during the PCR, electrophoresis showed for a product from cDNA template of all the stages reference gene HNRA. For cDNA from schistosomula 9 days p. t. incubated in SCM with duck serum, some product of size corresponding to the expected amplicon size was shown. However, no product was confirmed by sequencing so the product identity is not certain.

7 CONCLUSIONS

This thesis is focused on the evaluation of *T. regenti* cercaria/schistosomulum transformation methods followed by long-term cultivation.

First specific aim was to find the optimal method of c/s transformation for *T. regenti*, based on the number of obtained schistosomula and evaluation of the criteria for early schistosomulum (emptied penetration glands, shed glycocalyx). Short-term viability was also evaluated.

Second specific aim of this study was to find the optimal medium for long-term cultivations that would support growth and development of *T. regenti* (body growth and gut development).

Last specific aim of this thesis was to search for other markers of completed c/s transformation.

Following conclusions were made based on the results of this study:

- Tail loss is an important part of c/s transformation, because it accelerates the process.
- The largest quantity of schistosomula that survive in sufficient numbers at least 5 to 9 days p. t. can be obtained with the use of BeadBeater or passage through the syringe needle. Nevertheless, they mostly do not meet the criteria of early schistosomulum after 24 h incubation.
- Numbers of *T. regenti* schistosomula obtained by tested c/s transformation methods are much lower than reported for *S. mansoni*.
- The best transformation method for obtaining of long living schistosomula that meet the criteria for early schistosomulum is a duck skin penetration. However, the obtained number of these schistosomula is relatively small.
- *T. regenti* schistosomula survive better in complete media during long-term cultivations than in basic media.
- Addition of 10% heat-inactivated duck serum to the cultivation medium supports the growth of duck skin schistosomula. These schistosomula develop intestine similar to the adult one.
- Addition of 10% heat-inactivated duck serum also prolongs survival of duck skin schistosomula almost three times, compared to schistosomula living in the definitive host. Potential primordia of reproductive system were noted on day 58 p. t.
- Other potential markers of completed c/s transformation, including oral sucker development and evaluation of gene expression changes, need further optimizations.

To conclude, this thesis brings new information concerning application of methods suitable for *T. regenti* c/s transformation. The potential of duck serum to stimulate prolonged survival,

growth and gut development of *T. regenti* schistosomula was revealed. The results of this thesis could have major importance in realization of experiments where large number of schistosomula is needed; many of laboratory definitive hosts could be spared. In future, the schistosomula transformed *in vitro* and incubated with duck serum need to be compared in detail with *in vivo* developed schistosomula of the same age.

8 REFERENCES

Basch, P.F., 1984. Development and behavior of cultured *Schistosoma mansoni* fed on human-erythrocyte ghosts. The American Journal of Tropical Medicine and Hygiene 33 (5), 911–917.

Basch, P.F., 1981a. Cultivation of *Schistosoma mansoni in vitro*. I. Establishment of cultures from cercariae and development until pairing. The Journal of parasitology 1981 67 (2), 179–185.

Basch, P.F., 1981b. Cultivation of *Schistosoma mansoni in vitro*. II. Production of infertile eggs by worm pairs cultured from cercariae. The Journal of parasitology 67 (2), 186–190.

Blažová, K., Horák, P., 2005. *Trichobilharzia regenti*: the developmental differences in natural and abnormal hosts. Parasitology International 54, 167–172.

Bogitsh, B.J., Carter, O.S., 1977. Developmental studies on the digestive tract of schistosomules (*Schistosoma mansoni*) grown *in vitro.* I. Ultrastructure. Transactions of the American Microscopical Society 96 (2), 219–227.

Brink, L.H., McLaren, D.J., Smithers, S.R., 1977. *Schistosoma mansoni*: a comparative study of artificially transformed schistosomula and schistosomula recovered after cercarial penetration of isolated skin. Parasitology 74, 73–86.

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.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., others, 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 55, 611–622.

Caulfield, J.P., Cianci, C.M., 1985. Human erythrocytes adhering to schistosomula of *Schistosoma mansoni* lyse and fail to transfer membrane components to the parasite. Journal of Cell Biology 101, 158–166.

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.

Chanová, M., Horák, P., 2007. Terminal phase of bird schistosomiasis caused by *Trichobilharzia regenti* (Schistosomatidae) in ducks (Anas platyrhynchos f. domestica). Folia parasitologica 54, 105-107.

Clegg, J.A., 1965. *In vitro* cultivation of *Schistosoma mansoni*. Experimental parasitology 16, 133–147.

Clegg, J.A., 1959. Development of sperm by *Schistosoma mansoni* cultured *in vitro*. Bulletin of the Research Council of Israel 8E, 1–6.

Clegg, J.A., Smithers, S.R., 1972. The effects of immune rhesus monkey serum on schistosomula of *Schistosoma mansoni* during cultivation *in vitro*. International Journal for Parasitology 2, 79–98.

Clemens, L.E., Basch, P.F., 1989. *Schistosoma mansoni*: effect of transferrin and growth factors on development of schistosomula *in vitro*. The Journal of parasitology 75 (3), 417–421.

Colley, D.G., Wikel, S.K., 1974. *Schistosoma mansoni*: simplified method for the production of schistosomules. Experimental Parasitology 35, 44–51.

Coultas, K.A., Zhang, S.-M., 2012. *In vitro* cercariae transformation: comparison of mechanical and nonmechanical methods and observation of morphological changes of detached cercariae tails. The Journal of parasitology 98 (6), 1257–1261.

Cousin, C.E., Stirewalt, M.A., Dorsey, C.H., 1981. *Schistosoma mansoni*: ultrastructure of early transformation of skin-and shear-pressure-derived schistosomules. Experimental parasitology 51, 341–365.

Deng, J., Gold, D., LoVerde, P.T., Fishelson, Z., 2003. Inhibition of the complement membrane attack complex by *Schistosoma mansoni* paramyosin. Infection and immunity 71, 6402–6410.

Dolečková, K., Kašný, M., Mikeš, L., Cartwright, J., Jedelskỳ, P., Schneider, E.L., Dvořák, J., Mountford, A.P., Craik, C.S., Horák, P., 2009. The functional expression and characterisation of a cysteine peptidase from the invasive stage of the neuropathogenic schistosome *Trichobilharzia regenti*. International journal for parasitology 39, 201–211.

Dorsey, C.H., Cousin, C.E., 1982. *Schistosoma mansoni*: a rapid procedure for *in vitro* transformation of cercariae to schistosomules. The Journal of parasitology 68, 349–350.

Eveland, L.K., Morse, S.I., 1975. *Schistosoma mansoni*: *in vitro* conversion of cercariae to schistosomula. Parasitology 71, 327–335.

Gazzinelli, G., De Oliveira, C.C., Flgueiredo, E.A., Pereira, L.H., Coelho, P.M.Z., Pellegrino, J., 1973. *Schistosoma mansoni*: biochemical evidence for morphogenetic change from cercaria to schistosomule. Experimental parasitology 34, 181–188.

Gilbert, B., Da Rosa, M.N., Borojević, R., Pellegrino, J., 1972. *Schistosoma mansoni*: *in vitro* transformation of cercariae into schistosomula. Parasitology 64, 333–339.

Gobert, G.N., Tran, M.H., Moertel, L., Mulvenna, J., Jones, M.K., McManus, D.P., Loukas, A., 2010. Transcriptional changes in *Schistosoma mansoni* during early schistosomula development and in the presence of erythrocytes. PLoS Neglected Tropical Diseas 4, e600.

Gold, D., 1997. Assessment of the viability of *Schistosoma mansoni* schistosomula by comparative uptake of various vital dyes. Parasitology research 83, 163–169.

Gold, D., Flescher, E., 2000. Influence of mechanical tail-detachment techniques of schistosome cercariae on the production, viability, and infectivity of resultant schistosomula: a comparative study. Parasitology research 86, 570–572.

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. The Journal of parasitology 83 (6), 1079–1085.

Haas, W., Haeberlein, S., 2009. Penetration of cercariae into the living human skin: *Schistosoma mansoni* vs. *Trichobilharzia szidati*. Parasitology research 105, 1061–1066.

Haas, W., Schmitt, R., 1982. Characterization of chemical stimuli for the penetration of *Schistosoma mansoni* cercariae. Parasitology research 66, 293–307.

Hara, I., Hara, S., Fusco, A.C., Salafsky, B., Shibuya, T., 1993. Role of calcium ion in *Schistosoma mansoni* cercarial tail loss induced by unsaturated fatty acids. The Journal of parasitology 79 (4), 504–509.

Hockley, D.J., McLaren, D.J., 1973. *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercaria to adult worm. International journal for parasitology 3, 13–20.

Holtfreter, M.C., Loebermann, M., Frei, E., Riebold, D., Wolff, D., Hartung, G., Kinzelbach, R., Reisinger, E.C., 2010. Schistosomula, pre-adults and adults of *Schistosoma mansoni* ingest fluorescence-labelled albumin *in vitro* and *in vivo*: implication for a drug-targeting model. Parasitology 137, 1645–1652.

Horák, P., Kolářová, L., Dvořák, J., 1998a. *Trichobilharzia regenti* n. sp. (Schistosomatidae, Bilharziellinae), a new nasal schistosome from Europe. Parasite 5, 349–357.

Horák, P., Kovář, L., Kolářová, L., Nebesářová, J., 1998b. Cercaria–schistosomulum surface transformation of *Trichobilharzia szidati* and its putative immunological impact. Parasitology 116, 139–147.

Horák, P., Mikeš, L., Rudolfová, J., Kolářová, L., 2008. Penetration of *Trichobilharzia cercariae* into mammals: dangerous or negligible event? Parasite 15, 299–303.

Howe, S., Zöphel, D., Subbaraman, H., Unger, C., Held, J., Engleitner, T., Hoffmann, W.H., Kreidenweiss, A., 2015. Lactate as a novel quantitative measure of viability in *Schistosoma mansoni* drug sensitivity assays. Antimicrobial agents and chemotherapy 59, 1193–1199.

Howell, M.J., Bourns, T.K.R., 1974. *In vitro* culture of *Trichobilharzia ocellata.* International journal for parasitology 4, 471–476.

Hrádková, K., Horák, P., 2002. Neurotropic behaviour of *Trichobilharzia regenti* in ducks and mice. Journal of Helminthology 76, 137–141.

James, E.R., Taylor, M.G., 1976. Transformation of cercariae to schistosomula: a quantitative comparison of transformation techniques and of infectivity by different injection routes of the organisms produced. Journal of helminthology 50, 223–233.

Kašný, M., Mikeš, L., Dalton, J.P., Mountford, A.P., Horák, P., 2007. Comparison of cysteine peptidase activities in *Trichobilharzia regenti* and *Schistosoma mansoni* cercariae. Parasitology 134, 1599–1609.

Kemp, W.M., 1970. Ultrastructure of the cercarienhüllen reaktion of *Schistosoma mansoni*. The Journal of parasitology 56 (4), 713–723.

Kemp, W.M., Damian, R.T., Greene, N.D., 1973. *Schistosoma mansoni*: immunohistochemical localization of the CHR reaction in glycocalyx of cercaria. Experimental parasitology 33, 27–33.

Kouřilová, P., Hogg, K.G., Kolářová, L., Mountford, A.P., 2004. Cercarial dermatitis caused by bird schistosomes comprises both immediate and late phase cutaneous hypersensitivity reactions. The Journal of Immunology 172, 3766–3774.

Kusel, J.R., McVeigh, P., Thornhill, J.A., 2009. The schistosome excretory system: a key to regulation of metabolism, drug excretion and host interaction. Trends in parasitology 25, 353–358.

Lu, D., Zhao, X., Zhao, Y., Zhang, B., Zhang, B., Geng, M., Liu, R., 2011. Binding of Sudan II and Sudan IV to bovine serum albumin: comparison studies. Food and chemical toxicology 49, 3158–3164.

Mair, G.R., Maule, A.G., Day, T.A., Halton, D.W., 2000. A confocal microscopical study of the musculature of adult *Schistosoma mansoni*. Parasitology 121, 163–170.

Melo, A. de, Pereira, L.H., 1985. Kinetics of the cercaria-schistosomulum transformation *in vivo*. Revista da Sociedade Brasileira de Medicina Tropical 18, 17–21.

Michalick, M.S.M., Gazzinelli, G., Pellegrino, J., others, 1979. Cultivation of *Schistosoma mansoni* cercarial bodies to adult worms. Revista do Instituto de Medicina Tropical de Sao Paulo 21, 115–118.

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. Parasitology research 96, 230–241.

Milligan, J.N., Jolly, E.R., 2011. Cercarial transformation and *in vitro* cultivation of *Schistosoma mansoni* schistosomules. JoVE (Journal of Visualized Experiments) e3191–e3191.

Peak, E., Chalmers, I.W., Hoffmann, K.F., 2010. Development and validation of a quantitative, high-throughput, fluorescent-based bioassay to detect schistosoma viability. PLOS Neglected Tropical Diseases 4, e759.

Ramalho-Pinto, F.J., Gazzinelli, G., Howells, R.E., Mota-Santos, T.A., Figueiredo, E.A., Pellegrino, J., 1974. *Schistosoma mansoni*: defined system for stepwise transformation of cercaria to schistosomule *in vitro*. Experimental parasitology 36, 360–372.

Řimnáčová, J., Mikeš, L., Turjanicová, L., Bulantová, J., Horák, P., 2017. Changes in surface glycosylation and glycocalyx shedding in *Trichobilharzia regenti* (Schistosomatidae) during the transformation of cercaria to schistosomulum. PloS one 12 (3), e0173217.

Salafsky, B., Fusco, A.C., Whitley, K., Nowicki, D., Ellenberger, B., 1988. *Schistosoma mansoni*: analysis of cercarial transformation methods. Experimental parasitology 67, 116–127.

Samuelson, J.C., Caulfield, J.P., 1986. Cercarial glycocalyx of *Schistosoma mansoni* activates human complement. Infection and immunity 51, 181–186.

Shiff, C.J., Cmelik, S.H.W., Ley, H.E., Kriel, R.L., 1972. The influence of human skin lipids on the cercarial penetration responses of *Schistosoma haematobium* and *Schistosoma mansoni*. The Journal of parasitology 58 (3), 476–480.

Skelly, P.J., Shoemaker, C.B., 2000. Induction cues for tegument formation during the transformation of *Schistosoma mansoni* cercariae. International journal for parasitology 30, 625–631.

Skelly, P.J., Shoemaker, C.B., 1996. Rapid appearance and asymmetric distribution of glucose transporter SGTP4 at the apical surface of intramammalian-stage *Schistosoma mansoni*. Proceedings of the National Academy of Sciences 93, 3642–3646.

Stirewalt, M.A., Cousin, C.E., Dorsey, C.H., 1983. *Schistosoma mansoni*: stimulus and transformation of cercariae into schistosomules. Experimental parasitology 56, 358–368.

Stirewalt, M.A., Uy, A., 1969. *Schistosoma mansoni*: cercarial penetration and schistosomule collection in an *in vitro* system. Experimental Parasitology 26, 17–28.

Thornhill, J., Kusel, J., Oliviera, F.A. de, Ribeiro, F., Lima, S.F., Coelho, P.M.Z., McVeigh, P., Mattos, A.C.A., 2010. Uptake of macromolecules by cercariae during skin penetration and transformation to schistosomula (*Schistosoma mansoni*). Memórias do Instituto Oswaldo Cruz 105, 387–390.

Tiba, Y., Holanda, J.C., Ramalho-Pinto, F.J., Gazzinelli, G. t, Pellegrino, J., 1974. Schistosomula (*Schistosoma mansoni*) obtained *in vitro*: viability in culture and infectivity for mice. Transactions of the Royal Society of tropical Medicine and Hygiene 68, 72.

Wang, W., Kirschfink, M., Ruppel, A., 2006. *Schistosoma japonicum* and *S. mansoni* cercariae: different effects of protein in medium, of mechanical stress, and of an intact complement system on *in vitro* transformation to schistosomula. Parasitology research 99, 269.

Yasuraoka, K., Irie, Y., Hata, H., 1978. Conversion of schistosome cercariae to schistosomula in serum-supplemented media, and subsequent culture *in vitro*. The Japanese journal of experimental medicine 48, 53–60.

Zhong, C., Skelly, P.J., Leaffer, D., Cohn, R.G., Caulfield, J.P., Shoemaker, C.B., 1995. Immunolocalization of a *Schistosoma mansoni* facilitated diffusion glucose transporter to the basal, but not the apical, membranes of the surface syncytium. Parasitology 110, 383–394.

BOOKS AND THESES

Bulantová, J., 2012. Development of surface and body musculature of the bird schistosome *Trichobilharzia regenti* (Disertation thesis). Charles University in Prague, Prague.

Roberts, L.S., Janovy, J., 2009. Foundations of Parasitology. (8th edn). New York, USA. McGraw-Hill Inc. ISBN 978-007-131103-8, 251-5.

Vrbová, K., 2014. Cultivation of Trematodes (Bachelor thesis). Charles University in Prague, Prague, [in Czech].

Zar, J.H., 1999. Biostatistical analysis. Pearson Education India, 278.

Zídková, L., 2003. Indukce vyprazdňování penetračních žláz cerkárií *Trichobilharzia szidati* a *Trichobilharzia regenti* a částečná analýza jejich exkrečně-sekrečních produktů (Master's thesis). Charles University in Prague, Prague, [in Czech].

ONLINE REFERENCES

Grimes, S.E., 2002. Chapter 8. Preparation of washed chicken red blood cells, in: A Basic Laboratory Manual for the Small-Scale Production and Testing of I-2 Newcastle Disease Vaccine. RAP publication 2002/22. ISBN 974-7946-26-2.

9 SUPPLEMENT

The supplement file containing experimental data and results of statistical analysis is available online: https://drive.google.com/file/d/0B4Rj5ufwHIkWbThfNmhZMm1VNms/view?usp=sharing

LIST OF SUPPLEMENT FILE CONTENTS

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