CHARLES UNIVERSITY, FACULTY OF SCIENCE,

DEPARTMENT OF PARASITOLOGY

Ph.D. study programme: Parasitology

Summary of the Ph.D. Thesis

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Critical factors affecting pathogen development in sand flies

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Abstract

This thesis deals with barriers and factors critical for development of viruses, leishmania and gregarines in sand flies.

First, we focused on life cycle of sand fly-borne phleboviruses, especially possible routes of sand fly infection. As a laboratory model we chose Massilia virus (MASV), species closely related to Toscana virus, which is main causative agent of summer meningitis in Mediterranean area. We tested different ways of infection by MASV in various developmental stages of *Phlebotomus perniciosus*; infection of (i) first (L1) and fourth (L4) instar larvae through larval food, (ii) females by blood meal, (iii) both sexes by sugar meal. Infection of L1 and L4 by larval food and subsequent transstadial MASV transmission to adults were not efficient; from 875 adults only three were MASV-positive. Infection through bloodmeal led to high infection rate before defecation, nevertheless, post defecation the infection rate declined and only 5 out of 27 females were MASV-positive. The most efficient infection way was through the sugar meal: 72% of females (88 out of 122) and 51% of males (58 out of 113) were detected as MASV-positive. Moreover, both males and females infected by this way released MASV particles into the drop of sugar which stayed infectious for next 24 hours for other naïve sand flies; almost 30% *P. perniciosus* became infected after feeding on this sugar with regurgitated virus. We suppose that common feeding of infected and uninfected sand flies on the same sugar meal could be important part of *Phlebovirus* circulation in the nature.

Sand flies are well-known vectors of leishmania and sand fly peritrophic matrix (PM) was proposed as important barrier for leishmania development in some sand fly species, especially *Sergentomyia schwetzi.* We experimentally confirmed this theory by addition of *Beauveria bassiana* chitinase into infectious bloodmeal of *S. schwetzi*. In chitinase-treated *S. schwetzi* the PM was disrupted earlier and *Leishmania major* and *Leishmania donovani* had enough time to escape into ectoperithrophic space and develop mature infection with metacyclic forms and colonization of the stomodeal valve. In control group, no leishmania were able to survive defecation of bloodmeal remnants and all infections were lost.

As shown in mosquitoes, ambient temperature during both larval and adult life affects vector competence. We tested impact of different larval rearing temperature (27°C and 32°C) on susceptibility of *Phlebotomus sergenti* females to *Leishmania tropica*. Larvae

kept at higher temperature developed faster and produced smaller females, nevertheless infection rate or intensity of infection *L. tropica* did not differ between groups maintained at different temperature. Interestingly, increase of temperature during larval development eliminated gregarines *Psychodiella sergenti*; all sand flies emerged from larvae and pupae maintained at 27°C were infected with gregarines, with the mean number of gamonts per individual 29.5. In contrast, only in three adults out of 120 developed from larvae and pupae kept at 32°C were found positive for gregarines.

Finally, leishmania and gregarines may naturally co-occur in sand flies. In mosquitoes it was shown that the presence of gregarines affects development of other pathogens. Therefore, we decided to test whether the presence of gregarine *Ps. sergenti* in sand flies *P. sergenti* affects development of *L. tropica*. However, we did not find any significant differences in intensity of infection and infection rate of *L. tropica* between females infected and non-infected by gregarines.

Introduction

Insect-borne pathogens have to overcome various barriers during their development inside the vector; these are not just the physical barriers (i.e. peritrophic matrix, midgut wall or basal lamina), but also activity of immune system, natural gut microflora or the presence of another pathogen. Moreover, the ability to overcome these barriers could be influenced by environmental factors, most important of which is definitely temperature. The interaction between vector and pathogen is complex and different barriers play a role in various pathogen-vector pairs. In introduction of my thesis I described factors affecting development of arboviruses, leishmania and gregarines in sand flies.

Aims of the thesis

Aims of this thesis were to contribute to the study of barriers and critical factors which affect development of pathogens transmitted by sand flies, namely viruses, leishmania and gregarines.

The main objectives of the study were:

1. clarify the life cycle of sand fly-borne viruses by study different ways of infection *Phlebotomus perniciosus* by Massilia virus (MASV)

2. verify the hypothesis that peritrophic matrix of *Sergentomyia schwetzi* represents the main barrier for *Leishmania* development

3. test if ambient temperature during larval stage affects vector competence of *Phlebotomus sergenti* females to *Leishmania tropica*

4. study whether presence of gregarine *Psychodiella sergenti* in sand fly *P. sergenti* influences development of *L. tropica*

Summary and conclusions

This thesis summarizes results of three publications in peer-reviewed journals and one manuscript. It is focussed on natural barriers and factors which may limit development of viruses, leishmania and gregarines in sand flies.

Firstly, we were interested in the life cycle of sand fly-borne viruses of genus *Phlebovirus*. Phleboviruses are causative agents of human diseases with variety of clinical syndromes but very little is known about their circulation in nature. Their transovarial and sexual transmissions are not efficient enough to keep virus circulation (Tesh and Modi, 1987; Tesh et al., 1992) and no vertebrate reservoirs were found. To study the possible transmission routes of phleboviruses, we chose MASV, non-pathogenic species closely related to Toscana virus (TOSV), important human pathogen naturally transmitted by *P. perniciosus* (Charrel et al., 2009).

We proved that infection of first and fourth larval instars by larval food and subsequent transstadial MASV transmission to adults are rather ineffective. Very low infection rate was obtained also by feeding *P. perniciosus* females on blood mixed with virus. Surprisingly, the most efficient way of infection was using the sugar meal: 72% of females and 51% of males of *P. perniciosus* became MASV-positive. Moreover, infected sand flies repeatedly regurgitated the virus particles into the source of sugar which remained infectious at least 24 hours for other naïve individuals: almost 30% of *P. perniciosus* of both genders became positive for MASV after feeding on sugar with spitted virus. Interestingly, MASV infection was found in *P. perniciosus* salivary glands till day 7 post infection but virus particles were expectorated into sugar solution until day 21. These results suggest that virus is not released only through saliva but also by regurgitation from alimentary canal. Maybe, infection through the sugar meal led to different virus development than in sand flies infected through the blood meal and virus do not have to disseminate from midgut or infected salivary glands (Jancarova et al., manuscript).

We suppose that transmission by this type of co-feeding of infected and uninfected sand flies on the same sugar source may serve as an important part of the life cycle of MASV and probably also some other arboviruses (Jancarova et al., manuscript). For example, this might be true for TOSV, where various routes of infection studied (through blood meal, transovarially, venerally) seems to be nonefficient for maintenance of virus in nature (Tesh

and Modi, 1984; Tesh and Modi, 1987; Tesh et al., 1992; Maroli et al., 1993). Our results are in agreement with hypothesis that plants and sugar sources, like nectar, could be involved in circulation of other mosquito- and sand fly-borne viruses, namely vesiculoviruses (Johnson et al., 1969; Tesh et al., 1972) and negeviruses (Nunes et al., 2017). Moreover, our results support the theory that vertebrates probably do not have important role in sand fly virus life cycle and rather represent dead-end host (Tesh and Chaniotis, 1975). On the other hand, our findings oppose the hypothesis that the sand flies could be primary reservoirs of phleboviruses, including MASV (i.e. Alkan et al., 2013).

Sand flies belonging to genera *Phlebotomus* and *Lutzomyia* are proven vectors of human leishmaniasis (rewieved by Maroli et al., 2013) while members of the third main sand fly genus, *Sergentomyia* transmit reptile parasites of genus *Sauroleishmania* and their medical importance is questionable (Dvorak et al., 2018). Vector competence of sand flies is affected by number of physiological and molecular factors. In some sand fly species, particularly in *S. schwetzi*, an important role in *Leishmania* development is also played by PM, the chitin-containing layer secreted by midgut epithelial cells after bloodfeeding (Sadlova et al., 2013). Sadlova and Volf (2009) described positive correlation between the degree of PM disintegration and transformation from short procyclic promastigotes to long nectomonads, morphological stage attaching to midgut to avoid defecation and establish the midgut infection. In *S. schwetzi* the PM remained intact almost until defecation and was proposed to be a key factor responsible for *S. schwetzi* refractoriness to different leishmania species (Sadlova and Volf, 2009; Sadlova et al., 2013).

Our study has confirmed the crucial role of PM in *S. schwetzi*. Addition of *Beauveria bassiana* chitinase in to an infective bloodmeal caused weakened PM which disrupted earlier (24 hours PI: 94% vs 0%), allowing the early escape of *L. major* and *L. donovani* from the endoperitrophic space. Consequently, both *Leishmania* species transformed to metacyclic forms and colonized the stomodeal valve of *S. schwetzi*. On the other hand, in control group, none of the leishmania species established infection in *Sergentomyia* females and all infections were lost during defecation (Sadlova et al., 2018).

We expected that long persistence of PM in *S. schwetzi* could be caused by low midgut chitinase activity and thus we compared *S. schwetzi* with three other sand fly species differing in vector competence to *L. major* and *L. donovani*: *P. argentipes*, *P. papatasi* and *Phlebotomus orientalis*. Surprisingly, the dynamics and levels of exochitinase activity were

similar in *S. schwetzi*, *P. papatasi* and *P. orientalis*. Moreover, 72 hours after blood feeding the activity was highest in *Sergentomyia* (Sadlova et al., 2018). Therefore, we concluded that factors other than chitinase activity are responsible for long persistence of PM in *S. schwetzi*.

One of the most important extrinsic factors influencing various aspects of sand fly life is the ambient temperature. As shown in mosquitoes, temperature fluctuation during both larval and adult life affects also the vector competence, however, the results of such studies are contradictory, depending on the vector-pathogen combination. In sand flies the effect of temperature during larval development on vector competence of adults to *Leishmania* was not studied yet. Therefore, we decided to breed *P. sergenti* larvae at different temperature (27°C versus 32°C) and test the susceptibility of adult sand fly females to *L. tropica*, parasite naturally transmitted by this sand fly vector. Larvae kept at higher temperature developed faster and resulting females were smaller than in larvae maintained at lower temperature. Nevertheless, we did not observe any effect of different larval experimental conditions on the infection rates or intensities of *L. tropica* infections in adult females; both, larval breeding temperature and size of females did not affect these infection parameters (Jancarova et al., 2016).

Interestingly, the larval conditions significantly affected the development of gregarines *Ps. sergenti*, natural pathogen of *P. sergenti*: higher temperature significantly decreased infection rate and intensity of gregarine infection in both, larvae and adults. We hypothesized that this drastic negative effect on gregarines is caused by either: (i) accelerated metabolism of sand flies which provided suboptimal conditions for gregarines development, (ii) direct negative effect of higher temperature on oocysts (as shown in cockroach gregarines by Kolman et al., 2015) and/or (iii) enhanced immune response which may act against gregarines. While the mechanism of gregarine clearance requires further study, the increase of temperature during larval development represents an easy and an effective method how to rid off gregarines from sand fly laboratory colonies (Jancarova et al., 2016).

In nature, *Leishmania* and gregarines may occur together but nothing is known about the possible effect of gregarine presence on sand fly vector competence to *Leishmania*. In mosquitoes it was found that presence of different parasites in the host can modify the impact of pathogens. Thanks to that it might be possible to alter vector competence to another pathogen (Dong et al. 2012; Garza-Hernandéz et al., 2013; Vazeille et al. 2016). We

decided to study whether the presence of gregarine *Ps. sergenti* causes any barrier or negative effect for development of *L. tropica* in *P. sergenti*. We did not observe any significant differences in intensity of infection or infection rate of *L. tropica* between females *P. sergenti* infected and non-infected by gregarines (Jancarova et al., 2015).

In conclusion, we brought new information about factors critical for development of various pathogens in sand flies. Particularly we: i) proposed a new model of sand fly-borne viruses circulation in nature, ii) confirmed hypotheses that long persistence of PM is responsible for refractoriness of *Sergentomyia* to *Leishmania* species, iii) described that temperature during larval development and female size of *P. sergenti* did not affect infection parameters of *L. tropica* but effectively reduced gregarines *Ps. sergenti* and last but not least, iv) demonstrated that coinfection of *Ps. sergenti* did not have any visible effect on development of *L. tropica* in *P. sergenti*.

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Publications

Jancarova M., Bichaud L., Hlavacova J., Priet S., Spitzova T., Volf P., Charrel R. (manuscript): Experimental infection of sand flies by Massilia virus and viral transmission by co-feeding on sugar meal

Abstract

Massilia virus (MASV) belongs to phleboviruses and is closely related to Toscana virus, causative agent of human neuroinvasive disease. Circulation of phleboviruses in nature is poorly understood, experimental studies demonstrated that transovarial and sexual transmission are not enough efficient for maintenance of virus in nature and there is no convincing evidence for vertebrates as reservoirs of the virus. We studied various transmission routes of MASV isolated from *Phlebotomus perniciosus* and its development in various sand fly species. In *P. perniciosus* 4 types of infection were compared: in larval food to the first instar larvae (L1) or to the fourth instar larvae (L4), by blood meal to adult females and by sugar meal to adults of both sexes. From 875 adults emerged from infected L1 and L4 only three were positive. In females infected by bloodmeal the infection rate was high before defecation, then it decreased and MASV was detected in 5 out of 27 post defecation. Surprisingly, the most efficient route of infection was through sugar meal: 72% of females (88 out of 122) and 51% of males (58 out of 113) became virus-positive. Moreover, these sand flies regurgitated virus particules into a drop of sugar which remained infectious for naïve sand flies for at least 24 hours. Almost 30% of *P. perniciosus* (both males and females) get MASV from the sugar with expectorated virus. We suppose that transmission by co-feeding of infected and uninfected sand flies on a sugar source may represent an important part of the life cycle of MASV. Sugar meal infection was tested also in other sand flies species belonging to the three different genera: *Phlebotomus orientalis*, *Phlebotomus papatasi*, *Phlebotomus sergenti*, *Phlebotomus argentipes*, *Sergentomyia schwetzi* and *Lutzomyia longipalpis*. In males, no significant differences were found in intensity of infection and infection rate. In females *P. perniciosus* was the only species in which the infection rate grew steadily for the whole time of experiment duration.

Sadlova, J., Homola, M., Myskova, J., **Jancarova, M.**, & Volf, P. (2018). Refractoriness of *Sergentomyia schwetzi* to *Leishmania* spp. is mediated by the peritrophic matrix. *PLoS Neglected Tropical Diseases*, *12*(4), e0006382.

Abstract

Background

The peritrophic matrix (PM) is an acellular chitin-containing envelope which in most blood sucking insects encloses the ingested blood meal and protects the midgut epithelium. Type I PM present in sand flies and other blood sucking batch feeders is secreted around the meal by the entire midgut in response to feeding. Here we tested the hypothesis that in *Sergentomyia schwetzi* the PM creates a physical barrier that prevents escape of *Leishmania* parasites from the endoperitrophic space.

Methodology/Principal findings

Morphology and ultrastructure of the PM as well the production of endogenous chitinase in *S. schwetzi* were compared with three sand fly species, which are natural vectors of *Leishmania*. Long persistence of the PM in *S. schwetzi* was not accompanied by different morphology or decreased production of chitinase. To confirm the role of the PM in refractoriness of *S. schwetzi* to *Leishmania* parasites, culture supernatant from the fungus *Beauveria bassiana* containing chitinase was added to the infective bloodmeal to disintegrate the PM artificially. In females treated with *B. bassiana* culture supernatants the PM was weakened and permeable, lacking multilayered inner structure; *Leishmania* colonized the midgut and the stomodeal valve and produced metacyclic forms. In control females *Leishmania* infections were lost during defecation.

Conclusions/Significance

Persistence of the PM till defecation of the bloodmeal represents an important factor responsible for refractoriness of *S. schwetzi* to *Leishmania* development. *Leishmania major* as well as *L. donovani* promastigotes survived defecation and developed late-stage infections only in females with PM disintegrated artificially by *B. bassiana* culture supernatants containing exogenous chitinase.

Jancarova, M., Hlavacova, J., Votypka, J., & Volf, P. (2016). An increase of larval rearing temperature does not affect the susceptibility of *Phlebotomus sergenti* to *Leishmania tropica* but effectively eliminates the gregarine *Psychodiella sergenti*. *Parasites & Vectors*, *9*(1), 553.

Abstract

Background: In mosquitoes, it has previously been shown that rearing conditions of immature stages have an effect on the vector competence of adults. Here, we studied the impact of different larval rearing temperatures (27°C versus 32°C) on the sand fly *Phlebotomus sergenti* Parrot, 1917 and its susceptibility to two parasites: *Leishmania tropica* Wright, 1903, a dixenous trypanosomatid transmissible from sand flies to humans, and *Psychodiella sergenti* Lantova, Volf & Votypka, 2010, a monoxenous sand fly gregarine.

Results: Increased rearing temperature (32°C) affected the larval developmental times and size of *P. sergenti* adults but had no effect on the susceptibility of *P. sergenti* to *L. tropica*. No differences were found in *Leishmania* infection rates or in the intensities of *Leishmania* infection. Interestingly, increased larval rearing temperature significantly suppressed the development of gregarines. All 117 control sand flies tested were infected with *Ps. sergenti*, and the mean number of gamonts per individual was 29.5. In contrast, only three of 120 sand flies maintained at 32°C were infected and the mean number of gamonts per individual was just 0.04.

Conclusions: We demonstrated that the increased rearing temperature of *P. sergenti* larvae had no impact on the development of *L. tropica* in adult sand flies but had a profound effect on the gregarine *Ps. sergenti*. We suggest that increasing the larval rearing temperature by 5°C is a simple and effective way to clean sand fly colonies infected by gregarines.

Jancarova, M., Hlavacova, J., & Volf, P. (2015). The development of *Leishmania tropica* in sand flies (Diptera: Psychodidae): A comparison of colonies differing in geographical origin and a gregarine coinfection. *Journal of Medical Entomology*, *52*(6), 1378–1380.

Phlebotomus sergenti Parrot, 1917 is the main vector of *Leishmania tropica*; however, its broad geographical range and molecular heterogeneity suggest possible variability in vector competence. We infected laboratory-reared *P. sergenti* originating from Turkey and Israel to compare their susceptibility to *L. tropica*. In both tested groups, heavy late-stage infections with the presence of metacyclic forms and colonization of the stomodeal valve were observed. The similar development of *Leishmania* in both sand fly colonies indicates that the different geographical origin of *P. sergenti* is not reflected by a different vector competence to *L. tropica*. Additionally, we tested the effect of the gregarine *Psychodiella sergenti* on *L. tropica* coinfections; no apparent differences were found between *P. sergenti* infected or not infected by gregarines

Curriculum vitae

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Education:

2013–present	Ph.D. study in Parasitology, Department of Parasitology, Faculty of Science, Charles University, Prague Supervisor: prof. RNDr. Petr Volf, CSc. Ph.D. thesis: "Critical factors affecting pathogen development in sand flies"
2011–2013	Master in Parasitology, with honours, Department of Parasitology, Faculty of Science, Charles University, Prague Supervisor: prof. RNDr. Petr Volf, CSc. Master thesis: "*Leishmania*–gregarine coinfections in sand flies"
2008–2011	Bachelor degree in Systematic Biology and Ecology, with honours, Faculty of Science, University of Ostrava, Ostrava Supervisor: doc. RNDr. Petr Kočárek, Ph.D. Bachelor thesis: "The intraintestinal parasitization of genus *Tetrix* "

Awards:

2013-2017	STARS – Fellowship Supporting Talented Ph.D. Research Students

Internships:

2017	Research stay at UMR754 – IVPC (Infections Virales et Pathologie Comparée), Claude Bernard University Lyon 1, Lyon, France Laboratory of Frédérick Arnaud, Ph.D. Duration: 5 weeks
2014, 2015	Research stay at UMR190 Unité des Virus Emergents, Faculté de Médecine de Marseille, France Laboratory of Prof. Remi Charrel, MD, Ph.D. Duration: 4 months

Courses and trainings:

2016	Next - generation sequencing, SEQme s. r. o, Ledeč nad Sázavou, Czech Republic
2015	Certificate of professional competence to design experiments and experimental projects under Section 15d (3) of Act No 246/1992 Coll., on the Protection of Animals against Cruelty, number: CZ 03044
2015	Methods of Functional Genomics, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic
2015	Molecular Methods in Insect Physiology and Immunity, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic
2014	Training school, "Q-PCR on chips for high throughput detection of vectors", ANSES, Maisons-Alfort, Paris, France
2013	Practical courses on real-time PCR, SEQme s. r. o, Prague, Czech Republic

Work experience:

2015 – now	research scientist, Department of Parasitology, Faculty of Science, Charles University, Prague
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Publications:

Miglianico, M., Eldering, M., Slater, H., Ferguson, N., Ambrose, P., Lees, R. S., Koolen, K., Pruzinova, K., **Jancarova, M.**, Volf, P., Koenraadt, C. J. M., Duerr, H.-P., Trevitt, G., Schultz, P. G., Yang, B., Chatterjee, A. K., Wisler, J., Sturm, A., Bousema, T., Sauerwein, R. W., Tremblay, M. S., Dechering, K. J. (2018): Repurposing isoxazoline veterinary drugs for control of vector-borne human diseases, accepted in *Proceedings of the National Academy of Sciences* 12th June 2018

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