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**Mgr. Roman Leontovč**

**Molecular adaptations of neurotropic and visceral bird schistosomes  
during the infection of the avian definitive host.**

**Molekulární adaptace neurotropních a viscerálních schistosom během  
infekce ptačího hostitele.**

Ph.D. thesis/Dizertační práce

**Supervizor/Školitel: RNDr. Martin Kašný, Ph.D.**

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**V Praze dne**

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## **Table of Contents**

<b>Abstract.....</b>	<b>5</b>
<b>Abstrakt.....</b>	<b>7</b>
<b>Aims of the thesis .....</b>	<b>9</b>
<b>Introduction .....</b>	<b>10</b>
<b>Schistosomes of the genus <i>Trichobilharzia</i>.....</b>	<b>10</b>
<b>1. Cercarial stage .....</b>	<b>12</b>
1.1 Energy metabolism .....	12
1.2 Transcription.....	13
1.3 Translation and amino acid metabolism .....	14
1.4 Penetration - peptidases.....	16
1.5 Venom allergen-like proteins (VALs).....	18
<b>2. Schistosomulum stage .....</b>	<b>22</b>
2.1 Nutrient acquisition via tegument.....	23
2.1.1. Glucose uptake.....	23
2.2 Nutrients acquisition via the alimentary tract .....	24
2.2.1 Micro Exon Genes (MEGs).....	25
2.2.3 Peptidases.....	26
<b>Concluding remarks .....</b>	<b>31</b>
<b>Publication No.1.....</b>	<b>33</b>
<b>Publication No.2.....</b>	<b>34</b>
<b>Publication No.3.....</b>	<b>35</b>
<b>References .....</b>	<b>36</b>

## Abstract

Genus *Schistosoma* is one the most studied group of helminths due to the importance of several representatives in terms of veterinary and human health. The advent of the modern sequencing technologies, as well as the increasing computational capacities, enabled large-scale screening of nucleic acids and thus deep exploration of complex transcriptome and genome information.

To date the main attention of leading molecular parasitological “Schistosoma” research teams was focused on serious human pathogens *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium*. In the term of molecular/biochemical research, the other schistosomatids were mostly neglected and general knowledge was limited to characterization of particular genes/proteins without further link to biological functional complex.

Presented thesis summarises the first large-scale insights into the molecular basis of biological principles of two bird schistosomes *Trichobilharzia regenti* and *Trichobilharzia szidati* during their invasion of the definitive avian host. While *T. szidati* uses the “classical” visceral way of migration - bloodstream and lungs (same as human schistosomes), *T. regenti*, migrates trough the peripheral nerves and spinal cord. Neurotropic migration is unique among schistosomes and it is also extremely rare within helminths. We aimed to determine the molecular mechanisms linked with visceral and neurotropic life strategy of both *Trichobilharzia* species using transcriptomic profiling of two consecutive developmental stages – cercariae (free living stage) and schistosomula (tissues migrating stage).

Our work started with the transcriptomic analysis of cercariae and schistosomula of neurotropic *T. regenti* leading to the identification of protein classes and biological

pathways related to important physiological processes (publication No 1). In order to more accurately identify the molecular mechanisms linked to neurotropism of *T. regenti* schistosomula, we sequenced and reconstructed the transcriptome of visceral *T. szidati* and performed comparative analysis. The numerous links to particular visceral or neurotropic strategies of these two schistosomes were identified (publication No 2). Our further research was related to functional characterisation of the important proteolytic enzyme cathepsin B - peptidase of *T. regenti*, including also a detailed analysis of expression of different isoforms (publication No 3).

## Abstrakt

Rod schistosoma je jednou z nejvíce studovaných skupin helmintů vzhledem k významu některých zástupců z hlediska humánní a veterinární medicíny. Rozvoj moderních sekvenačních technologií a se stále se zdokonalující výpočetní technika umožnily globální pohled na molekulární biologii schistosom za použití genomiky a transkriptomiky. Dosud byla pozornost předních vědeckých týmů zabývajících se schistosomózou zaměřena na významné lidské patogeny *Schistosoma mansoni*, *Schistosoma japonicum* a *Schistosoma haematobium*. Ostatní zástupci čeledi Schistosomatidae byli z hlediska molekulární biologie/biochemie většinou opomíjeni a výzkum byl zaměřen především na jednotlivé geny/molekuly, bez celkového přehledu. Předkládaná práce prezentuje komplexní transkriptomickou analýzu ptačích schistosom - neurotropní *Trichobilharzia regenti* a viscerální *Trichobilharzia szidati* během nákazy kachny jakožto definitivního hostitele. Zatímco *T. szidati* využívá běžnou viscerální migraci (stejně jako lidské schistosomy), *T. regenti* využívá k migraci periferní nervy a centrální nervovou soustavu. Způsob neurotropní migrace je mezi schistosomami unikátní a také velmi vzácný v rámci všech helmintů. Práce se zaměřuje na molekulární mechanismy spojené s viscerální a neurotropní životní strategií obou druhů pomocí transkriptomické analýzy dvou vývojových stádií – cercárií (volně žijící stadium) a schistosomul (parazitické stadium).

Naše práce začala transkriptomovou analýzou cercárií a schistosomul neurotropní *T. regenti*, která vedla k identifikaci biologických drah a proteinových skupin důležitých pro každé vývojové stadium se zaměřením na stadium schistosomuly (publikace č. 1). Aby bylo možné identifikovat molekulární mechanismy spojené s neurotropním chováním schistosomul *T. regenti*, provedli jsme analýzu transkriptomu viscerální schistosomy *T. szidati* a provedli mezidruhovou srovnávací analýzu. Identifikovali

jsme řadu procesů, které mohou být spojeny s viscerální a neurotropní schistosomózou (publikace č. 2). Třetí autorský článek je zaměřen na funkční charakteristiku významné peptidázy *T. regenti* - cathepsinu B, včetně detailní analýzy genové exprese jednotlivých isoform (publikace č. 3).



## **Aims of the thesis**

Five years of the research aimed to provide the first complex overview of the molecular biology of the two avian schistosomes differing in the life strategy during the infective phase of the definitive host. Specific focus was on the molecular mechanisms linked to visceral and neurotropic schistosomiasis using the transcriptomic-bioinformatic approach.

### **Specific aims**

1. Sequence, reconstruct and annotate the first transcriptomes of visceral and neurotropic avian schistosomes *T. szidati* and *T. regenti*.
2. Identify the key biological and metabolic pathways as well as the protein classes specific to cercarial and schistosomulum stages.
3. Identify the molecular mechanisms possibly linked to visceral and neurotropic schistosomiasis with special focus on nutrition preferences of *T. szidati* and *T. regenti* schistosomula.

## Introduction

Introductory part aims to biology of genus *Trichobilharzia* including the aspects of medical/economic importance. Following sections describe the first part of the infection of the definitive hosts by schistosomes and these are divided into two main parts dedicating to cercarial and schistosomulum stage – transfer of the parasite from free water environment to the host body. For cercariae and schistosomula the global picture of the molecular processes, as described in attached publications, is not provided here. The focus is rather given on particular pathways/protein classes or specific molecules, which are important in the term of parasite survival. Specific attention is also paid on selected processes/molecules, which are intensively studied on human schistosomes to provide the insight outside the genus *Schistosoma*. Our observations on *Trichobilharzia* schistosomes are confronted and discussed with the knowledge obtained on human schistosomes.

## Schistosomes of the genus *Trichobilharzia*

Genus *Trichobilharzia* (Schistosomatidae; Trematoda) is the most numerous within the Schistosomatidae family comprising ~35 species (Brant and Loker, 2013). As a whole family schistosomatidae, flukes of the genus *Trichobilharzia* possess two-host life cycle utilising the snail and the bird as an intermediate and definitive host (Horák, Kolářová and Adema, 2002). Mammals including man can be also infected by *Trichobilharzia* sp. and cercariae exhibit even higher affinity to human skin than the duck skin (Haas and van de Roemer, 1998). Invasion of human skin by *Trichobilharzia* sp. cercariae is associated with a cercarial dermatitis - host immune reaction eliminating the transformed cercarial stage in the skin and prevent its further

migration. Cercarial dermatitis occurs worldwide and in recent years new outbreaks were recorded (Veleizán, Flores and Viozzi, 2016; Gordy, Cobb and Hanington, 2018) therefore the cercarial dermatitis is considered as an emerging disease (for review see Horák *et al.*, 2015). Despite the fact the cercariae are generally entrapped and destroyed in the mammalian skin, the experiments performed in our lab showed, that cercariae of *T. regenti* can avoid the immune response of primo-infected hosts, leave the skin and migrate to internal organs (Horák *et al.*, 2008; Lichtenbergová *et al.*, 2011). Besides the medical importance, bird schistosomes have an economic impact on commercial recreational areas, often being closed for swimming due to the outbreaks of cercarial dermatitis (e.g. seasonal statements of local Institutes of public health)

**Lifecycle of the schistosomes** During the two–host lifecycle, parasite alternates between three distinct environments - freshwater, intermediate host, and definitive host. In freshwater, miracidia (larvae hatched from eggs) seek and infect an intermediate snail host, undergo asexual reproduction that results in thousands of cercariae (invasive larvae) being shed into the water column to locate and infect the definitive host. Invasion of the definitive host is accompanied by the radical morphological, molecular-biochemical, metabolic changes. Within the host schistosomula (transformed cercariae) migrates to the final location where matured adults mate and lay eggs. Migratory route and final localization in the definitive host are species specific.

## **Infection of the definitive host**

### **1. Cercarial stage**

The aim of the cercariae is to find and infect the definitive host. Cercariae are well adapted for the aquatic environment and consist of the body and the bifurcated tail serving as the propeller. Cercariae of the schistosomatids reflect the biotic (chemical) and abiotic (light, gravity, turbulence) signals to maximize the chance to meet and infect the definitive host - duck. The life of the cercariae is time-limited because free-living cercariae do not obtain any food or substrates other than oxygen ([Armstrong et al., 2001](#)). Cercariae are fully dependent on the endogenous reserves obtained in the previous molluscan intermediate host. The main source of energy is glycogen, which enables to span the lack of nutrients on the way between the intermediate and the definitive host ([Tielens, A. G. M., Hellemond, 2006](#)). The following section describes selected biological processes related to the cercariae when seeking and penetrate the definitive host.

#### **1.1 Energy metabolism**

To date, the experimental “wet lab” research of schistosomatids’ metabolism is limited only to human schistosomes, whereas for bird schistosomes only transcriptomic data and *in silico* predictions are available ([Leontovyč et al., 2016](#); [Leontovyč et al., 2019, publication No.1, 2](#)). It was proved that cercariae of *S. mansoni* maintain the aerobic metabolism where limited stores of glycogen are utilised by glycolysis and the Krebs cycle is the main terminal process of the carbohydrate breakdown ([Coles, 1972](#); [Bruce et al., 1974](#); [Von Kruger et al., 1978](#)) followed by oxidative phosphorylation covering high energy demands of cercariae ([Santos et al., 1999](#)). The results of *T. regenti* and *T.*

*szidati* cercariae transcriptomic data analysis showed high upregulation of genes related to aerobic metabolism and it was recorded that the gain of the energy from glycogen reserves is similar for both species (Leontovyč *et al.*, 2019, publication No.2). It is not a surprise that cercariae of human and bird schistosomes utilise the same processes of energy metabolism, which were also previously defined for many other free-living stages of other trematodes (Tielens, van den Heuvel and van den Bergh, 1984; Boyunaga *et al.*, 2001; Prosdocimi *et al.*, 2002).

## 1.2 Transcription

Messenger RNA can be detected in cercarial stage of schistosomatids in high amounts and a large number of studies of schistosomes is focused on gene expression and quantification of mRNA in cercarial stage either focused on particular genes/transcripts (Zeraik *et al.*, 2013; Pereira *et al.*, 2014; Farias *et al.*, 2019) or dealing with large-scale profiling (Jolly *et al.*, 2007; Fitzpatrick *et al.*, 2009; Parker-Manuel *et al.*, 2011; Picard *et al.*, 2016) (see the supplementary table S1 for overview of large-scale transcriptomic studies of schistosomes). However recent research revealed, that the transcription in the cercarial stage of *S. mansoni* is epigenetically silenced and the detected mRNA predominantly comes from the gene expression in the molluscan intermediate host (Roquis *et al.*, 2015). On the other hand, Cai *et al.* (2017) reported about the transcription in cercariae of *S. japonicum* based on detection of mRNA of proteins linked with the transcription machinery. Thus they speculate, that the transcription occurs in cercariae in certain level. However such hypothesis was raised based on detection of transcription factors and repressors in the cercarial transcriptome of *S. japonicum* (Cai *et al.*, 2017), without any direct wet lab proof such as labelling of newly transcribed RNA by e.g. 5-ethynyl-uridine. Therefore there is no

significant evidence that such mRNA was synthesized by sporocysts in snail host or in free living cercariae. There is no experimental data for transcription silencing/regulation in bird schistosome cercariae, however number of transcripts encoding transcription factors were detected in cercariae of *T. regenti* and *T. szidati* and some of them were upregulated compared to schistosomula (Leontovyč *et al.*, 2019, publication No.2). Nevertheless it is not clear whether such mRNA comes from the intramolluscan stages or is synthesized in the cercariae. Since the transcription is energy-demanding process it is likely that silencing of the transcription is also present in cercariae of the bird schistosomes as proved in *S. mansoni* (Roquis *et al.*, 2015). It is likely that cercariae as a not growing and short-lived developmental stage do not waste the limited energy resources for transcription, however, carry the mRNA synthesized in the snail host and is fully prepared for rapid translation in the definitive host where the source of nutrients is “unlimited”.

### **1.3 Translation and amino acid metabolism**

Cercariae of schistosomes synthesize proteins during their life to keep all biochemical processes running (Atkinson and Atkinson, 1982; Blanton and Licate, 1992). Based on the whole transcriptome study of three life forms/stages of *S. mansoni* - germ balls, cercariae and 3-day schistosomula, cercariae were identified as the least translationally active. This was demonstrated based on the differential transcription of initiation, translation and elongation factors, tRNA synthetases and ribosomal proteins (Parker-Manuel *et al.*, 2011). Contrary, cercariae of *T. regenti* and *T. szidati* showed a much higher amount of mRNA linked to translation compared to 7 days schistosomula of *T. regenti* or 4 days schistosomula of *T. szidati* (Leontovyč *et al.*, 2019, publication No.2). For example, ribosomal proteins were highly upregulated in the cercarial stage

of both species. Out of 56 significantly differentially expressed transcripts ( $\log_2$  fold change  $>2$  or  $<-2$ ,  $FDR \leq 0.05$ ) annotated as ribosomal proteins, 55 were up-regulated in the cercarial stage of *T. szidati*. Data from *T. regenti* showed the same trend where 26 out of 28 significantly differentially expressed transcripts were up-regulated in cercariae and only 2 in the schistosomula stage. Similarly, other genes related to translation are upregulated in cercariae of both species such as translational factors or tRNA synthetases (Leontovyč *et al.*, 2016, 2019 publication No.1, 2). Interestingly cercariae of *T. szidati* and *T. regenti* contains a high amount of transcripts related to translation compared to schistosomula, however, it is not necessarily mean that not-growing cercariae translate more proteins than schistosomulum stage which is growing, developing, interacting with host and actively feeding. The high amount of translation-related genes may be linked with the radical transformation of the cercariae after penetration of the definitive host, which is associated with rapid protein synthesis (Leontovyč *et al.*, 2016, 2019 publication No.1, 2). As already mentioned cercariae need to synthesize proteins during their life and as the free-living, not-feeding stage have to rely on endogenous reserves or the biosynthesis. Therefore amino acids used in protein synthesis have to be obtained from the sources inside the intermediate host body or cercariae must rely on biosynthesis (Tielens, A. G. M., Hellemond, 2006). Biosynthetic capacities of amino acids are known in parasitic stages of *Fasciola hepatica* and *S. mansoni*, but limited in free-living stages (Tielens, A. G. M., Hellemond, 2006). Unfortunately in free-living stages of helminths this problematics have been poorly studied or were not highlighted in large-scale transcriptomic studies. In avian schistosomes, *T. regenti* and *T. szidati* it was disclosed that biosynthesis of amino acids in the cercarial stage play an important role. In total 31 and 27 transcripts belonging to amino acid biosynthesis according to KEGG brite database (Kanehisa

and Goto, 2000) were upregulated in cercariae of *T. regenti* and *T. szidati*. Contrary 0 (*T. regenti*) and only 2 (*T. szidati*) transcripts related to amino acid biosynthesis were upregulated in the schistosomulum stage (Leontovyč *et al.*, 2019 publication No.2). It is evident that the cercaria/schistosomulum transformation significantly decrease the amino acid biosynthesis and the parasitic schistosomulum obtain the amino acids from the host.

#### **1.4 penetration - peptidases**

The cercarial stage is determined for survival until the active finding, penetration and infection of definitive host. The life of the free-living cercariae of avian and human schistosomes is limited to 1- 1.5 days (Neuhaus, 1952; Lawson and Wilson, 1980). If the definitive host is not reached, the endogenous resources are drained and cercaria dies. The finding and invasion of the definitive host is the critical step in the lifecycle of the schistosomes. Once cercariae find the definitive host, the invasive processes are started – attachment on hosts' surface, loss of the tail, emptying the penetration glands, glycocalyx surface reduction, etc. (Horák *et al.*, 1998). In order to break thru the host skin barriers the cercariae adopt various mechanisms where the functional protein molecules play a leading role.

**Peptidases** as the proteolytic enzymes are the key compound are stored in the different types of penetration glands in the body of the cercariae and in the case of cercariae of schistosomes their release is invoked by specific chemical stimuli such as unsaturated fatty acids (MacInnis, 1969; Austin, Stirewalt and Danziger, 1972; Shiff *et al.*, 1972). It has been shown, that the repertoire of the peptidases differs not only between avian and human schistosomes, but within the whole genus *Schistosoma*. While the most abundant penetration related enzyme in *S. mansoni* cercariae is the



serine peptidase - cercarial elastase (Ingram *et al.*, 2012), *T. regenti* possesses the high activity of cysteine peptidases predominantly cathepsin B (Mikeš, Zídková, *et al.*, 2005; Kašný *et al.*, 2007; Dolečková *et al.*, 2009, Dvořáková *et al.*, 2020 publication No.3). Similarly, *S. japonicum* cercariae utilise the cysteine peptidase cathepsin B as the main penetration enzyme (Dvořák *et al.*, 2008). Cercarial elastase mRNA was not confirmed in the transcriptome of *T. szidati* and *T. regenti* cercariae (no single read was mapped to reference across 4 replicates) (Leontovyč *et al.*, 2016, 2019, publication No.1, 2), however the coding gene of the cercarial elastase is present in the genome of *T. regenti* (Howe *et al.*, 2017), which suggests the transcription of the cercarial elastase in the snail host and cercariae carry the translated protein. Nevertheless elastase-like enzyme was not detected in *T. regenti* cercariae by using of *S. mansoni* elastase antibodies (Mikeš, Zídková, *et al.*, 2005) or cleavage of elastase specific substrates (Kašný *et al.*, 2007). As mentioned above during the penetration of the definitive host, cercariae of *T. regenti* and *T. szidati* mostly rely on cysteine peptidase specifically cathepsin B, which was detected in cercariae on protein level (Mikeš, Zídková, *et al.*, 2005; Kašný *et al.*, 2007). Transcriptomic profiling of the *T. regenti* and *T. szidati* cercariae revealed no or very low expression of cathepsin B in the cercarial stage of both species, which suggests the expression of the cathepsin B in the intramolluscan stage and cercariae carry the translated protein. Among those low expressed cathepsin B transcripts (several orders of magnitude lower compared to schistosomula), the most abundant transcript of cathepsin B2 was identified in cercarial transcriptome of *T. regenti* and *T. szidati*. It was proved, that cathepsin B2 is the main penetration enzyme of the *T. regenti* cercariae (Dolečková *et al.*, 2009) (experimental data for *T. szidati* are missing). Differential gene expression analysis between cercariae and schistosomula of *T. regenti* and *T. szidati* defined the

upregulated peptidases in cercarial stage e.g. Calpain, Methionine aminopeptidase, Prolyl endopeptidase, Caspase 8, ADAMTS-like, carboxypeptidase N, Sentrin-specific protease, leishmanolysin-like peptidase, proteasome subunit beta 4, beta-aspartyl-peptidase, mitochondrial-processing peptidase subunit beta, cathepsin A.

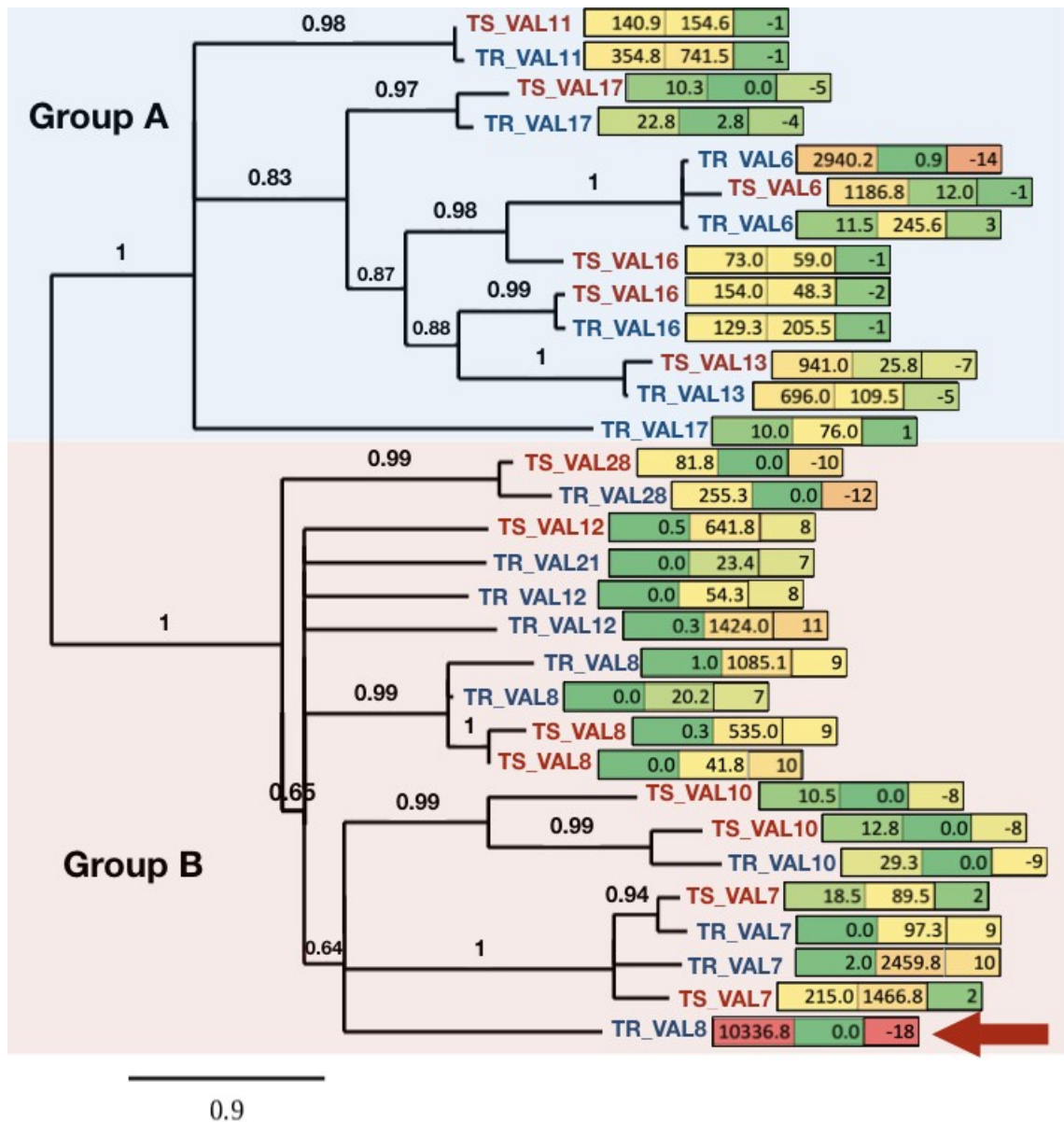
Equally for both species the calpain peptidase was the most expressed peptidase in cercarial stage (Leontovyc *et al.*, 2016; Leontovyč *et al.*, 2019, publication No.1, 2). Calpains are not considered as the degradation enzymes and rather serve as the enzymes to cleave the target proteins in response to calcium signalling. Calpains are also known for the ability to cut off the cytoskeletal proteins to facilitate the cell migration (Campbell and Davies, 2012). Calpain was recently identified as the enzyme preventing the blood clotting in schistosomula and adults of *S. mansoni* (Wang, Dai and Liu, 2017; Wang *et al.*, 2018), however high expression of calpains was detected also in neurotropic *T. regenti* cercariae which after transformation to schistosomula migrate through/along the peripheral nerves (Lichtenbergová *et al.*, 2011) so it is unlikely that calpain serves as the anticoagulant in that case. In *S. mansoni* it was disclosed that calpain may regulate the surface membrane synthesis (Siddiqui *et al.*, 1993) given the fact that during the transformation from cercariae to schistosomula the rapid formation of surface membrane takes place (Horák *et al.*, 1998), it is likely that the high dosage of calpain transcripts are linked to this surface membrane formation.

### **1.5 Venom allergen-like proteins (VALs)**

Apart of proteolytic enzymes which play the key role during the penetration of the cercariae into the definitive host, the highly upregulated genes in cercarial stage are in the focus of this thesis. The most upregulated transcript of cercariae of *T. regenti* belongs into the family of venom allergen-like proteins (VALs). Venom allergen-like

proteins (VALs) are structurally conserved proteins belonging to the sperm coating protein/Tpx-1/Ag-5/Pr-1/Sc-7 (SCP/TAPS) superfamily (Cantacessi *et al.*, 2009; Cantacessi and Gasser, 2012). They are generally believed to act as the modulators of the host response therefore they are in focus of recent research as the candidates for the protection strategies against helminths (Wilbers *et al.*, 2018). To date 29 VALs have been identified in *S. mansoni* (Chalmers *et al.*, 2008; Farias *et al.*, 2012) and can be divided into 2 groups. Members of the larger group (VALs 1-10, 12, 14, 15, 18-29) supposed to be secreted due to the presence of the signal peptide and the conserved cysteine residues forming the disulphide bridges. Representatives of the second smaller group (VALs 6, 11, 13, 16, 17) lack the signal peptide as well as the conserved cysteine residues and therefore are predicted as the non-secreted. The knowledge about the specific function of the VALs is limited to the several particular representatives, e.g. lipid binding function (SmVAL4) (Kelleher *et al.*, 2014), plasminogen binding (SmVAL18) (Fernandes *et al.*, 2018), host matrix metalloprotease modulation (SmVAL9) (Yoshino *et al.*, 2014) and targeting the SmVAL6 by IgE, IgG4, and IgG1 (Farnell *et al.*, 2015). In schistosomes VALs are differentially expressed during the life cycle, where the expression is limited in the intra mammalian stages, infective stages or VALs are expressed ubiquitously across the lifecycle (Chalmers *et al.*, 2008; Parker-Manuel *et al.*, 2011; Farias *et al.*, 2019), there is no evidence of the direct involvement of the VALs in the penetration of the cercariae into the definitive host, however high expression of some representatives in cercarial stage (e.g. SmVAL1, 4, 7, 10, 13, 16, 21, 16, 19) indicate the important role of VALs during invasion into the definitive host (Farias *et al.*, 2019). In total 22 and 15 transcripts of venom allergen-like proteins were identified in cercarial and schistosomula transcriptomes of *T. regenti* and *T. szidati* respectively, representing 14 and 11

different VALs. Differential gene expression analysis between cercariae and 7 days old schistosomula of *T. regenti* showed the enormous up-regulation of transcript annotated as VAL 8 (probably VAL 10 based on phylogenetic and differential expression analysis - Fig. 1) in the cercarial stage. With log<sub>2</sub>FC 18 it was the second most differentially expressed gene in the whole transcriptome (Leontovyc *et al.*, 2016, Publication No. 1). In general, one half of all VALs identified in cercaria/schistosomula transcriptome of *T. regenti* and *T. szidati* were up-regulated in cercarial stage (VAL, 6, 10, 13, 16, 17, 28) and some of them were expressed exclusively in this stage (VAL 10, 17, 28). Phylogenetic analysis and differential gene expression analysis (Fig. 1) clearly showed the distribution into two groups same as for human schistosomes and also that the expression of VALs is stage specific and the same VALs are up/down regulated in *T. regenti* and *T. szidati*.



**Fig. 1 - Phylogenetic analysis of Venom Allergen Proteins (VALs) of *Trichobilharzia regenti* (TR) and *Trichobilharzia szidati* (TS).** Dendrogram of protein sequences of *T. regenti* and *T. szidati* annotated as VALs, coloured rectangles shows the gene expression in counts per million (CPM) of cerariae (1. rectangle) and schistosomula (2. rectangle) and differential gene expression in Log2 fold change (3. rectangle). CPM values for cercariae and schistosomula are the mean of four replicates.

Maximum likelihood tree constructed from protein sequences aligned by MUSCLE v3.8.31 in PhyML program v3.1 with WAG substitution model, 4 gamma-distributed rate categories (gamma shape parameter estimated from the data, gamma=1.887).

## 2. Schistosomulum stage

During the penetration into the definitive host cercariae of schistosomes undergoes the rapid biological transformation accompanied by a loss of tail, the formation of double membrane covering the tegument, switch from aerobic to anaerobic metabolism, followed by gut development and activation (Mclaren and Hockley, 1977; Horemans, Tielens and van den Bergh, 1992; Skelly, Stein and Shoemaker, 1993; Horák *et al.*, 2008). Schistosomulum (transformed cercaria) as a parasitic stage migrates through the definitive host to the final localisation where mature into adult worms. During this process the schistosomula grow and change the different niches/tissues of the definitive host body. According to the general life strategy, two types of schistosomes are known. 1. Visceral - Schistosomula migrate via the circulatory system to lungs, then re-enter the veins to reach the final localisation depending on the species and 2. neurotropic – Schistosomula migrate through/along the peripheral nerves to the spinal cord, brain to end up in the nasal cavity (Horák, Kolářová and Dvořák, 1998; Lichtenbergová *et al.*, 2011). *Trichobilharzia regenti* is the only one known schistosome possessing the neurotropic migration.

The next section is describing the life of the schistosomula when residing the lungs in the case of visceral schistosomes and spinal cord in case of *T. regenti* with the special focus is on the different acquisition of the nutrients. Visceral schistosomes reach the lungs within days after penetration depending on species. The highest concentration of lung schistosomula in mice is 5-6 days post infection (dpi) for *S. mansoni* and 3 dpi for *S. japonicum* (Gui *et al.*, 1995) lung infection of *S. haematobium* in hamsters is peaking in 9 dpi (Ghandour, 1978). The highest concentration of lung schistosomula of *T. szidati* in duck is 2-4 dpi (Haas and Pietsch, 1991; Chanová, Vuong and Horák, 2007). *Trichobilharzia regenti* is only known

schistosome with the neurotropic migration in the definitive host. Schistosomula reach the spinal cord via peripheral nerves 2 dpi and can be found in different parts of the spinal cord till the 15 dpi with the peak infection around 6 dpi (Hrádková and Horák, 2002) while migrating through the spinal cord schistosomula actively feed on the nervous tissue (Lichtenbergová *et al.*, 2011; Leontovyč *et al.*, 2019, publication No.2).

## **2.1 Nutrient acquisition via tegument**

In the opposite of cercariae, schistosomula have the “unlimited” source of nutrients once enter the definitive host. Schistosomula (in lungs or the nervous tissue) are “bathing” in the nutrients and are well adapted to maximize the uptake – the direct uptake via tegument and active feeding via oral sucker, followed by digestion in the gut.

### **2.1.1. Glucose uptake**

Schistosomes, as the members of the Neodermata, are covered by a layer called tegument or neodermis. It is a parasite-host syncytial interface consisting of two lipid membranes with nuclei situated under the basal membrane. The tegument is not only passive barrier but it is the biologically active place with many important processes including protection, excretion, signalling, osmoregulation and the nutrition (Jones *et al.*, 2004). In terms of the nutrients uptake, mechanisms or quantitative estimates of tegumental uptake of lipids and amino acids are not well known, however, tegument plays the major role in glucose uptake of *S. mansoni*. (Skelly *et al.*, 2014). It has been discovered, that the glucose uptake across the tegumental hydrophobic surface membranes is facilitated by Schistosome Glucose Transporter

Proteins (SGTPs) (Skelly *et al.*, 1994; Krautz-Peterson *et al.*, 2010). In total 3 different SGTPs were detected in *S. mansoni* - SGTP 1, 2 and 4 and only SGTP1 and SGTP2 exhibited the glucose transport activity (Skelly *et al.*, 1994), for details see reviews (Skelly *et al.*, 2014; You *et al.*, 2014). Specifically, SGTP4 has been identified only in intramamalian stages (Skelly and Shoemaker, 1996). Orthologues of SGTPs identified by Skelly and colleagues (Skelly *et al.*, 1994) were identified also in transcriptomes of *T. regenti* and *T. szidati*. However, the SGTP4 previously detected on protein level only in intramammalian stage, was upregulated in cercarial stage compared to 7 and 4 dpi schistosomula of *T. regenti* and *T. szidati* respectively (log<sub>2</sub>FC= -3.56 and -0.95) (Leontovyc *et al.*, 2016; Leontovyč *et al.*, 2019, publication No. 1, 2). Thus it seems that SGTP4 is rapidly transcribed in snail host, mRNA is carried by cercariae and ready for immediate translation once inside the definitive host.

## **2.2 Nutrients acquisition via the alimentary tract**

Within the life cycle of the schistosomes, only the life stages schistosomula and adults have functional alimentary tract. It consists of the oral cavity, oesophagus and the bifurcated blind intestine (caeca) (Jamieson, 2017). After cercarial/schistosomula transformation, schistosomula start to actively feed. The main source of nutrients for visceral schistosomes is blood (Caffrey *et al.*, 2004), to date only one species of bird schistosomes *T. regenti* is known for feeding on nervous tissue in schistosomulum stage rather on blood (Lichtenbergová *et al.*, 2011; Leontovyč *et al.*, 2019, publication No.2). The first phase processing of blood takes place in the oesophagus (Hall *et al.*, 2011) and it is facilitated by the molecular products of the oesophageal glands (Li *et al.*, 2013; Wilson *et al.*, 2015). One of the most studied proteins possibly involved in the oesophageal blood digestion are transcriptional/translational products of Micro



Exon Genes (MEGs) (DeMarco *et al.*, 2010; Hall *et al.*, 2011; Li *et al.*, 2013, 2015, 2018; Orcia *et al.*, 2017).

### 2.2.1 Micro Exon Genes (MEGs)

Micro exon genes were for the first time described in *S. mansoni* genome and as the name suggests they are consisting of the number of very short exons, which comprise 75% of the coding sequence (Berriman *et al.*, 2009). They seem to be unique for the family schistosomatidae since no similarity with annotated genes outside of *Schistosoma* spp. and *Trichobilharzia* spp. were found (Berriman *et al.*, 2009; Leontovyč *et al.*, 2016; Leontovyč *et al.*, 2019, publication No.1, 2). Almost all products of MEGs encode the signal peptide and they are likely to be secreted (Berriman *et al.*, 2009). Immunohistochemical localisation of MEGs combined with differential gene expression analysis of *S. mansoni* confirmed MEGs in the oesophageal gland and were predicted to be involved in the blood digestion including erythrocyte lysis, leucocyte tethering and killing and disposal of platelets preventing the coagulation (Wilson *et al.*, 2015). Products of MEGs seem to be essential for the survival of the schistosomes in the definitive host. For example, macaques infected by *S. mansoni* exhibit the self-curing process by blocking the SjMEGs 4.1, 8.2, 9, 11 by the IgG which stops the blood processing in the oesophagus, leading to the death of the worms by starvation (Li *et al.*, 2015). Taken together MEGs are important for the blood digestion of human schistosomes, however recent transcriptomic data of bird schistosomes *T. regenti* and *T. szidati*. confirmed the presence of the MEGs outside of the genus schistosoma and identified 4 and 10 transcripts of MEGs in *T. regenti* and *T. szidati* respectively. Detailed gene expression analysis showed the high expression of the MEGs in blood-feeding *T. szidati* schistosomula compared to *T. regenti*

schistosomula feeding on neural tissue (Fig. 2). In addition two transcripts annotated as the MEG-3.1 were the first and the sixth most expressed transcripts in *T. szidati* schistosomula. In opposite *T. regenti* schistosomula, feeding on nervous tissue, had markedly lower expression of MEGs than those in *T. szidati* (Fig.2, panel b) Therefore it seems that MEGs are not strictly linked with the visceral schistosomiasis, however, they are differentially involved in the feeding of neurotropic and visceral schistosomes.

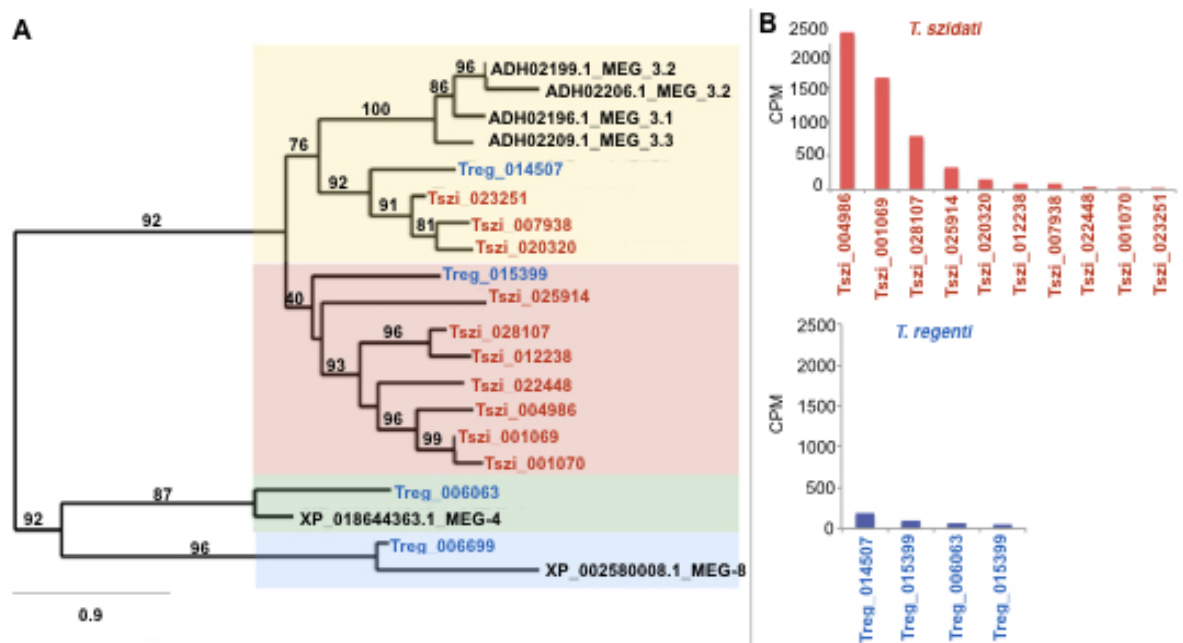


Figure 2. Phylogenetic relationships of protein sequences encoded by microexon genes (MEGs) among *Trichobilharzia szidati* (red), *T. regenti* (blue) and *Schistosoma mansoni* (reference taxon; black) constructed using a maximum likelihood (ML) tree building method, with nodal support values indicated (a), and a comparison of transcription levels (in counts per million reads, CPM) between *T. szidati* and *T. regenti* (b). (Leontovyč *et al.*, 2019, publication No.2).

### 2.2.3 Peptidases

The pre-processed food goes from the oesophagus to the bifurcated, blind-ended gut, where it is further processed. The predominant digestive function taking place in the schistosome gut is degradation of the macromolecular substrates obtained from the ingested food and extracellular absorption of the nutrients (Delcroix *et al.*, 2006; Hall

*et al.*, 2011). The gut is covered by the syncytial gastrodermis. It is few  $\mu\text{m}$  thick single layer of epithelial cells with numerous sheet-like lamellae extending the surface (Jamieson, 2017). The molecular composition of the gastrodermis is poorly known whereas the isolation methods are not well developed in contrast to tegument. Therefore the alternative approaches for the identification of the molecular composition have been performed such as mass spectrometry analysis of the vomitus (Hall *et al.*, 2011) or laser microdissection (Nawaratna *et al.*, 2011). Among the molecules analysed in the vomitus of the *S. mansoni* adults numerous hydrolases including peptidases and lysosomal carrier proteins were identified. This could suggest the extracellular secretion of lysosomal contents into the gut lumen. Apart of other identified molecules in gastrodermis, peptidases play the key role in the macromolecular substrate degradation (Caffrey *et al.*, 2004; Delcroix *et al.*, 2006). Peptidases are also important molecules for parasite-host cross-talk. Apart of interactions with the immune system and tissue lysis during migration, peptidases degrade the macromolecular substrates facilitating the acquisition of the nutrients (Caffrey *et al.*, 2004). With minor exceptions cysteine peptidases are synthesized in the gastrodermis as the inactive enzymes, then transported via the vesicles in to the gut lumen and activated by acidic pH (Dalton *et al.*, 2006), transactivated by another peptidases (Sajid, 2003) or activated by sulphated polysaccharides (Jílková *et al.*, 2014). During the breakdown of the ingested food, peptidases act as the network/cascade rather than as the individual proteins. In *S. mansoni* (3 weeks old worms), it was proposed, that initial cleavage of haemoglobin is facilitated by cathepsin D resulting two peptides further processed by cathepsins B and L which results into small peptides break down by cathepsin B, C, and leucine aminopeptidase. Final products are absorbable peptides and amino acids. During this proteolytic

cascade individual proteases may have a redundant role (Delcroix *et al.*, 2006). Aspariginyl endopeptidase (legumain) was proposed as the enzyme trans-activating the other peptidases (Dalton *et al.*, 1996; Sajid, 2003; Delcroix *et al.*, 2006), however, the activation is not fully dependent on legumain (Krautz-Peterson *et al.*, 2010). To date, most of the studies were focused on proteolytic digestion of the blood-feeders. Direct comparison of closely related schistosomes (genus *Trichobilharzia*) with different diet - nervous tissue vs. blood revealed different expression profiles of peptidases possibly linked to different feeding preferences (Leontovyč *et al.*, 2019, publication No.2). Among the peptidases previously described as acting in the degradation of the haemoglobin (Delcroix *et al.*, 2006), blood-feeding *T. szidati* schistosomula expressed in higher levels cathepsin B, D, L, C and legumain. Interestingly legumain showed the highest expression. Contrary schistosomula of *T. regenti* showed the highest expression of cathepsin B and other hemoglobinolytic enzymes were expressed in much lower rates and legumain showed the lowest expression among these peptidases (Leontovyč *et al.*, 2019, publication No.2). The different expression of the legumain between *T. szidati* and *T. regenti* may be explained by multiple roles of legumain in *Trichobilharzia* sp. While blood-feeding schistosomula of *T. szidati* express legumain in high rates it may play role in direct haemoglobin processing as previously described in *Paragonimus westrmani* and hard tick *Ixodes ricinus* (Choi *et al.*, 2006; Sojka *et al.*, 2007) and also may play role in trans-processing other peptidases - cathepsin B (Sajid, 2003) and cathepsin D (Sajid, 2003; Dalton *et al.*, 2006; Delcroix *et al.*, 2006). Schistosomula of *T. regenti* feed on neural tissue therefore high amount of the legumain for haemoglobin digestion is probably not needed and therefore legumain is expressed in much lower rates.

**Cathepsin B** is probably the most studied peptidase among schistosomes and was also along with legumain the first sequenced peptidase of schistosomes (Klinkert *et al.*, 1989). Specifically, cathepsin B1 was identified as the enzyme enabling degradation of macromolecular substrates such as haemoglobin, albumin and IgG in *S. mansoni* (Sajid, 2003) and albumin, fibrinogen, IgG, Collagen I and IV, myosin, myelin basic protein and poorly haemoglobin, in *T. regenti*. (Dvořák *et al.* 2005, Dvořáková *et al.*, 2019, publication No.3). In depth analysis of cathepsin B1 of *T. regenti* schistosomula identified 6 different isoforms of cathepsin B, two of them TrCB1.5 and TrCB1.6 appeared to be inactive due to the substitution of the catalytic cysteine for glycine in the active site (Dvořák *et al.*, 2005). First insight into larval transcriptome of *T. regenti* pointed out on the interesting highest expression of inactive isoforms TrCB1.5 and TrCB1.6 among all isoforms in schistosomula (Leontovyč *et al.*, 2016, publication No.1). According to further detailed study of transcriptomic data of *T. regenti* schistosomula it was observed that active TrCB1.1 and inactive TrCB1.6 are the top expressed isoforms of TrCB and exhibit the comparable expression rate (Dvořáková *et al.*, 2019, publication No.3). Beside the TrCB1.5 and TrCB1.6, the inactive forms of parasite peptidases are known also from other parasites e.g. legumain of *S. mansoni* (Caffrey *et al.*, 2000) or serine peptidase of *Sarcoptes scabiei* (Holt *et al.*, 2004) and one of the hypothesis is that inactive isoforms have a competitive regulatory role binding the peptidase substrates or inhibitors (Merckelbach *et al.*, 1994; Holt *et al.*, 2003, 2004; Dvořák *et al.*, 2005). In the light of new information about the high expression of TrCB1.1 in schistosomula of *T. regenti*, poor ability to degrade haemoglobin and high ability to breakdown the myelin basic protein it seems, that TrCB1.1 evolved into the molecule facilitating the migration and the nutrition uptake of *T. regenti* schistosomula which is adopted to the neural tissue environment.

Supplementary table

**S1 table** - Summary of large-scale transcriptomic studies of schistosomes focused on differential gene expression within the life cycle. Dpi - days post infection; Wk - weeks; HR - hours post infection

Organism sequenced	technology	eggs	miracidia	germ balls	sporocysts	cercariae	schistosomula <i>ex vivo</i>	schistosomula <i>in vitro</i>	adults	adults male	adults female	Study
<i>Schistosoma japonicum</i>	microarray	no	no	no	no	no	yes (3dpi)	yes (3dpi)	yes (mixed)	no	no	Chai et al., 2006
<i>S. japonicum</i>	Sanger	yes	yes	no	no	yes	yes (14dpi)	no	yes	yes (42-45dpi)	yes (42-45dpi)	Liu et al., 2006
<i>Schistosoma mansoni</i>	microarrays	no	no	no	yes	yes	no	no	yes (mixed)	no	no	Jolly et al., 2007
<i>S. mansoni</i>	microarrays	no	no	no	no	no	no	no	yes (mixed)	no	no	Verjovski-Almeida et al., 2007
<i>S. mansoni</i>	Long SAGE	no	yes	no	yes (6dpi, 20dpi in vitro)	no	no	no	no	no	no	Taft et al., 2009
<i>S. mansoni</i>	microarrays	yes	yes	no	yes (mother/dauther)	yes	no	yes (3-Hr, 24-Hr, 3dpi, 6dpi, 24dpi)	yes (2Wk, 3Wk, 5Wk, 7Wk)	7-Wk	7-Wk	Fitzpatrick et al., 2009
<i>S. japonicum</i>	microarrays	yes	yes	no	yes	yes	yes (3dpi)	no	yes	yes (4Wk, 6Wk, 6.5Wk, 7Wk)	yes (4Wk, 6Wk, 6.5Wk, 7Wk)	Gobert et al., 2009
<i>S. mansoni</i>	microarrays	no	no	no	no	no	no	yes (5-Hr, 5dpi)	no	no	no	Gobert et al., 2010
<i>S. mansoni</i>	microarrays	no	no	yes	no	yes	no	yes (3dpi)	no	no	no	Parker-Manuel et al., 2011
<i>S. mansoni</i>	Roche 454	no	no	no	no	no	no	no	yes (mixed)	no	no	Almeida et al., 2012
<i>S. mansoni</i>	Illumina	no	no	no	no	no	no	yes (skin x mechanically transformed)	no	no	no	Protasio et al., 2013
<i>S. mansoni</i>	Illumina	no	no	no	no	no	no	no	yes	yes (6-wk)	yes (6-wk)	Piao et al., 2014
<i>S. japonicum</i>	Illumina	no	no	no	no	no	yes (21dpi)	no	yes (mixed, 42 dpi)	no	no	Wang et al., 2015
<i>S. mansoni</i>	Roche 454	yes	no	no	no	no	no	no	no	yes (6-8Wk)	yes (6-8Wk)	Anderson et al., 2015
<i>S. mansoni</i>	Illumina	no	no	no	no	yes (unisex clones)	yes (3-5Wk)	no	yes	yes (49dpi)	yes (49dpi)	Picard et al., 2016
<i>Trichobilharzia regenti</i>	Illumina	no	no	no	no	yes	no	no	no	no	no	Leontovyc et al., 2016
<i>S. japonicum</i>	microarray	yes	no	no	no	yes	yes (14dpi)	no	yes (6Wk)	no	no	Cai et al., 2017
<i>Trichobilharzia szidati</i>	Illumina	no	no	no	no	yes	yes	no	no	no	no	Leontovyc et al., 2019
<i>S. mansoni</i>	Illumina/Roche 454/Microarrays	no	no	yes	yes	yes	no	no	no	no	no	Buddenborg et al., 2019

## Concluding remarks

Schistosomes have a complex life cycle requiring dramatic changes of particular developmental stages. One of the critical steps is the infection of the definitive host, where the cercariae have to swiftly react on transition between the aquatic environment and the environment inside the definitive host. It has to deal with the different osmotic pressure, different aerobic conditions, nutrition uptake, host immunity etc. Schistosomula migrate through the various tissues, actively feed, grow and develop and finally navigate through the tissues to reach the final localisation. During this process schistosomula are under constant pressure of the host immune system. The adaptations enabling successful infection of the definitive host can be observed on the molecular level using transcriptomic profiling of the different developmental stages and differential gene expression analysis. To date the large-scale transcriptomic studies were focus on few representatives of the genus *Schistosoma*. Presented thesis broadened the knowledge of the molecular biology of the schistosomatids with avian species. We explored the transcriptomic landscape of cercarial and schistosomulum stages of two schistosomes – visceral *T. szidati* and neurotropic *T. regenti* and compared the data with already published findings on human schistosomes. We found that the molecular mechanisms of cercarial stage possess common molecular mechanisms across different species such as carbohydrate and energy metabolism which is linked with the same aim of cercariae (regardless on species) – to cover the energy demands during the active seeking of the host and utilising same source of energy – glycogen stores. The differences between the cercariae of the human and avian schistosomes may be observed in mechanisms linked to different definitive hosts they penetrate to. For example different proteolytic enzymes used for penetration into mammal (*S. mansoni*) or bird (*T. regenti*).

Regarding to schistosomula we confirmed the different modes of nutrient acquisition using of light and electron microscopy – nervous tissue and blood for *T. regenti* and *T. szidati* respectively. We linked the microscopic observations with the transcriptional data and we observed different expression profiles of peptidases as well as the products of Micro Exon Genes (MEGs) and therefore we predicted the different role of these enzymes in the digestion of the blood and the nervous tissue.

We used transcriptomic-bioinformatics approach for our investigations, which is powerful tool for global overview, however subtle details may stay hidden. Also the predictions and hypothesis should be put into the context with parasite biology and already published wet-lab experiments. Nevertheless we strongly believe that our data provides new and significant insights into molecular biology of avian shistosomes, elucidate some aspects of the visceral and neurotropic schistosomiasis and can serve as a valuable resource for comparative studies of schistosomatids and other trematodes.



## Publication No.1

**Leontovyč R., Young N. D., Korhonen P. K., Hall R. S., Tan P., Mikeš L., Kašný M., Horák P., Gasser R. B. (2016): Comparative transcriptomic exploration reveals unique molecular adaptations of neuropathogenic *Trichobilharzia* to invade and parasitize its avian definitive host. PLoS Neglected Tropical Diseases 10: e0004406. DOI: 10.1371/journal.pntd.0004406 [IF2016=3.834]**

*Trichobilharzia regenti* is unique among schistosomes due to its neurotropic migration in the definitive host. Infection may cause serious neuromotor disorders of the permissive avian and accidental mammalian hosts. This publication is the first large-scale analysis utilizing transcriptomic-bioinformatic approach to identify key biological pathways and protein classes specific to two consecutive stages - free-living cercariae and parasitic schistosomula of both species.

Based on gene expression analysis cercariae of the *T. regenti* didn't much differ from well-characterized human schistosomes. They use the aerobic metabolism where limited stores of glycogen are utilised by glycolysis where the Krebs cycle is the main terminal of the carbohydrate breakdown followed by oxidative phosphorylation to cover high-energy demands of cercariae. Screening of cercarial peptidases didn't confirm the transcription of cercarial elastase - the main penetration enzyme of *S. mansoni*. The transcriptome of schistosomula revealed the microaerobic metabolism and an overview of the schistosomula peptidases provided valuable information about enzymes involved in digestion and migration of the worm.

### **My contribution:**

- Conceived and designed the experiments
- Performed the experiments
- Analyzed the data
- Wrote the manuscript

## Publication No.2

**Leontovyč R., Young N., Korhonen P., Hall R., Bulantová J., Jeřábková V., Kašný M., Gasser R., Horák P. (2019): Molecular evidence for distinct modes of nutrient acquisition between visceral and neurotropic schistosomes of birds. Scientific Reports 9: 1347. DOI: 10.1038/s41598-018-37669-2 [IF2018=4.011]**

The results of the larval transcriptome of *T. regenti* indicated mechanisms/molecules possibly involved in neurotropic life strategy of the *T. regenti* schistosomula. We constructed the larval transcriptome of related visceral schistosome *T. szidati* to identify the biological processes linked to visceral and neurotropic schistosomiasis. In this study, we particularly focused on distinct modes of nutrient acquisition. We linked the light microscopy and transmission electron microscopy (TEM) with *in silico* analysis and predicted the molecular mechanisms of nutrients uptake by blood-feeding and neurotropic schistosomes.

Using comparative transcriptomics we obtained data of different expression of micro exon genes (MEGs) between the *T. regenti* and the *T. szidati* to date only known from human schistosomes. Our data support the hypothesis of the involvement of the MEGs in blood digestion of visceral schistosomes. Detailed analysis of expression of peptidases in both species revealed the expression profiles of peptidases linked to the different life strategies of *T. regenti* and *T. szidati*.

### **My contribution:**

- Conceived and designed the experiments
- Performed the experiments
- Analyzed the data
- Wrote the manuscript

## Publication No.3

Dvořáková H., Leontovyč, R. Macháček T., O'Donoghue A., Šedo O., Zdráhal Z., Craik Ch., Caffrey C., Horák P., Mikeš L. (2020) **Isoforms of Cathepsin B1 in Neurotropic Schistosomula of *Trichobilharzia regenti* Differ in Substrate Preferences and a Highly Expressed Catalytically Inactive Paralog Binds Cystatin.** *Frontiers in Cellular and Infection Microbiology* 10: 66. doi:10.3389/fcimb.2020.00066 [IF2020=3.518]

Long-term research of *T. regenti* pointed out the prominent role of the cathepsin B1 (TrCB1) by schistosomula. It is possibly involved in migration and the nutriment digestion by the parasitic developmental stages. Previously several isoforms of TrCB1 have been identified including two TrCB1.5, TrCB1.6 with amino acid substitution of the catalytic cysteine in the active site. Those two isoforms appeared to be inactive.

This part of our research is focused on the in-depth characterization of the active and inactive recombinant forms of isoforms of the TrCB1 including substrate specificity, macromolecular substrate digestion and inhibition tests by host inhibitors. Transcriptomic data obtained from previous part of the research were used for detailed analysis of gene expression of particular isoforms, which revealed the surprising result of high expression of the inactive isoform.

### **My contribution:**

- Bioinformatic analysis – gene expression of cathepsin B isoforms

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