Wilfried Haas (Prof. Dr.)
Institut für Biologie
der Universität Erlangen-Nürnberg
Staudtstr. 5
D-91058 Erlangen
Tel. xx49-913164439
e-mail: whaas@biologie.uni-erlangen.de
homepage: http://www.biologie.uni-erlangen.de/parasit

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Assessments on the Ph D thesis of **Martin Kasny**: "Peptidases of Trematodes"

As I work with the infective stages of schistosomes and bird schistosomes, I followed up the work of Martin Kasny on peptidases of cercariae, and my group members and I are now happy to have his Ph D thesis in hands. A very important and useful part of this thesis is the comprehensive, complete review on the up-to-date status of the knowledge on trematode peptidases. In this review Martin Kasny not only summarizes the crowd of published facts. Important is that he in addition evaluates the published results and that he pulls the data together with their biological function and evolution. This is a difficult task, as the various proteases contribute to an enormous diversity of biological processes. Martin Kasny solves this problem by inserting much background information into comprehensive tables, into footnotes and figures. I like footnotes such as on missinterpretations (p. 37) or skilful informative figures such as Fig. 4, p. 36 and Fig. p. 38 (an unimportant exception is Fig. 1, p. 13, where parasite stages and hosts are mixed up). Such a professional review on trematode proteases is desired by many parasitologists and it would be worth to be published.

The practical research work of Martin Kasny is presented in 4 published papers. The treated topic fills an important gap in the research on schistosomes and on trematodes at large. A main problem of schistosome work is that most researchers deal with *Schistosoma mansoni* (a main reason is, that this species is easy to maintain in the laboratory), and that the results on *S. mansoni* are often considered to be valid for all trematodes. The schistosome research group of Prague deals with (among other species) 2 bird schistosome species, and most of the results of this group relativize the generalised *S. mansoni* results. An important conclusion from this work is that parasite biology and parasite-host relationships are much more diverse than expected. This fully applies also to the work of Martin Kasny. The advantage of this work is that

it was performed comparatively (considering different species) and that Martin applied a high diversity of methods (including purification and biochemical characterization of peptidases, application of specific inhibitors, immunhistochemistry, sequencing of peptidases by mass spectrometry methods, cloning and expression of recombinant enzymes etc. etc.). Among the results in the papers of Martin Kasny, I consider the following as the most important:

The contents of the cercarial acetabular glands were obtained by exposing the cercariae of the bird schistosomes to various stimuli. The two species responded very differently to the stimulus Praziquantel, and this indicates that each of the species uses its own host-invasion strategy. Moreover, the secreted products did not react to a serum raised against *S. mansoni* elastase and this suggests that bird schistosomes obviously have evolved a fully different strategy of skin invasion than the model organism *S. mansoni*. This was confirmed by the succeeding comprehensive studies with cercarial extracts and secretions of penetration glands of *Trichobilharzia regenti* and *S. mansoni*. Important was that the work was carried out with both species comparatively. *T. regenti* cercariae obviously do not dispose on the well known elastase, but they secrete a cysteine peptidase, which was carefully characterized as catepsin B-like enzyme. As the enzyme was also secreted by *S. mansoni* cercariae, and as it degrades the skin compounds keratin and collagens, it seems to have an important function in the skin invasion of both species. Therefore, this work with the bird schistosome and its individual strategy of skin invasion provides also new insights into the skin invasion of the intensively studied *S. mansoni*.

Martin Kasny has, by applying a high diversity of modern methods, skilfully elaborated a high amount of new findings on schistosome cercarial proteases. Therewith he has contributed considerably to a better understanding of schistosome skin invasion. Nevertheless, a great part of the complex skin invasion processes is still not understood and we are in a state where we only can speculate and formulate hypotheses. As an opponent, I would like to ask questions on such topics, which are still not understood. It would be interesting to hear Martins ideas on the following topics where he is the expert, and my group and I speculate with much less expertise: (1) Schistosome cercariae have these two types of acetabular glands with different contents. We found that *T. szidati* cercariae are able to secrete mainly the circumacetabular or mainly the postacetabular gland contents (by specific contractions, sorry, not yet published). However, the gland ducts are assemblied together in bundles, and the parasites secrete always also certain

amounts of the other gland type. What are your speculations on the function of this strategy? Is there an advantage to always secrete also a bit from the other gland type?

(2) We observed the entry of *T. szidati* and *S. mansoni* cercariae into our "living human skin" (sorry, *S. mansoni* not yet published). We were surprised, that the bird schistosome *T. szidati* entered the human skin significantly faster than *S. mansoni*, the specialist for humans (*T. szidati* within a mean of 4.0 min, *S. mansoni* within 6.4 min). Can you speculate, why *T. szidati* is the better skin-invader?

(3) Much is known on schistosome skin invasion, and you contributed nice data for a better understanding of this topic. However, much future research work is still necessary until we really know what happens in skin invasion of the different species. My question to you is: When you would be provided with unlimited funding (a dream of most researchers), on which questions would you focus, and which methods would you apply?

Finally I want to strongly and without any reservation recommend that Martin Kasny should obtain the Ph D degree and I (and I am shure other Schisto researchers also), hope very much that Martin will have the chance to continue his successful research, and I am looking forward to his future results!

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(Wilfried Haas, Prof. Dr.)

Mgr. Martin Kašný
Charles University in Prague
Faculty of Science
Department of Parasitology
Viničná 7
Prague
12800, Ctech Republic
Tel. 0042-221951816
e-mail: kasa@post.cz

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Reply to questions asked in the assessment of Ph.D. thesis of Martin Kasny: "Peptidases of Trematodes" by the reviewer Prof. Dr. Wilfried Haas.

1st question: *Schistosome cercariae have these two types of acetabular glands with different contents. We found that T. szidati cercariae are able to secrete mainly the circumacetabular or mainly the postacetabular gland contents (by specific contractions, sorry, not yet published). However, the gland ducts are assembled together in bundles, and the parasites secrete always also certain amounts of the other gland type. What are your speculations on the function of this strategy? Is there an advantage to always secrete also a bit from the other gland type?*

For some schistosome cercariae, e.g., *Schistosoma mansoni* or *Trichobilharzia szidati* it was documented that the contents of particular penetration glands (circum- and postacetabular) could be released separately. Partly independent gland release system is probably based on specific chemical stimuli. It was reported that the hydrophobic compounds (lipids, fatty acids) stimulate mainly secretion from cercarial circumacetabular glands and hydrophilic compounds stimulate secretion from postacetabular glands.

Above all, I believe that it is probable that vehement muscle contraction of cercarial body after specific chemical stimulation leads to the release of a bit of content of the non-stimulated gland type. That is because the interspace of each duct is usually filled up by content of appropriate gland. Due to this fact and due to morphological bundling (connection) of penetration glands ducts it is likely that during stimulation and release of one gland type a residual amount of the content originated from the duct of the non-stimulated gland is released, too.

Although our results (Mikeš et al. 2005) showed that the contents of both gland types are released simultaneously, it might be an artefact caused by testing *in vitro*. Yet we believe that separation of different gland contents and interconnection of ducts leads rather to the vision of "at the right time" blending of appropriate substances. There are several hypotheses explaining the possible significance of two separate gland systems.

The particular components of *S. mansoni* or *T. szidati* cercarial separate gland compartments (circum- and postacetabular) could be stored in their inactive forms until the point of their release.

We hypothesized (Mikeš et al. 2005), that some of the gland biomolecules could be activated simply by the influence of external conditions (e.g. pH) after release. But also other gland molecules could be necessary for their activation, which could be reached after the interfusion with components originating from the different gland type. Although this activating system has not been satisfactorily described, it could be useful for protection of cercarial body structures against the damage during the storage of molecules in their active forms (such as peptidases).

The other function of separate cercarial penetration glands and mixing of their components could be the possible cross-linking of substances from one gland type (circumacetabular) by some agents from the second gland type (postacetabular). This may be the case of postacetabular mucopolysaccharides of *S. mansoni*, which probably could be polymerized by circumacetabulat calcium ions - this would form the well known sticky glue-like substance enabling tight attachment of cercariae to the host surface.

2nd question: *We observed the entry of T. szidati and S. mansoni cercariae into our "living human skin" (sorry, S. mansoni not yet published). We were surprised, that the bird schistosome T. szidati entered the human skin significantly faster than S. mansoni, the specialist for humans (T. szidati within a mean of 4.0 min, S. mansoni within 6.4 min). Can you speculate, why T. szidati is the better skin-invader?*

It was published that the penetration of *S. mansoni* or *T. szidati* cercariae is similarly stimulated by free fatty acids originated from upper layers of the host skin. The low amount of free fatty acids among duck-foot skin lipids was revealed in contrast to human skin. It could imply that *T. szidati* has developed more sensitive system for perception of penetration stimuli (free fatty acids). Therefore, the stimulation of *T. szidati* cercariae by fatty acid-rich environment of human skin could lead to increased signalization for release of cercarial gland content, followed by faster penetration into the host skin than in the case of *S. mansoni* stimulated by standard dose of free fatty acids.

The faster penetration of *T. szidati* than *S. mansoni* cercariae could cohere also with different composition of human and duck-foot skin. Duck-foot skin possesses thicker keratinized surface layer, which is probably more difficult to disrupt, although *T. szidati* cercariae are equipped to penetrate this barrier relatively fast. Therefore, it is probable that *T.*

szidati cercariae would penetrate even faster through the "more delicate" human skin than *S. mansoni* cercariae for which is the penetration through human skin natural.

Finaly a serine peptidase - elastase - is thought to be the main penetration enzyme of *S. mansoni* cercariae. Although elastase was not identified in the related species *S. japonicum*, it was proved that *S. japonicum* cercariae can migrate through the human skin faster than those of *S. mansoni*. For the bird schistosomes *T. regenti* and *T. szidati*, no elastase gene was identified and no elastase activity was recorded, too. It implies that *S. japonicum*, *T. regenti* and *T. szidati* probably use other highly-active peptidase(s) which consequently enable faster penetration of these cercarial species into their hosts compared to *S. mansoni*. Based on our results we suggest that at least in bird schistosomes these peptidase may be members of papain-like family of the cysteine peptidase class (cathepsins B).

3rd question: *Much is known on schistosome skin invasion, and you contributed nice data for a better understanding of this topic. However, much future research work is still necessary until we really know what happens in skin invasion of the different species. My question to you is: When you would be provided with unlimited funding (a dream of most researchers), on which questions would you focus, and which methods would you apply?*

I suppose that for better understanding of parasite (cercarial) penetration process it could be very effective to combine several modern approaches and techniques such as mass spectrometry proteomics, genomics or computational genomic, 3D computational modeling, transgenomics or biomonitoring. The routine, fast and progressive application of all these combined approaches could lead to fast-mined robust results subsequently exploited for development of e.g. effective chemotherapeutics against various parasites (a dream of each parasitologist).

For a better understanding I will demonstrate it on a hypothetical example with e.g. *T. regenti* cercariae.

A dominant 34 kDa protein antigen from *T. regenti* cercarial excretory–secretory products was determined on Western blots with sera from infected mice and patients with cercartial dermatitis (that is real).

Although the genome of *T. regenti* is not characterized, by use of modern mass spectrometry methods (MALDI TOF-TOF, LC MS/MS) we are able to determine the partial amino acid sequence of the unknown 34 kDa protein antigen. After the yield of at least 6-7 aminoacids we could prepare degenerate primers and in few steps we would be able to clone the part of appropriate gene followed by obtaining of the whole gene sequence coding for the 34 kDa antigen. Applying the computational analysis to DNA or amino acid sequence

combined with computational 3D modeling, we could estimate several important biochemical characteristics (e.g. pI, 3D protein folding, potential glycosylation sites etc.) and the theoretical function of this protein in cercarial body. Using other molecular techniques, we could than express the recombinant 34 kDa protein and raise the specific antibodies against it to localize the 34 kDa protein in the cercarial body. The ultrastructural localization could as well closely determine the protein function estimated according to genomic data in the step before.

Theoretically, it would be also possible to prepare transgenic *T. regenti* cercariae (by e.g. biolistic technique using miracidia) or silence some genes to (bio-)monitor cercarial life accomplishment like the ability to penetrate or changes in parasite(schistosomula)-host interactions with the down-regulated gene.

The results of all these combined techniques would help to determine the real biological function of various proteins. On this base it would be then possible to construct e.g. effective inhibitors (in the case of bioactive molecules such as peptidases) or develop an effective vaccine. The effectiveness of potential inhibitors could be tested by computational 3D modeling.

I mean that understanding of biological functions of particular essential molecules is very important and could subsequently help to complete the mosaic of understanding the whole complicated parasite life processes, like the invasion by cercariae too.