Title of the thesis: ***In vitro* cultivation of the trematode species *Trichobilharzia regenti*** (*In vitro* kultivace motolice *Trichobilharzia regenti)*

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Brief Summary Evaluation:

This thesis evaluates cercaria/schistosomulum transformation methods involving *T. regenti* as well as later parasite cultivation methods. Cercarial tail separation using the bead beating or the syringe method followed by cultivation in medium supplemented with some duck serum is the most optimal method described so far. Under these conditions worm growth and prolonged survival is evident. This is a key and valuable finding and should help in many future studies of *T. regenti* biology and biochemistry.

RECOMENDATION: I recommend this work for defense and give it an "**A**" evaluation.

Some comments and questions follow. Several are listed to provoke discussion and debate about various aspects of the described experiments. Some of these questions (e.g. numbered 1-3 and in bold text below) could be posed during the oral examination session at the thesis defense. The thesis is fine as is but the candidate should feel free, if time allows, to incorporate changes into a final version based on the comments below. Consider this report as containing suggestions only.

In the Abstract you write: "*In vitro* cultivation of trematodes enables closer understanding of their biology and parasite-host interactions". How exactly does cultivating the parasites enable a better understanding of parasite-host interaction?

English: "problem" not "problematic" here

Page 3: Similar question to the earlier one, how useful is transcriptomic analysis of parasites in culture (since they are outside their normal environment)?

Why is the "complex body structure" relevant in regards to cultivation?

Can you provide some primary source references for the statement that: "Cercariae then actively search for the definitive host"?

1. **The Haas et al 1997 paper does not examine skin penetration, only penetration of agar. Is this a good model for invasion? What do you**

make of the fact that this paper reports that acetabular gland contents were released not just by skin lipids but also "when the cercariae were damaged" or treated with toxins? The Haas paper also reports that the skin lipids "had a damaging effect on the cercariae". Why should this be? Why do you think that not all cercariae penetrate (only ~40% at best)?

Add a reference for the "about 60%" statement.

Page 4: The Haas and Haeberlein (2009) paper cited notes that "full skin penetration" occurs by about 6.5 min (paper fig 1c). Why do you say here that it "is completed within 30 min"?

Haas and Haeberlein had exposed themselves some time earlier to infection with *T. szidati* (Haas and van de Roemer 1998) before later conducted experiments with *S. mansoni* (reported in the Haas and Haeberlein (2009) paper). Might this impact their *S. mansoni* results?

While I have many questions about the results reported, the following papers might also be relevant in this section of the thesis:

W. Haas, R. Schmitt Characterization of chemical stimuli for the penetration of Schistosoma mansoni cercariae. I. Effective substances, host specificity Z. Parasitenkd., 66 (1982), pp. 293–307

W Haas, R. Schmitt Characterization of chemical stimuli for the penetration of Schistosoma mansoni cercariae. II. Conditions and mode of action Z. Parasitenkd., 66 (1982), pp. 309–319

W. Haas Schistosoma mansoni: cercaricidal effect of 2-tetradecenoic acid, a penetration stimulant Exp. Parasitol., 58 (1984), pp. 215–222

F.G. Austin, M.A. Stirewalt, R.E. Danziger Schistosoma mansoni: stimulatory effect of rat skin lipid fractions on cercarial penetration behavior
Exp. Parasitol., 31 (1972), pp. 217–224

Do you believe that <u>all</u> "schistosomula enter lungs and stay there till day 7 p. i."?
(See: Parasitol Res. 1998;84(4):338-42. *Schistosoma haematobium, S. intercalatum, S. japonicum, S. mansoni,* and *S. rodhaini* in mice: relationship between patterns of lung migration by schistosomula and perfusion recovery of adult worms. Rheinberg CE1, Moné H, Caffrey CR, Imbert-Establet D, Jourdane J, Ruppel A.

Also, this paper might help flesh out this section: The saga of schistosome migration and attrition. Wilson RA. Parasitology. 2009 Oct;136(12):1581-92. doi: 10.1017/S0031182009005708).

As you later point out, we found that neither of the fatty acids linolenic acid (1 mM) nor capric acid (0.1 mM) promotes the morphogenesis of a normal *S. mansoni* schistosomulum tegument (Int J Parasitol. 2000 Apr 24;30(5):625-31). This makes me suspicious that a lot of the results involving parasites and fatty acid exposure are artefactual. What is your opinion? (Likewise, what does the fact that "crude egg lecithin" impacts acetabular gland depletion (as you note on page 6) tell us?).

Add references for the timing in the nerves and migration rates.

Page 5: How many of them die during migration? How do you know? Is there a reference?

Why do you need worms clean of host tissue?

Mostly the cercariae swim. It is probably a good idea to describe “inching movements” here.

Page 6: Define “p.t.” here (then you do not need to on page 7).

Add a reference for the schistosomulum survival time.

Page 7: I’m not sure what “ca” means.

Do you think that the purpose of the loss of the cercarial glycocalyx is “to avoid immune reaction of host”? Is this the only purpose?

Is the SCIP-1 protein “paramyosin-like” or is it paramyosin?

I don’t fully understand the difference in esophageal morphology of cercariae versus schistosomula.

Add a reference to more/less tail shedding under different circumstances.

Page 11: I would add the composition of SCM here.

Say what the staining is in the figure 2 legend. Why exactly is the MC stained here?

Page 12: Do the papers you reference (Coultas and Zhang, 2012; Milligan and Jolly, 2011) rigorously compare the needle method versus e.g. plain vortexing to find the “best” method?

I think we need to be a little careful about the skin penetration method. My memory is that cercariae only penetrate when the skin is placed atop a high osmolar solution. They don’t penetrate if there is water on both sides of the skin barrier. Right? This makes it almost impossible to distinguish the impact of the skin alone versus exposure to the high osmolar solution. (My bet is that the latter is pretty much all that you need (and time). What do you think?).

Page 13: What does “gain” mean here exactly (and on page 11)? When do cercarial bodies become schistosomula? Do you think that the CBs generated by the different methods are the same thing? Are they comparable? Do we know how viable the different CBs listed in the table are?
In table 1 what does e.g. “59,5” mean? (or 35,8?). Explain in the legend.

Page 14: Add a reference for ELAC and *T. szidati*.

Page 15: Can you include some indication regarding the time frame involved in the different images shown in figure 3?

Page 16: Say what the "x" marks in table 3 mean. Were the *in vitro* worms never measured beyond 5 days or did they just not get bigger in culture?

Page 17: Tiny spelling error: "therefore".

Page 18: *Radix peregra* is first listed as the intermediate host used but then *R. lagotis* snails are mentioned (next paragraph). Is just one species used or both?

Do you wish to report here how many snails were routinely used and how much water? Also it might be useful to know how long previously the snails were infected (and how that was carried out)?

Page 22: Section 4.4. Should you put here how many cercariae in what volume?

How were you sure that any transformation occurred in the duck skin experiment. Perhaps everything happened only once you put the skin into medium? (I.e. Cercariae stuck to the skin but transfer to medium induced transformation?)

Page 25: 4.5.2, How many worms?

Figure 6 is great. (Can you indicate which were the 5 best in the figure?)

2. **Page 26: Why do you assume that tail removal is an important trigger for further development? (Also, page 75: Speculate as to how tail loss might accelerate c/s transformation.)**

Page 28: Say (as you do elsewhere) "fixed' not "fixated" here.

Don't you wash again after adding the labeled UEA I? If not, how do you know that the reagent is not just passively adsorbed (and not really bound)? (Ditto for FITC-phalloidin, page 33)

Page 31: How does the $MgCl_2$ solution relax the musculature? What does it look like if you do not do this?

Page 34: Say what "IHC" is here.

Page 37: Note how the schistosomula (whose gene expression we are examining here) were prepared. What criteria were used to assess that schistosomula were present (in the gene expression work).

3. **Page 44: How come there is no measure of variation in table 12? Without this, how can you know that anything is significantly best? In the legend, explain what significantly best means.**

Can you say if the parasites that had shed the glycocalyx were also (in general) the ones with empty glands? Would it be useful to add a column labeled "both"?

4. **Page 45: Might it be interesting to uncover what the duck serum coat is? What might it be? How might you figure out what it is? (Likewise, what do you think is in FBS but not duck serum (page 49) that kills the parasites?)**

Page 48: What are the bubbles? Why not seen with all methods?

Page 51: Since you mention the eye spots, indicate them in figure 23.

Did you notice any relationship between any particular treatment and the multiple pigment spots? Is it ok (correct) to call them all eyespots?

Page 52: What does it mean to say that "Ingestion of RBCs was observed on day 5"? Do all parasites ingest? Can you say what proportion?

Page 63: Sorry to hear that the anti-SGTP antibodies did not behave well – especially anti-SGTP1 not reacting with the *S. mansoni* positive control. (Page 73: the antibodies are polyclonal, not monoclonal, but against short SGTP peptides. They are >20 years old and may be losing potency).

Page 64: There's probably no need to list these genes again here. Just combine all information about them into either table 8 or table 15 (and eliminate the second table).

Page 65: Some of the PCR data are hard to interpret. Do you think that the GAPDH band is real? The qPCR data suggest that it is detectable but why does the 9dS GAPDH band seem bigger than the rest? (Or is that the only real positive here?). Also, detecting Axon guidance gene expression at 2d but not 5d, maybe a bit at 9d and certainly at 9dS is confusing.

Page 69: Do you mean "is dependent" rather than "in dependence"?

Page 71: Can you propose some experiments that might uncover what the "coat" exactly is and how come it still forms even after duck-skin penetration?