**Charles University**
**Faculty of Science**
**Department Parasitology**

# INTERACTION *PHLEBOTOMUS–LEISHMANIA*

**Ph.D. Thesis**

**MARTINA HAJMOVÁ**

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**Thesis advisor: prof. RNDr. Petr Volf, CSc.**

**Curriculum vitae:**

**Martina Hajmová**
January 29, 1974, Vrchlabí, Czech Republic

1992 – 1997 - MSc. studies, Dept. Zoology, Faculty of Sciences, Charles university Prague (Topic of Diploma Thesis: Anatomy, biology and ecology of *Tectocepheus velatus* (Acari, Oribatida)).

1999 – 2003 - postgraduate study, Dept. Parasitology, Faculty of Sciences, Charles University Prague (Topic of Thesis: Interaction *Phlebotomus - Leishmania)*

2002 – 3 months of external laboratory experience in Centro de Pesquisas Rene Rachou, Belo Horizonte (prof. P.F.P. Pimenta)

2003 – present – Sales and Product Manager, Olympus C&S, Prague

**Address**: Martina Hajmová
Vítězná 1282
Vrchlabí 543 01
**e-mail:** hajmova@centrum.cz
**phone:** +420 602 566 293

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**Thesis advisor:**
prof. RNDr. Petr Volf, CSc., Department of Parasitology, Faculty of Science, Charles University, Prague

**Reviewers:**
prof. RNDr. Libor Grubhoffer, CSc., Faculty of Biological Sciences, University of South Bohemia, České Budějovice

RNDr. Oleg Ditrich, CSc., Academy of Science of The Czech Republic, České Budějovice

**Examination commitee:**
doc. RNDr. Jan Tachezy, Ph.D., Faculty of Science, Charles University, Prague - head

prof. RNDr. Libor Grubhoffer, CSc., Faculty of Biological Sciences, University of South Bohemia, České Budějovice

prof. RNDr. Petr Horák, Ph.D., Faculty of Science, Charles University, Prague

doc. RNDr. Václav Hypša, CSc., Academy of Science of The Czech Republic, České Budějovice

RNDr. Eva Nohýnková, Ph.D., 1st Faculty of Medicine, Charles University, Prague

prof. RNDr. Jiří Vávra, DrSc., Faculty of Science, Charles University, Prague

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Selected abstracts

**Hajmova, M.,** Volf P., Kolli B. K., Chang K.-P. (2001) GP63 expression of *Leishmania amazonensis* affects their early infection in the midgut of Lutzomyia longipalpis. Abstract in Abstract Book, WorldLeishII, Hersonissos, Crete, May 2001, p.124.

**Hajmova, M.**, Volf P., Kolli B. K., Chang K.-P. (2002) The Role of *Leishmania* Surface Metaloprotease gp63 in Sand Fly Vector. Journal of Eucaryotic Microbiology 58, p.17A.

**Hajmova, M.**, Votypka, J., Volf, P. (2002) Blocked stomodeal valve: similar mechanism of transmission in two trypanosomatid models. Entomología y vectores 9 (Supl. 1) ISOPS IV. p. 131.

Volf P., Dvorakova E., **Hajmová M.**, Skarupova S. and Svobodova M. (2002) Lectins and glycoproteins in sand fly midgut. Entomología y vectores 9 (Supl. 1) ISOPS IV. p. 80.

## Introduction

The leishmaniases are diseases with wide epidemological and clinical diversity caused by different parasite species belonging to the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). *Leishmania* have dimorphic life cycle consisting of extracellular promastigotes that multiply and develop within the alimentary tract of the sand fly vector, and intracellular amastigotes that reside and multiply within the phagolysosomal vacuoles of their host macrophages. Phlebotomine sand fly (Diptera: Psychodidae) are the only known natural vectors of all *Leishmania* species. During the life cycle in the vector parasites have to undergo several potential barriers. The barriers include the digestive enzymes in the gut, that might inhibit the early growth of parasites in the blood meal; the peritrofic matrix, that might behave as physical barrier to parasite migration out of the abdominal midgut; the excretion of digested bloodmeal, that might result in loss of infection from the gut; the anatomy and physiology of the anterior gut, that might prevent the forward movement of metacyclic promastigotes and their egestion during bloodfeeding into the host tissue.

This work was concerned with some aspects of interaction *Phlebotomus – Leishmania.* Three main topics were studied in this work – susceptibility of sand flies for different *Leishmania* species, the role of metaoproteases gp63 during the development in the vector and the mechanism of “regurgitation” theory.

Different species of phlebotomine sand flies have different microhabitat of the gut - *Leishmania* adaptation to this environment is species-specific. Some sand fly species transmitt one *Leishmania* species only while the others support development of various parasite species. Subgenus *Adlerius* (Diptera: Psychodidae) comprises about 20 known species in Eurasia; some are suspected vectors of visceral leishmaniasis and at least one has been implicated as a vector of cutaneous leishmaniasis. Species specifity has not been determined yet, because the taxonomy of subgenus Adlerius is extremely

difficult and some species are recognized only on the basis of male morphology. Furthermore the experimental colonies of subgenus *Adlerius* are very rare worldwide. The *Phlebotomus (Adlerius) halepensis* vector competence for *Leishmania major* and *L. tropica* was carefully studied in this work and was compared to natural models.

The factors which are involved in adaptation mechanism of *Leishmania* during colonization of the sand fly gut are not clearly known. Surface metalloprotease gp63 is supposed to be involved in this interaction. It has been suggested that gp63 may degrade hemoglobin and other proteins in the blood meals, thereby providing nutrients needed for the growth of promastigotes. On the other hand, in laboratory model *Phlebotomus papatasi / L. major* the gp63-deficient parasites developed similarly to the controls. We tested *Lutzomyia longipalpis* females fed with promastigotes of a *Leishmania amazonensis* clone whose gp63 was up- and down-regulated and compared infections in early and late stages.

Transmission from an insect vector to a vertebrate host is a key moment in the life cycle of heteroxenous parasites. The regurgitation of metacyclic stages of *Leishmania* from the sand fly cardia is thought to be prevailing mechanism of *Leishmania* transmission. We studied if this regurgitation results from the damage of the stomodeal valve caused by *Leishmania* parasites.

**Aims of the work**

- **Study the susceptibility of *Phlebotomus (Adlerius) halepensis* to *Leishmania major* and *L. tropica***

- **Accomplish the experimental transmission of *L. major* by *Phlebotomus (Adlerius) halepensis, Ph. duboscqi* and *Lutzomyia longipalpis***

**List of Publications**

Original Papers


**Hajmová, M.** and Smrž, J. (2001) Reproductive and Nutritional Biology of *Tectocepheus velatus* (Acari : Tectocepheidae) in different biotopes. Proceedings of the 10th International Congress of Acarology: 255 - 261.

Sadlova, J., **Hajmova, M.**, Volf, P. (2002) *Phlebotomus halepensis* : susceptibility to *Leishmania major* and *L. tropica* infections and host feeding preferences. Medical and Veterinary Entomology: 17: 244-250.

**Hajmová, M.**, Chang, K.P., Kolli, B., Volf, P. (2004) Down regulation of gp63 in *Leishmania amazonensis* reduces its early development in *Lutzomyia longipalpis.* Microbes and Infection 6: 646-649.

Volf, P., **Hajmová, M.**, Sádlová, J., Votýpka, J. (2004) Blocked and damaged stomodeal valve of the vector: similar mechanism in two Trypanosomatid – Diptera (Nematocera) models. International Journal for Parasitology 34: 1221-1227.

Secundino, N.F.C., Nacif-Pimenta, R., **Hajmova, M.**, Volf, P., Pimenta, P.F.P. (2005) Midgut muscle network in *Lutzomyia longipalpis* and *Phlebotomus duboscqi* sand flies: spatial organization and structural modification after blood meal. Arthropod Structure & Development 34: 167-178

and an unidentified avian *Trypanosoma* from *Trypanosoma corvi* clade, respectively. Females with the late-stage infections were processed for the optical and transmission electron microscopy. Localization of the parasites and changes to the stomodeal valve were in some aspects similar in all vector–parasite pairs studied: (i) a large plug of flagellates was observed in cardia region, (ii) parasites were attached to the chitin lining of the stomodeal valve by the formation of zonal hemidesmosome-like plaques. *Leishmania* promastigotes were found both attached to the valve as well as unattached in the lumen of midgut. The stomodeal valve of infected sand flies was opened, its chitin lining was destroyed and the unique filamentous structures on the apical end of cylindrical cells were degraded. In the *Culex*– *Trypanosoma* model, the whole population of epimastigotes was found in close contact with the chitin lining, and degenerative changes of the valve were less pronounced. We suggest that the phenomenon involving a blocked valve facilitating the regurgitation of parasites into the vertebrate host may occur generally in heteroxenous trypanosomatids transmitted by the bite of nematoceran Diptera.

- **Identify the host preference of *Phlebotomus (Adlerius) halepensis***

- **Study the role of gp 63 in a vectorial part of the *Leishmania* life cycle using *L. amazonensis* mutants differing in gp63 production**

- **Study the pathological changes in stomodeal valve of infected sand flies**

- **Describe the possible mechanism of transmission of some trypanosomatid parasites into the host**

## Materials and Methods

The *Phlebotomus (Adlerius) halepensis* colony (Jordan origin), *Lutzomyia longipalpis* (from Jacobina, Brazil), *Phlebotomus (Phlebotomus) duboscqi* (Senegal origin), *Phlebotomus (Paraphlebotomus) sergenti* (Turkish origin) and *Culex pipiens quinquefasciatus* (India) were used for experiments. Sand fly larvae were fed on composted and powdered mixture of rabbit faeces and rabbit food pellets with addition of dried Daphnia powder, mosquito larvae were kept under standard conditions. Adults were maintained at >70% relative humidity and 25–26C in 14 : 10 h light : dark photoperiod. Both sexes had access to dietary choice of 50% sucrose and 50% honey. Once or twice a week, females were allowed to feed on mouse, hamster, rabbit or human volunteer.

The parasite strains *Leishmania major* LV561 (MHOM/IL/67/LRC-L137 Jericho II), *L. tropica* (MHOM/TR/99/Vedha), *L. chagasi* (syn. *infantum*) M4192 (MHOM/BR/76/150406), *L. amazonensis* LV78 (MPRO/BR/72/M1845) and *Trypanosoma sp.* Cul1 (ICUL/CZ/1999/CUL1) isolated from *Culex pipiens pipiens* were used for experiments and were maintained alternatively

in SNB-9 blood agar and BALB/c mice (*L. major*) or hamster (*L. tropica*).

Sand fly females were infected by feeding through a chickskin membrane on suspension of amastigotes or promastigotes (final concentration $10^6$ parasites/ml in sand flies infections and $10^7$ in mosquito infections). Engorged females were separated, maintained in the same conditions as the colonies and dissected in intervals described in each experiment separately. The location was determined by dissection and examination under a light microscope. The infection intensity was estimated by scoring the proportions of sand flies with light (<100 parasites/gut), moderate (100–500 parasites/gut), heavy (500–1000 parasites/gut) or very heavy (>1000 parasites/gut) infections in the gut lumen. This method is only semiquantitative, but for weak infections provides more accurate data than the hemocytometer counting and also incorporates the promastigotes attached to the midgut tissue.

For light and transmission electron microscopy the engorged female sand flies and mosquitoes were anaesthetized on ice and fixed in 4% glutaraldehyde in PBS for 24 h at 4 C. Then, the samples were washed with PBS and post-fixed in 1% osmium tetroxide for 1h, dehydrated in a graded ethanol series and propylene oxide and embedded into Epon. For light microscopy, semithin sections (1 mm thick) were stained with toluidine blue. Thin sections of the cardia region were mounted on carbon-coated copper grids with Formwar film and stained with uranyl acetate and lead citrate and observed with 1200 JEOL electron microscope. Uninfected females, 10–12 days post blood meal, were processed in the same way and used as controls.

## Results

We tested *Phlebotomus (Adlerius) halepensis* Theodor (Jordan strain) for vector competence, compared with three standard vectors of cutaneous leishmaniases: *Phlebotomus (Phlebotomus) duboscqi, Phlebotomus (Paraphlebotomus) sergenti* and *Lutzomyia longipalpis. Phlebotomus halepensis* showed high susceptibility to both, *Leishmania major* and *L. tropica*, supporting typical suprapylarian parasite development similar to the other vectors. Development of infections was relatively fast, colonizing the thoracic midgut by 6 days post-bloodmeal in every case and reaching the stomodeal valve in >80% of flies. Host choice experiments in the laboratory showed that *P. halepensis* females fed readily on rat or rabbit and preferred the human forearm. In view of its vector competence and partial anthropophily, we infer that *P. halepensis* is a potential vector of cutaneous as well as visceral leishmaniases.

The zinc protease (gp63) of promastigotes was found to play a role in the sand fly part of the *Leishmania* life cycle. *Lutzomyia longipalpis* females were fed with promastigotes of a *Leishmania amazonensis* clone whose gp63 was up- and down-regulated by directional cloning into P6.5 for sense- and anti-sense transcription. Early development was found to differ significantly between the sense- and anti-sense transfectants 2 days post-feeding. The sense transfectants overexpressing gp63 were found similar to those with the vector alone: both developed in the gut at high rates of ~90–100% and at a high density with moderate to heavy parasite loads in >70% of the infected females. In contrast, the anti-sense transfectants with gp63 down-regulated developed at a lower rate (~70%) and, significantly, at a very low density, with moderate to heavy parasite loads only in ~30% of the infected females. On day 9 post-feeding, all three groups of transfectants developed at a similar rate of ~50% with comparable parasite loads. Thus, gp63 plays a role at the early stage of *L. amazonensis* establishment in *L. longipalpis.*

The regurgitation of metacyclic stages from the sand fly cardia is thought to be the prevailing mechanism of *Leishmania* transmission. This regurgitation may result through damage of the stomodeal valve and its mechanical block by the parasites. We found this phenomenon in three sand fly–*Leishmania* models and also in avian trypanosomes transmitted by *Culex* mosquitoes. *Phlebotomus duboscqi, Phlebotomus papatasi, Lutzomyia longipalpis,* and *Culex pipiens* were membrane-fed on blood containing *Leishmania major, Leishmania chagasi* (syn. *infantum*)