

## Description of the main research directions investigated by the institute

The Biology Centre of the Czech Academy of Sciences, v. v. i. (a public research institution; hereafter referred to as the BC) was founded on January 1, 2006 by a merger of five research institutes focused on biological and ecological research of microorganisms, free-living and parasitic animals, plants and their interactions, and aquatic and terrestrial ecosystems. These institutes include the Institute of Entomology, the Institute of Parasitology, the Institute of Hydrobiology, and the Institute of Plant Molecular Biology. In 2016, a large research infrastructure SOWA was established to develop cooperation between the Czech and international research community in a comprehensive study of interactions between soil and water on various spatio-temporal scales from micro-scale through river basin level to landscape level.

The foundation of the BC enables us to strengthen research capacity and extend our scope of research through synergy and joint use of high-level facilities such as those devoted to electron microscopy, confocal microscopy, and analytical biochemistry. The growing scientific potential of the BC created conditions amenable to applying for large projects supported by the EU. At present, the BC is a significant institution in the Czech Republic devoted to bioecological research, especially in entomology, parasitology, hydrobiology, soil biology, and plant molecular biology.

BC is a research institution of the edge-cutting non-university research in the area of evolutionary and developmental biology and ecology respected in the domestic and international context and with the ambition to be one of the leading research institutions in Europe and in the world. The way to achieve a top position is to strengthen its international character, with a more significant opening to the world community of evolutionary and developmental biology and ecology, including senior positions for foreign experts from abroad. Furthermore, to strengthen the international character of the School of Doctoral Studies in Biological Sciences in cooperation with the Faculty of Science of the University of South Bohemia in České Budějovice is being developed.

The **principal objective of the BC** is to **perform high-quality research on organisms and ecosystems** using cutting-edge methods of molecular biology, genetics, evolutionary and developmental biology, ecology, mathematical modeling, and other fields of contemporary biology. Due to the extremely wide range of organisms and ecosystems covered by the BC, scientific work in the BC is organized predominantly within the framework of individual research institutes. On the other hand, a **growing number of joint research activities has arisen in recent years, connecting established research teams, ad-hoc project teams, and individuals across the BC**, regardless of the formal institute-based structure of the BC. One of the most recent examples includes the large research infrastructure SoWa (Soil and Water) and active participation of the BC in the Strategy AV21 of The Czech Academy of Sciences (CAS), described in detail below.

The BC has significant international project experience. BC is the fourth most successful institute of the Czech Academy of Sciences in obtaining projects of the H2020 program in the field of Life Sciences and Chemical Sciences. The BC became the solver of 3 ERC grants from the European Research Council Executive Agency, i.e. ERC Advanced grant **Ecological determinants of tropical temperate trends in insect diversity - Diversity6continents** (Proposal No. 669609, duration 2015-2020); ERC Consolidator grant **Insect Photoperiodic Timer - InPhoTime** (Proposal No.

726049, duration 2017-2022); ERC Starting grant **Why is the world green: testing top-down control of plant-herbivore food webs by experiments with birds, bats and ants - BABE** (Proposal No. 805189, duration 2018-2023). Another successful area is the MSCA program. Here BC solves four successful grants. Examples of other large international projects include **Advanced Tools and Research Strategies for Parasite Control in European farmed fish - ParaFishControl** (Proposal No. 634429, duration 2015-2020) or **Co-creating a decision support framework to ensure sustainable fish production in Europe under climate change – ClimeFish** (Proposal No. 677039, duration 2016- 2020) or **Development of Generic Strategies for the Construction of Genetic Sexing Strains in Pest Lepidoptera** (Proposal No. 23379, duration 2019 – 2024). The BC received a total of 19 international grants from 2015 to 2019 from H2020, ESF, EFRR, SNSF. The BC has its own Project Department that consists of skilled and experienced project and financial managers. Managers are well suited for preparing and managing large international projects and coordinating all project partners.

The BC is a “**smart scientific institution**” in the true sense. A great deal of emphasis is placed on two-way communication with the general public in the South Bohemian region and across the Czech Republic. We work to positively influence the lives of the population of the region and the Czech Republic as a whole through encounters and dialogue with the general and professional public and stakeholders. In science communication, we focus on topics in biology and ecology, which are directed toward children and the education system. In this way, we are contributing to the effort of raising a new generation of first-class scientists. Our goal is to conduct **research whose results are in and for society**.

It is a great academic challenge to **transfer research results into practical applications**. The BC works intensively on turning its results into inventions, methods and working procedures. There is the **UTT (Knowledge and Technology Transfer)** to facilitate the preparation of applications for suitable research projects and to direct negotiations with industrial partners and researchers in order to support their cooperation.

The BC maintains a **close relationship with the Faculty of Science**, University of South Bohemia in České Budějovice. BC researchers are engaged in teaching activities in BSc, MSc and PhD programs at the University. Practical experience in research and preparation of theses are facilitated by student participation in established research programs at the BC. An integral part of the institute's activities is the **organization of scientific events** such as international symposia and workshops. BC researchers provide expert opinions to national and international agencies, professional societies, and grant agencies. They also serve as editors of international scientific periodicals and as members of their editorial and advisory boards, in addition to being referees of peer-reviewed journals.

The BC is aware of the great importance of **Large Infrastructures for Research, Experimental Development and Innovation** (LIREDI) on national and EU levels. Therefore, BC intensively prepared a project of Large Infrastructure for Research, Experimental Development and Innovation in environmental sciences called **SoWa (soil & Water)**. The SoWa research infrastructure was established in 2016 to develop the cooperation of the Czech and international research community in a comprehensive study of interactions between soil and water on various spatio-temporal scales from the microscale through the river basin level to the landscape

level. SoWa works closely with the Institute of Soil Biology BC and the Institute of Hydrobiology BC. Eleven SoWa laboratories are developing six research programs that integrate an overview of nutrient depletion at the soil-water interface and then address specific key biotic and abiotic processes, biodiversity and productivity in terrestrial, aquatic ecosystems. SoWa provides the scientific community, state administration, companies, and the non-profit sector with several open access services. The SoWa Research Infrastructure is co-funded by the Ministry of Education, Youth and Sports of the Czech Republic by the targeted support for large infrastructures and by the European Union through the Operational Programme Research, Development and Education. SOWA is included in the Roadmap of Large Infrastructures for Research, Experimental Development and Innovation of the Czech Republic for 2016 - 2022.

Two other proposals of LIREDI have the BC as a partner institution: **Czech-Biolmaging** (Modernization and support of research activities of the national infrastructure for biological and medical imaging Czech-Biolmaging) and **ELIXIR CZ** (ELIXIR-CZ: Capacity building). The research infrastructure ELIXIR CZ (<https://www.elixir-czech.cz>) is responsible for sustainable infrastructure for storing, processing, and analyzing life science data in the Czech Republic. It also provides related training to researchers. The national infrastructure for biological and medical imaging, **Czech-Biolmaging** (<http://www.czech-bioimaging.cz>), is a distributed research infrastructure of leading imaging facilities that provide users with open access to a broad portfolio of imaging methods. Making these technologies available to the scientific community is an absolute prerequisite for maintaining the competitiveness of biological and medical sciences in the Czech Republic. Another positive feature of Czech-Biolmaging is that it combines top research institutes of the Academy of Sciences of the Czech Republic (CAS) with leading universities in the Czech Republic that have many years of experience in teaching and education. The project **ELIXIR-CZ: Capacity building** focuses on providing the underlying physical and virtualized compute and data infrastructure and its further improvement through specific research activities targeting essential aspects of the RI ELIXIR CZ. In this, it complements the separate RI ELIXIR-CZ project that deals primarily with the operational aspects of the infrastructure, services administration and direct user support and training. Both projects are also to be supported by the ERDF and MEYS in the period 2015 – 2022.

The BC responded successfully to a recent initiative of the CAS called **Strategy AV21** (Strategy of the Czech Academy of Sciences for the 21<sup>st</sup> century). The involvement of BC in this program is described in more detail in a separate section below.

**As previously mentioned, scientific life is still, to a great extent, organized in individual research institutes within the BC and their respective teams. Therefore, it is practical to characterize major research programs and achievements of the BC according to the 5 institutes and 17 research teams (departments). Furthermore, the SoWa research infrastructure is integrated as a separate part of our organizational structure. SoWa is presented below in a separate section of the application on research infrastructures and also as a part of team 11. Research teams cooperate with one another closely and intensively. These cross-team cooperations have consistently led to better research output. The teams also frequently cooperate with other institutes of the CAS and other national and international institutes and universities, detailed in the individual team reports.**

## **Institute of Entomology**

The Institute of Entomology conducts basic research on insects in the fields of molecular biology and genetics, biochemistry, physiology and developmental biology, systematics and biodiversity, ecology and ecosystem conservation. The selected research results are directed towards application to the protection of nature and the environment, to the preparation of integrated strategies to control insect pest populations, and to act as the basis for developing knowledge in the biomedical sciences. The research program focuses on four main areas: Molecular and genetic mechanisms of insect development, Physiology and biochemistry of stress and the seasonal responses of insects, Biodiversity of insects and conservation biology, Ecology and evolution of insect communities.

### **Team 1: Insect Molecular Biology and Genetics**

This team is using various insect species as model organisms to study relevant biological phenomena including cell growth and energetic metabolism regulation, hormonal and genetic regulation of insect development, metamorphosis and oogenesis, the molecular, anatomical and genetic basis of insect circadian biological clocks and photoperiodic timers, structure and function of nuclear genomes, chromosome biology, the role of telomeres and telomerase in the regulation of lifespan and stress resistance.

### **Team 2: Insect Biochemistry and Physiology**

The team studies insect responses and adaptations to physiological and environmental stress. By integrating molecular, biochemical, physiological, and whole animal levels, the team aims to complex understanding of mechanisms that help insects to survive and perform in stressful situations ranging from thermal challenge or freezing during overwintering; through exposure to insecticides, toxins, or oxidative stress; to immune challenge, exposure to biological control agents, or genetically modified plants.

### **Team 3: Insect Biodiversity and Conservation Biology**

The main goal of this team is to document and understand the principles of insect biodiversity in non-tropical regions in order to conserve this fundamental component of ecosystems. From polar regions across woodlands to arid steppes, we relate insect life histories, population dynamics and community structure to land use, conservation policies and climate changes, seeking for ecologically rational solutions of pressing problems and answering fundamental questions regarding community assembly and ecosystem functions.

### **Team 4: Insect Ecology**

The research focuses on the fundamental principles of the assembly and function of terrestrial ecosystems, including the dynamics of food webs and their response to environmental change and anthropic disturbance. We study ecological and evolutionary mechanisms of biodiversity origin and function, using a combination of field and laboratory observations, experiments and modeling on local to global scales, with a wide variety of taxa and globally distributed networks of study sites.



## **Institute of Parasitology**

The Institute of Parasitology deals mainly with basic and partially applied research into human and animal parasites. It focuses on studying life cycles, host relationships, the evolution and molecular aspects of unicellular and multicellular parasites of humans, livestock and fish. Another important part is the study of ticks and their transmitted diseases. The common goal is methodically diverse, internationally successful, comprehensive research on parasitic organisms. Research at the Institute of Parasitology has long been organized according to the thematic areas of individual laboratories in the following 4 sections: Molecular parasitology, Biology of vectors and transmitted diseases, Evolutionary parasitology and Medical and veterinary parasitology.

### **Team 5: Aquatic Parasitology**

The team includes three laboratories. Fish Protistology focuses on myxozoan fish parasites, their diversity and evolution, host-parasite interactions and fish immune responses. Helminthology combines organismal approaches with molecular methods in studies on helminths in aquatic environments, including emerging fish-borne parasitic diseases. Electron Microscopy provides high-quality service in biological electron microscopy and participates in ultrastructural research on host-parasite interactions.

### **Team 6: Evolutionary Parasitology**

The team is, in general, interested in the evolution of parasites and related organisms. It includes the evolution of apicomplexan parasites and their photosynthetic relatives, the evolution of organisms hosting complex plastids (alveolates, stramenopiles) and their organelles (mitochondria and plastids), the evolution of protists living in low oxygen conditions, reductive evolution of mitochondria, the evolution of diplomonads, and population genetics and coevolution of parasites and their hosts.

### **Team 7: Veterinary Parasitology and Zoonotic Diseases**

Diversity, genetic variability, epidemiology of parasites in the livestock and wildlife. Transmission of zoonotic parasites at the animal-human interface. Host-parasite relationship based on experimental inoculation, the study of the host and age specificity, the parasite-host metabolic exchange and the host immune response. Ecology of gastrointestinal parasites of non-human primates, the impact of host and environmental factors. The beneficial role of eukaryotic symbionts in health and disease.

### **Team 16: Functional Genomics of Trypanosomes**

The team study functions of proteins in trypanosomes and leishmanias pathogenic for humans; focus on proteins involved in the processing of transfer RNAs, mitochondrial metabolism and maturation of iron-sulfur clusters and editing and processing of mitochondrial transcript; interested in the evolution and diversity of these parasitic flagellates.

### **Team 17: Ticks and Tick-borne Pathogens**

The “Ticks and Tick-borne Pathogens” team (T&TBP) consists of five mutually co-operating research groups investigating a broad spectrum of topics dealing with the biology of ticks and tick-transmitted pathogens, such as tick-borne encephalitis virus and Lyme disease spirochetes. Studies are mainly directed towards understanding the pathogenesis of tick-borne diseases, molecular biology and ecology of their causative

agents, mechanisms involved in disease transmission, and tick physiology and immunology.

### **Institute of Hydrobiology**

The Institute of Hydrobiology conducts research on the interrelationships between aquatic organisms and their interactions with abiotic factors in standing water, especially in artificial reservoirs and lakes. We conduct surveys from sub-processes to the ecosystem level. The specialization of the Institute staff ranges from hydrochemistry through biochemistry, microbiology, algology, protozoology, and the ecology of zooplankton to ichthyology. This structure enables the study of relationships in food networks using both basic approaches: "bottom-up" (from bottom to top - nutrients to fish – the production of living matter) and "top-down" (from top to bottom - fish to nutrients - feedback control of processes from higher trophic levels).

#### **Team 8: Aquatic Microbial Ecology**

The team studies aquatic microorganisms with considerably different functions, i.e. viruses, bacteria, picocyanobacteria, protozoa, and phytoplankton. The team combines classical methods and state-of-the-art techniques of molecular biology and bioinformatics to expand our understanding of microbial ecology and evolution. For decades, lakes and reservoirs of the various trophy have been the main study sites, but recently the scope has been broadened to include, e.g., shallow ponds or post-mining lakes.

#### **Team 9: Ecology of Fish and Zooplankton**

The team is focused on the highest trophic levels in freshwater ecosystems, zooplankton and fish. The team investigates different aspects of fish and zooplankton ecology using diverse methodological approaches and advancements. The main interest is oriented towards an ecology of early life stages of fishes, spatio-temporal, trophic and behavioral ecology of fishes, fish population dynamics, the ecology of fish and zooplankton communities and conservation biology.

#### **Team 15: Hydrochemistry and Ecosystem Modelling**

The team investigates biogeochemical nutrient cycles and processes that influence the chemical composition and quality of surface waters. The team is focused especially on processes in mountain lakes, reservoirs, and ponds, with considerable attention to the hydrology and water chemistry of runoff from different types of catchments and to selected processes in the soil environment that influence the transport of mineral and organic substances into surface waters.

### **Institute of Plant Molecular Biology**

The Institute of Plant Molecular Biology deals with complex plant research at the molecular level that includes the plant genome, cell structure and function, the understanding of epigenetic mechanisms, the functional genomics of plant metabolites, the molecular principles of photosynthesis, biophysics, the biochemistry and molecular biology of the metabolism of metals and the molecular biology of plant pathogens.

### **Team 10: Plant Virology**

The research focuses on microbial and viral pathogens of plants – viruses, phytoplasmas, and bacteria. Studies on plant viruses cover all stages of the research, including their discovery, molecular, biological, and electron microscopy characterization. Ongoing projects aim on the discovery of novel virus species, plant-virus-vector interactions, virus characterization, transmission and elimination, and usage of phages against phytopathogenic bacteria.

### **Team 12: Molecular Cytogenetics of Plants**

the team explores sequence organization and evolution of plant genomes and chromosomes, with a major focus on repetitive DNA. The activities are centered around three topics: (1) Development of bioinformatics tools to characterize repetitive DNA elements in sequencing data, (2) Application of these tools in combination with molecular-cytogenetic approaches to investigate sequence composition and evolution of repeats in plants, and (3) Functional and structural analysis of plant centromeres.

### **Team 13: Biophysics and Biochemistry of Plants**

The main research fields are (1) Trace metal metabolism in higher plants and algae in terms of metal trafficking, sequestration, complexation and hyperaccumulation, metal toxicity, metal detoxification and metal deficiency, and (2) Mechanisms and regulation of photosynthetic light harvesting. In both fields, biophysics, biochemistry and ecophysiology of photosynthetic organisms (higher plants, algae, bacteria) are analyzed using physico-chemical and molecular biological methods.

### **Team 14: Molecular Genetics of Plants**

The team activities build upon the use of molecular biology and Computational biology to (1) Decipher the gene regulatory network associated with secondary metabolite production and charting the role of transcriptional control of glandular trichome initiation and development in hop *Humulus lupulus* and (2) Investigation of parasitic RNA dysregulating hop gene expression, the viroid disease and molecular mechanism(s) of its propagation, adaptation induced pathogenesis and elimination.

### **Institute of Soil Biology**

The institute develops the multidisciplinary field of soil biology, i.e. soil zoology, microbiology, chemistry and micromorphology, and deals with basic issues related to the creation, fertility and regeneration of soils. Activity is focused on research into the structure and dynamics of soil organism communities in natural and anthropogenically affected ecosystems, the interaction between soil animals, microorganisms and abiotic components of the soil environment, the study of humus formation and its transformation, and the cycle of biogenic elements in soil.

### **Team 11: Soil Ecology**

Research is focused on a better understanding of the composition and functioning of soil biota communities in natural and disturbed ecosystems. The research includes biotic-abiotic interactions, stress tolerance, adaptation mechanisms and biogeochemical cycles. Emerging environmental issues are addressed, such as the effect of land-use and climate change on soil functioning and the role of biota in soil

structure formation, carbon sequestration, nutrient cycling and pollutant spread and degradation.

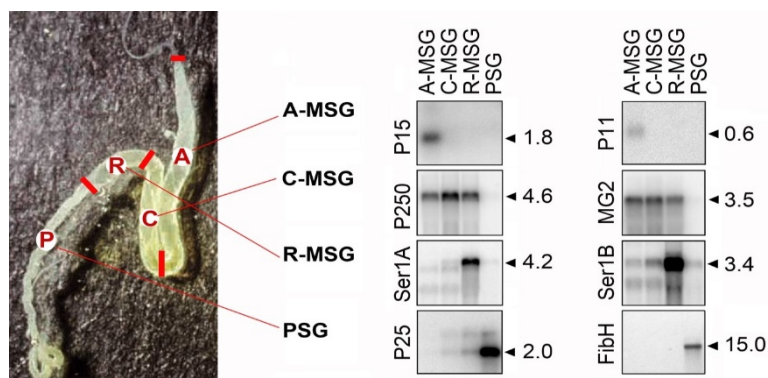
### **Soil and Water Research Infrastructure (SOWA)**

The SoWa research Infrastructure conducts its own research and provides mainly services to other researchers on key issues of the interaction of soil and water. Emphasis is placed on interactions determining the flows of water and major nutrients (especially N and P) in the ecosystem. Emphasis is placed on ecosystems under strong anthropogenic pressure. Sowa's many laboratories work closely with the laboratories at the Institute of Soil Biology and the Institute of Hydrobiology. Research activity is a part of the team 11: Soil Ecology along with the Institute of Soil Biology.

## Research activity and characterisation of the main scientific results

### Structure and evolution of lepidopteran silk

Lepidopteran silk is a complex assembly of proteins produced by a pair of highly specialized labial glands called silk glands (Fig. 1, left). Silk composition has been examined only in a handful of species. In attempt to detect all components of silk from greater wax moth *Galleria mellonella* (Pyralidae), we analysed silk-gland-specific transcriptomes prepared from three developmental periods: penultimate instar larvae, wandering stage larvae, and prepupae. Our results revealed at least 27 novel candidate silk components, which were further characterized (Fig. 1, right). Eight candidates were verified by proteomic analysis or microsequencing of cocoon proteins, and several others were confirmed by similarity with known silk genes and their expression patterns. We show that most candidates encode abundant secreted proteins produced by middle silk glands including ten sericins, two seroins, one or more mucins, and several sequences without apparent similarity to known proteins. We did not detect any novel posterior silk gland-specific protein, confirming that there are only three fibroin subunits. Our data not only show that the number of sericin genes in the greater wax moth is higher than in other lepidopteran species thus far examined, but also the total content of soluble proteins in silk is twice as high in *G. mellonella* than in *Bombyx mori* or *Antheraea yamamai* (Zurovec et al., 2016, Biomacromolecules 17, 1776–1787; Kucerova et al., 2019, Sci. Rep. 9, 3797; Kludkiewicz et al., 2019, *Insect Biochem. Mol. Biol.* 106, 28–38). The expansion of soluble silk components in *G. mellonella* might contribute to the compact character of the cocoon, which provides protection against parasitoids and bees. Our data will serve as a foundation for future identification and evolutionary analysis of silk proteins in the Lepidoptera.

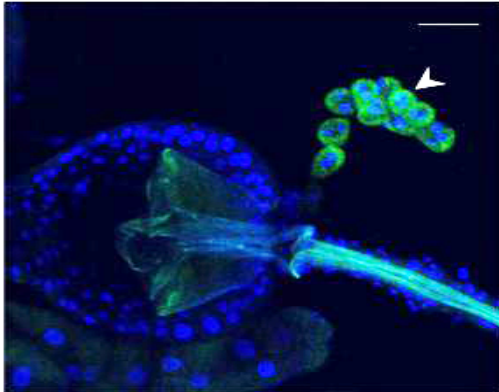


**Figure 1. *Galleria mellonella* silk glands and tissue specificity of silk protein transcripts.** Fibroins are produced in the posterior silk gland section (PSG) and sericins in different parts of middle section (MSG). Northern blots on right show examples of *G. mellonella* silk transcripts.

### Genetic analysis of signal transduction in *Drosophila*

*Drosophila* Imaginal Disc Growth Factors (IDGFs) are a family of chitinase-like glycoproteins abundantly secreted into the haemolymph by fat body and haemocytes. We show that the prototypical member, IDGF2, is an insulin-independent trophic and protective factor in cultured insect cells as well as in intact *Drosophila* larvae and adults. It protects cells from death caused by serum deprivation, toxicity of xenobiotics or high concentrations of extracellular adenosine (Ado) and deoxyadenosine (dAdo). Recombinant IDGF2 can renew the mitochondrial and cellular energy homeostasis and prevent the dissipation of mitochondrial membrane potential ( $\Delta\Psi_m$ ) in cultured cells. Transcriptional profiling supported the role of IDGF2 in energy metabolism, detoxification and innate immunity. We also show that IDGF2 is induced by injury in larval stages. The highest IDGF2 accumulation found at garland (Fig. 2) and pericardial

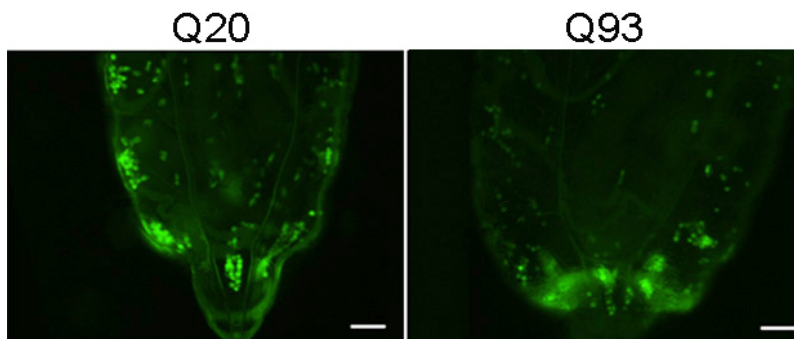
nephrocytes is also consistent with its role in organismal detoxification (Broz et al., 2017, *Sci. Rep.* 7, 43273). Taken together, IDGF2 has similar overall homeostatic and protective effect as mammalian serum and chitinase-like glycoproteins recently associated with pathogenic processes related to inflammation, extracellular tissue remodelling, fibrosis and solid carcinomas.



**Figure 2. Localization of IDGF2 protein in garland cells.** Localization of IDGF2::GFP (green) was driven by the IDGF2 promoter in garland cells attached to proventriculus. Blue - DAPI stain. Scale 50  $\mu$ m.

### Expression of human mutant protein huntingtin in *Drosophila* causes neurodegeneration and damages the immune system

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an expansion of CAG trinucleotide in the Huntingtin (*htt*) gene. Mutant HTT protein (mHTT) contains an expanded polyglutamine tract which causes cytotoxicity and leads to neurodegeneration.



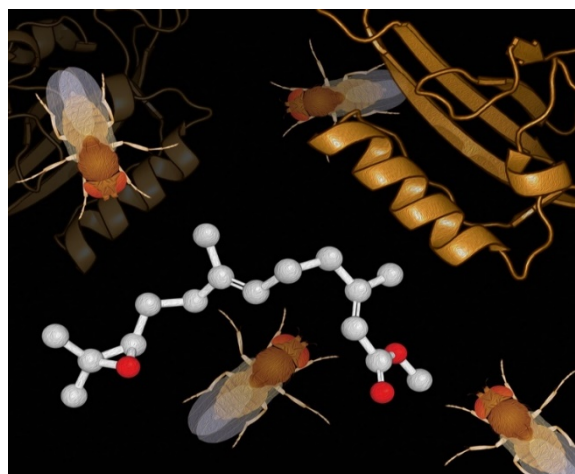
**Figure 3. Ectopic expression of mutant (Q93) and wild-type (Q20) huntingtin in *Drosophila* larva.** The larval abdomens expressing mutant HTT (Q93) show decreased number of circulating and sessile hemocytes (stained in green). Scale 100  $\mu$ m.

While most of described HD symptoms are related to neuronal dysfunction, emerging evidence indicates that the expression of mHTT in other tissues also contributes to the pathogenesis of HD. We used transgenic *Drosophila* expressing mutant HTT protein specifically in the hemocytes to examine its effects on fly viability, hemocyte numbers, and sensitivity to infection. We found that expressing mHTT in the hemocytes does not directly cause a lethal effect, although it reduces the number of circulating hemocytes and affects ATP synthesis in these cells (Fig. 3). We also observed the induction of antimicrobial peptides and impairment of the immune response against different pathogens in mHTT-expressing *Drosophila*. Our findings provide an insight into the effect of innate immunity impairment during HD progression (Lin et al., 2019, *Front. Immunol.* 10, 2405).

## Juvenile hormone receptor signalling

Juvenile hormones (JHs) are sesquiterpenoids that play major roles in development and reproduction of insects and other arthropods. Synthetic compounds that mimic JH serve as insecticides against agricultural pests and disease vectors. However, the molecular action of this unique hormone or its insecticidal mimics has long been obscure as a receptor for JH remained unknown. The prime JH receptor candidate is a bHLH-PAS transcription factor, represented in the fruit fly *Drosophila melanogaster* by two paralogous proteins, Gce and Met. We employed the *Drosophila* model to demonstrate that the JH-binding capability of Gce/Met is essential for the proteins to function *in vivo*. Mutated Gce/Met variants that do not bind JH fail to activate transcription of JH-induced genes and cannot sustain normal development of the fly. This genetic evidence unequivocally establishes Gce/Met in its JH receptor role. Knowledge of a JH receptor impacts our understanding of arthropod biology and defines a molecular target for development of improved insecticides. The study (Jindra et al., 2015, *PLoS Genet.* 11, e1005394) received over 80 citations and the following review on the subject (Jindra et al., 2015, *Curr. Opin. Insect Sci.* 11, 39–46) was cited about 90 times.

Upon binding of an agonist ligand, the receptor (Gce or Met) forms a heterodimer with another bHLH-PAS domain protein Taiman and the resulting complex activates target genes. How the Gce/Met recognizes JH was not known. Several homologs of native JH have been found in different insect and related arthropod species. We systematically examined the affinity of the *Drosophila* JH receptor Gce for a set of native and synthetic JH analogues with altered stereochemistry. We characterized the potency of each ligand in terms of Gce binding, its activity in a cell-based reporter assay, and its morphogenetic effects on the fly development. Both *in vitro* and *in vivo* results mutually confirmed a remarkable stereoselectivity of the Gce protein and its strong preference for the native ligand conformation (Fig. 4). Proper double-bond geometry of the natural hormonal ligand is critical for its tight receptor binding, dimerization of Gce with Taiman, and the ensuing induction of a JH-responsive target gene. Interestingly, Gce can also be activated by chemically unrelated synthetic agonists that are used as JH-mimicking insecticides, some of which surpass the native JH in all agonist activities. The published study (Bittova et al., 2019, *J. Biol. Chem.* 294, 410–423) has become a milestone to our further research addressing the molecular basis of the receptor-agonist interaction.



**Figure 4. Specific binding of juvenile hormone (JH) to its intracellular receptor Gce/Met is essential to sustain insect development.** The rendition depicts fruit flies crawling around the aliphatic molecule of their native JH whose double bond geometry and epoxide moiety determine the high-affinity binding to the receptor protein Gce, represented by a structural model of its ligand-binding pocket. Either mutations within the pocket or altering the ligand geometry both disable the receptor function *in vitro* and during development. (Illustration: [www.biographix.cz](http://www.biographix.cz))

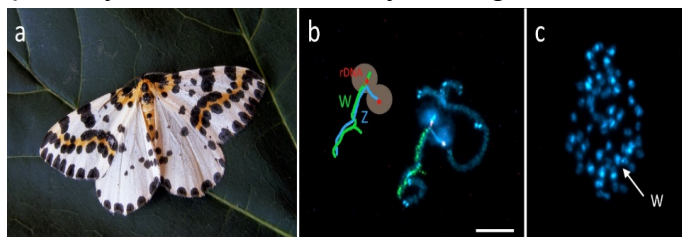


## Origin of the W chromosome and its role in sex determination in Lepidoptera

Moths and butterflies (Lepidoptera) represent the largest animal group with female heterogamety with WZ/ZZ (or Z0/ZZ) sex chromosome systems. The W chromosome is an evolutionary novelty in this species-rich group of insects. To understand driving forces in the evolution of the W chromosome and significance of its acquisition for the adaptive radiation of Lepidoptera, we traced its evolutionary origin and studied its properties and functions in the ancestral and derived sex chromosome systems.

We examined sex chromosomes in lower Ditrysia and non-Ditrysia and confirmed WZ/ZZ systems in Tischeriidae, which is a sister group of Ditrysia, Gracillariidae and Plutellidae. Whereas 2 basal lineages of Ditrysia, Psychidae and Tineidae, lacked W and showed a Z0/ZZ system. We also showed conserved synteny of Z-linked genes between Tischeriidae and Ditrysia. Our results suggest independent W origins in non-Ditrysia and advanced Ditrysia, likely by adoption of a B chromosome, thus supporting a hypothesis of parallel evolution of the W chromosome in Lepidoptera (Dalíková et al., 2017, *J. Hered.* 108, 709–719; Hejníčková et al., 2019, *Genes* 10, 1016).

For the first time, we described the WZ sex chromosomes of *Abraxas grossulariata*, an iconic species in which female heterogamety was discovered at the beginning of the 20<sup>th</sup> century. We showed that although *A. grossulariata* and its congener *A. sylvata* split only around 9.5 million years ago, their W chromosomes differ greatly. Our results



suggest rapid molecular divergence of W chromosomes by independent spreading of female-specific repetitive DNA (Fig. 5; Zrzavá et al., 2018, *Genes* 9, 279).

**Figure 5. Sex chromosomes of the magpie moth, *Abraxas grossulariata*.** (a) Wild-type moth. (b) WZ bivalent at pachytene. W is marked by FISH with a W-painting probe (green), rDNA (red) clusters are immersed in nucleoli (grey circles in the drawing). (c) Mitotic metaphase chromosomes. Bar = 10  $\mu$ m.

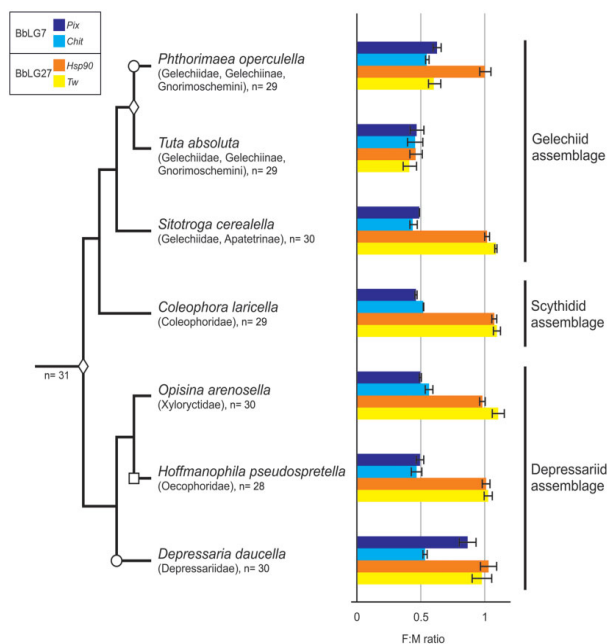
In the silkworm *Bombyx mori*, the W chromosome plays a dominant role in sex determination. But our study revealed that this role is not conserved in wild silkmoths, *Samia cynthia* ssp. We performed crosses between two subspecies of wild silkmoths and showed that their W chromosomes are transmitted to both sexes in offspring of F<sub>1</sub> hybrids without any effect on sexual traits and fitness. Our results clearly suggest that the W chromosome of *S. cynthia* plays no role in sex determination and reproduction (Yoshido et al., 2016, *Heredity* 116, 424–433).

## Role of sex chromosomes in adaptive evolution and speciation of moths

Accumulating evidence suggests that sex chromosomes play a central role in genetics of speciation and it also applies to Lepidoptera. In cooperation with David Heckel's group (Jena, Germany), we identified a Z-linked sterility locus which is responsible for behavioural sterility of female hybrids between two races of the fall armyworm, *Spodoptera frugiperda*, promoting thus their divergence towards speciation (Kost et al., 2016, *Evolution* 70, 1418–1427). In this regard, even more importance is given to the turnover of sex chromosomes by fusion with autosomes, resulting in neo-sex chromosomes. We have previously shown that in Tortricidae, the neo-Z chromosome formation brought two genes for insecticide resistance and clusters of detoxification genes under sex-linked inheritance, increasing the adaptive potential of tortricids



(Nguyen et al., 2013, *PNAS* 110, 6931–6936). This finding was recently confirmed using linkage-mapping and NGS methods in the studies we participated (Picq et al., 2018, *G3* 8, 2539–2549; Wan et al., 2019, *Nat. Commun.* 10, 4237). We also analyzed sex chromosomes in 7 species of Gelechioidea by linkage-mapping of genes using qPCR and in all confirmed neo-Z chromosomes. Our results suggest that the ancestral autosomal LG7 fused to sex chromosomes in a common ancestor of all Gelechioidea (Fig. 6). This brought under sex-linkage UDP-glucosyltransferases, involved in the detoxification of plant secondary metabolites (Carabajal Paladino et al., 2019, *Genome Biol. Evol.* 11, 1307–1319). The turnover that predated two large radiations, Tortricidae and Gelechioidea, supports the hypothesis that sex chromosome-autosome fusions may indeed contribute to ecological specialization and speciation in moths.

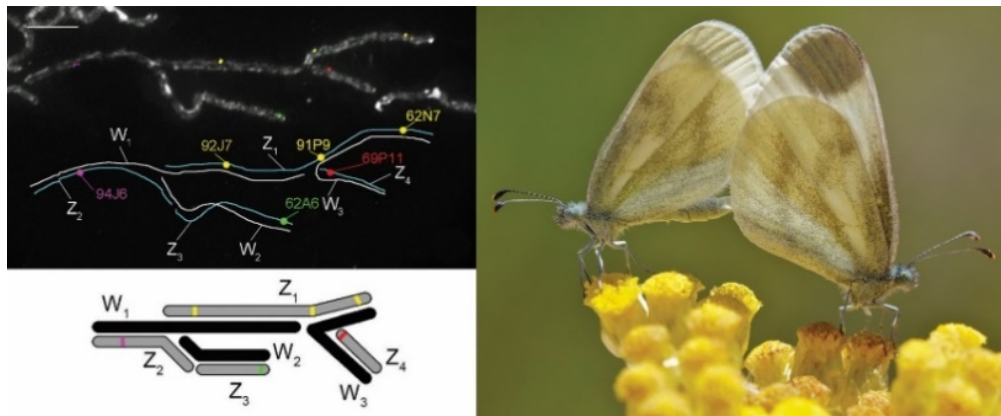


**Figure 6. qPCR linkage test of 2 LG7 and 2 LG27 genes in 7 Gelechioidea species.** Values near 0.5 indicate sex-linked, close to 1 autosomal gene. F:M, female-to-male ratio.

## Origin and evolution of multiple sex chromosomes in *Leptidea* butterflies

Multiple sex chromosomes formed by rearrangements with autosomes are rare in female heterogametic taxa (WZ/ZZ), hampering studies on their evolutionary drivers and significance. We analysed sex chromosomes in three Western Palearctic species of wood white butterflies – *Leptidea juvernica*, *L. sinapis* and *L. reali* – and found unique species-specific sex chromosome systems with 3–4 W and 3–4 Z chromosomes (Šichová et al., 2015, *BMC Evol. Biol.* 15, 89). We even found 3 W and 6 Z chromosomes in the Eastern Palearctic species *L. amurensis* (Šichová et al., 2016, *Biol. J. Linn. Soc.* 118, 457–471). Using microarray-based comparative genomic hybridisation (array-CGH) and a library of bacterial artificial chromosomes (BACs), developed in *L. juvernica*, we identified Z-linked *Leptidea* orthologs of *Bombyx mori* genes, mapped them by BAC-FISH on multiple Z chromosomes, and reconstructed the evolution of these complex sex chromosome systems in three Western species (Fig. 7). We also demonstrated, for the first time in species with female heterogamety, gradual molecular degeneration of originally autosomal parts translocated onto W chromosomes that represent evolutionary strata of various age and levels of differentiation (Yoshido et al., 2020, *Heredity* 125, 138–154). These findings are important for understanding the general mechanisms of evolution of multiple sex chromosomes and their role in population divergence and speciation.

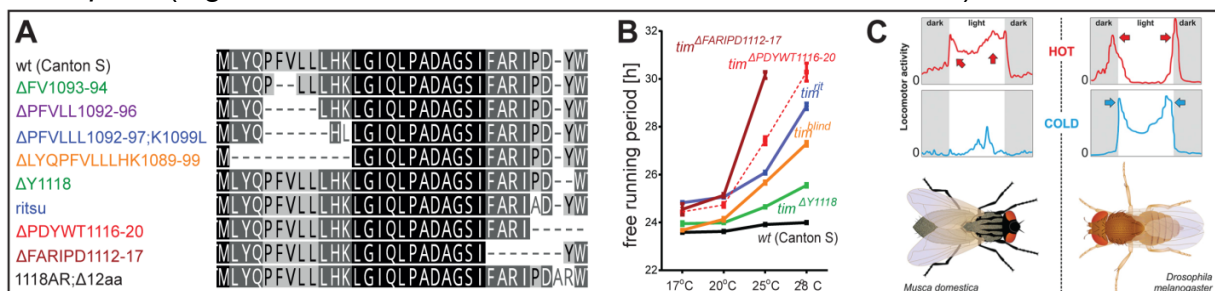
In addition, we contributed to evidence for a mechanism – inverted meiosis – that rescues fertility of chromosomal hybrids of species with holocentric chromosomes, as shown by our results in *L. sinapis* (Lukhtanov et al., 2018, *PNAS* 115, E9610–E9619).



**Figure 7. Analysis of multiple sex chromosomes in *Leptidea*.** Upper left: BAC-FISH mapping and schematic drawing of the sex-chromosome multivalent in *L. juvernica* female; bar = 10 µm. Lower left: scheme of multiple sex-chromosomes in *L. juvernica* female. Right: mating of *L. sinapis*.

### Circadian clock genes in insects

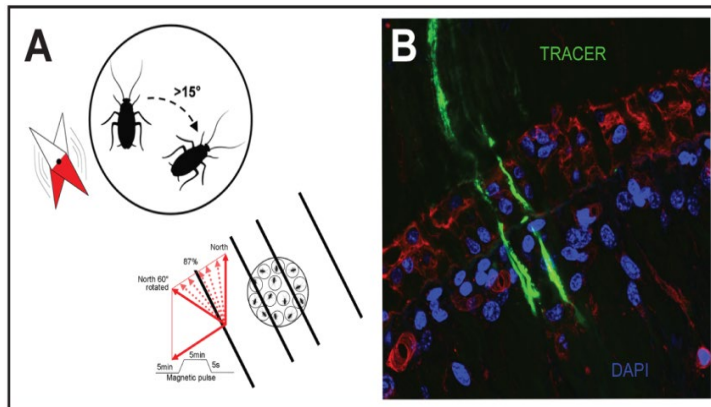
Nearly all organisms synchronize their metabolism, physiology and behaviour to daily changes of day and night using their internal circadian clocks. Despite remarkable success of circadian research, some key aspects of this time-measuring device remain elusive. One of them is temperature compensation, a phenomenon when the entire clock “keeps ticking” with the same pace in relatively large temperature range, whereas the individual biochemical reactions are temperature sensitive. To shed more light on genetic basis of this phenomenon, we performed systematic screen in *Drosophila* and identified evolutionarily conserved region of protein Timeless particularly essential for temperature compensation (Fig. 8A, B; Singh et al., 2019, *Front. Physiol.* 10, 1442). We further explored role of temperature using the housefly, *Musca domestica*, and revealed conserved behavioural patterns of temperature-dependent activity profiles that are orchestrated by mechanism which evolved independently in *Musca* and *Drosophila* (Fig. 8C; Bazalová and Doležel, 2017, *G3* 7: 2637–2649).



**Figure 8. Insect circadian clocks.** (A) Examples of obtained mutant sequences in Timeless protein resulting in (B) temperature compensation phenotypes. (C) Comparison of locomotor activity profiles of *Musca domestica* and *Drosophila melanogaster* at cold (blue) and hot (red) temperatures, respectively.

Circadian clock genes were postulated to be involved in insect magnetoreception. However, the actual experiments were undertaken at intensities of magnetic fields ~10times stronger than the natural geomagnetic field (GMF). In collaboration with

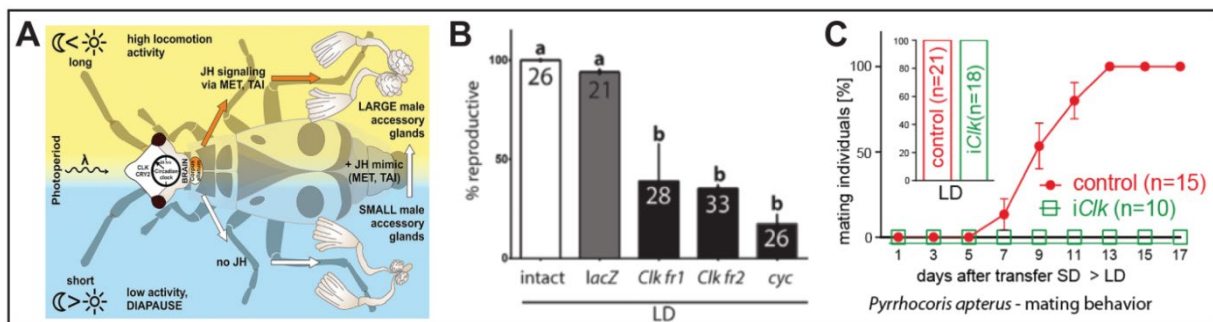
Martin Vácha (MU, Brno) we established behavioural assay for magnetoreception in cockroaches. Not only were we able to work with intensities comparable to GMF, but the assay also addressed directionality of the magnetic field. Using RNA interference, we confirmed role of the circadian clock protein Cryptochrome 2 in the magnetoreception and using immunohistochemistry we localized Cry2 in compound eyes (Fig. 9; Bazalová et al., 2016, *PNAS* 113, 1660–1665). The follow up study (Bartoš et al., 2019, *J. R. Soc. Interface* 16, 20190285) describes the influence of man-made radiofrequency fields on the insect circadian clock.



**Figure 9. Insect magnetoreception.** (A) Rotating magnetic field induces activity in cockroaches (recognized as body turns). (B) Detail of *Periplaneta* eye. The neuronal projections (green) from the eye ommatidia penetrate through the layer of Cry2-positive cells (red) localized underneath the retina. Blue, cell nuclei (DAPI stained).

### Insect photoperiodic timers

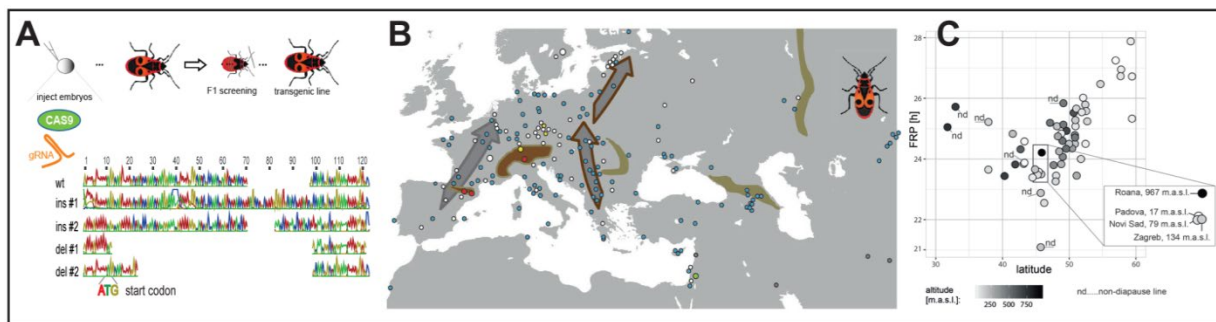
Organisms living in temperate zones often anticipate seasons and adapt to unfavourable conditions in advance. The most reliable seasonal cue is the day length and seasonality of many insect species relies on the so-called photoperiodic timer (photoperiodic clock), a device that measures the ratio of day-to-night. Despite the remarkable importance of photoperiodic timers for insect fitness, their underlying molecular mechanism and genetic basis remains elusive (Doležal, 2015, *Curr. Opin. Insect Sci.* 7, 98–103; Doležal, 2019, *Curr. Biol.* 29, R1176–R1178.). We took advantage of robust reproductive phenotype in *P. apterus* and using RNAi confirmed role of two key clock transcription factors in diapause regulation (Fig. 10A, B; Kotwica-Rolinska et al., 2017, *Physiol. Entomol.* 42, 266–273). Furthermore, our data indicate role of circadian clock genes in photoperiodic time measurement when *Clk* silencing abolished animals' ability recognize transition from short to long photoperiod (Fig. 10C; Urbanová et al., 2016, *Insect Biochem. Mol. Biol.* 70: 184–190).



**Figure 10. Insect photoperiodic timers.** (A) Scheme of the photoperiod-dependent phenotypes. (B) Role of circadian clock genes in female diapause. (C) Ability to discriminate transfer from short and long photoperiod requires circadian clock gene *Clk*.

### ***Pyrrhocoris apterus* as an emerging insect model**

The Linden bug, *P. apterus*, served as an excellent model organism of classical insect endocrinology for more than five decades. Its size permits microsurgical operations (Hejníková et al., 2016, *J. Insect Physiol.* 93-94, 72–80) and relatively robust RNA interference is used for gene silencing (Ibrahim et al., 2017, *J. Insect Physiol.* 107, 167–174). To further expand the repertoire of experimental tools we have established genome editing (Fig. 11A; Kotwica-Rolinska et al., 2019, *Front. Physiol.* 10, 891). We took advantage of *P. apterus* broad distribution and exploited its rhythmicity (Fig. 11B, C; Pivarčiová et al., 2016, *J. Biol. Rhythms* 31, 568–576) and cold tolerance (Ditrich et al., 2018, *Insects* 9, 144).



**Figure 11. *Pyrrhocoris apterus* model.** (A) Example of gene editing relying on CRISPR/Cas9-induced deletions. (B) Remarkable distribution of *P. apterus* illustrated by our collection of field lines. Arrows highlight two major colonization routes to Northern Europe. (C) Example of phenotypic variability, cline in free running period, recorded in our collection.

### **Oxidative stress affects the length of *Drosophila* telomeres in hormetic fashion**

Telomeres play a key role in chromosome stability as they distinguish their natural ends from chromosomal breaks and compensate for chromosome shortening, which is caused by cellular processes, such as incomplete DNA replication or oxidative damage. Oxidative stress is regarded as the main cause of telomere shortening.

Paraquat is a redox cycling compound widely used to induce oxidative stress in various experimental paradigms including telomere length analyses, organismal aging, and activity of antioxidant defence. Most studies performed shock experiments with sublethal paraquat concentrations but still very close to lethal dose. We focused on the chronic effect of wide range of paraquat concentrations in *Drosophila*. When we tested the paraquat effect on antioxidant activity, a biphasic curve rather than linear dose-response was recorded between the paraquat treatment and level of antioxidant reaction (Kruček et al., 2015, *Arch. Insect Biochem. Physiol.* 88, 235–248). This is consistent with hormesis, which is the effect when harmful agents affect an organism over a long period but in a low dose, resulting in a beneficial “eustress” effect on cellular or physiological functions rather than harm (Fig. 12A).

We used the concept of hormesis as a presumption for our next study (Korandová et al., 2018, *J. Insect Physiol.* 104, 1–8) where we tested chronic exposure of a wide range of paraquat concentrations on *Drosophila* telomeres. Telomeres are highly sensitive to oxidative DNA damage, and oxidative stress substantially contributes to telomere shortening. In contrast, we found that a long-term treatment of *Drosophila* with optimal concentrations of oxidizing agent not only induces resistance against



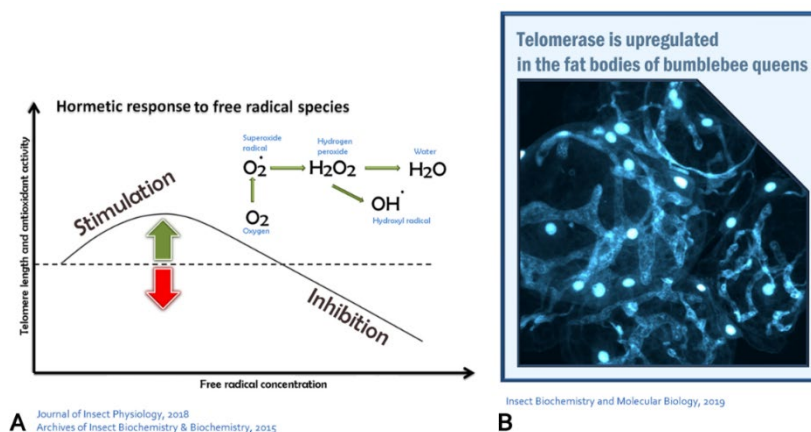
lethal doses, but it is associated with progressive telomere lengthening and boosting of transcriptional activity at telomeres, being in agreement with the concept of hormesis (Fig. 12A).

### Telomeres as a clue to explain the extreme lifespan extension of reproductive individuals in eusocial insects

Numerous theories have been proposed to explain the process of aging, but none of the theories appear to be fully satisfactory. Nevertheless, telomeres and telomerase are one of the factors that associate with lifespan regulation and resistance to age-associated diseases as insufficient telomerase activity limits the stem cell division and thus decreases regeneration capacity of organism.

The organism's lifetime is inversely correlated with reproduction rate/potential of the individual in relation to differential allocation of resources between somatic maintenance and the reproductive process. Although this phenomenon (named cost of reproduction) is generally valid, it is contradicted by advanced eusocial insects, where the lifespan of reproductive individuals is up to 100-fold longer than that of non-reproductive individuals. It makes eusocial insects excellent model systems for research of aging.

In our studies, we tested telomerase activity in several species of eusocial insects, such as honeybees (*Apis mellifera*) and several termite species, to determine whether the caste-related lifespan differences correlate with levels of telomerase activity, and indeed, in all of the species we consistently found elevated telomerase activity in the somatic tissues of reproductive individuals (Korandová and Frydrychová, 2016, *Chromosoma* 125, 405–411; publication on termites in preparation). Also, we tested the telomerase activity in the bumblebee *Bombus terrestris*, which is the species with primitive social organization showing annuality of the colonies and queen winter diapause. We found that telomerase activity together with the intense DNA synthesis are upregulated only in the fat bodies of *B. terrestris* queens prior to their diapause (Fig. 12B), which seems to be associated with the enhanced metabolic activity of the tissue to build-up a sufficient energy reserve. Moreover, we found that activity of telomerase in the fat body is decreased with the mating process, thus resembling the cost of reproduction effect. These data indicate that there is a different mechanism for longevity regulation between the primitive social and eusocial insects (Koubová et al., 2019, *Insect Biochem. Mol. Biol.* 115, 103241).

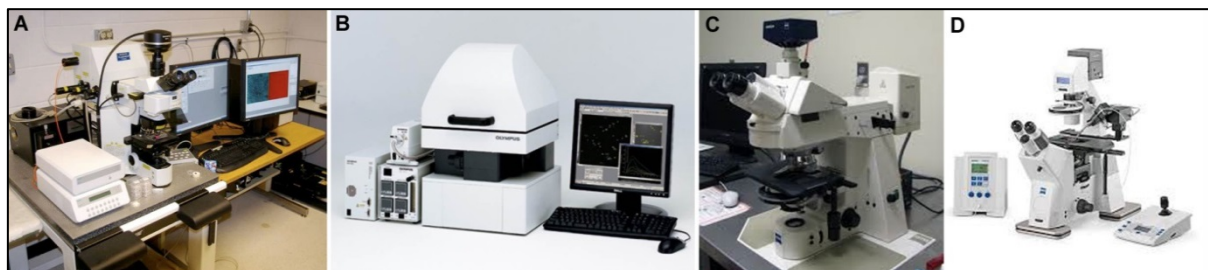


**Figure 12. Pictures from paper-highlights, published along with our original papers. (A)** Schematic picture of hormetic effect on telomere length and antioxidant activity. **(B)** The fat body cells of *Bombus terrestris* queens.

Along with the caste-related lifespan differences, honeybees display a remarkable plasticity in aging rate as a response to social context in the colony and season in the year. Aging is decelerated in generation of winter bees, which hatch at the end of summer, and can live up to eight months compared to around 8-week lifespan of summer bees. We showed that winter bees are associated with the increase of telomerase activity in the fat bodies, correlating with the gradual increase in the fat body mass and level of vitellogenin, which is produced in the fat body and serves as the honeybee longevity marker. Importantly, the elevated levels in the winter bees are not set at the time when bees hatch but gradually evolve over the course of the year depending on environmental factors (manuscript in preparation).

### Bioscience imaging and histology unit

Over the past several years, our team has also established a state-of-the-art biological imaging, histology, and microinjection facility with the multiline laser scanning confocal microscopes, Olympus FV1000 (Fig. 13A) and FV3000, the unique bioluminescence imaging system for non-invasive long-time *in vivo* imaging with sub-cellular resolution (Olympus LV200; first such instrument in the Central Europe; Fig. 13B), 2 fluorescence and DIC microscopes (Zeiss Axioplan 2, Fig. 13C; fully automated Olympus BX51) with high sensitivity cooled CCD cameras, automated microinjection system (Eppendorf Transjector; Fig. 13D), and sophisticated 2D, 3D, and 4D computer image analysis system (Imaris Bitplane). In the frame of this facility, we are collaborating and/or providing an expertise for more than 20 laboratories of the Biology Centre as well as the University of South Bohemia. This activity resulted in more than 21 joint publications in the evaluating period.

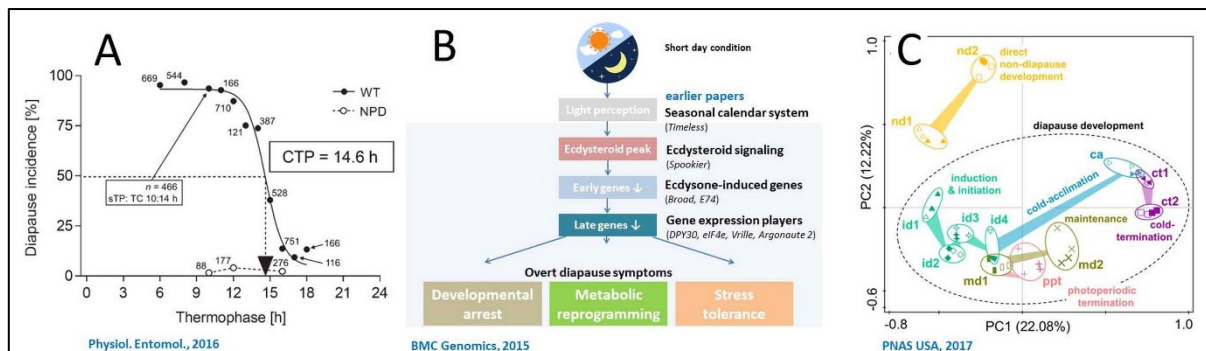


**Figure 13: Biological imaging and microinjection facility.** (A) Laser scanning confocal microscope Olympus FV1000. (B) Bioluminescence imaging system Olympus LV200. (C) Fluorescence and DIC microscope Zeiss Axioplan 2. (D) Automated microinjection system Eppendorf Transjector.

## Research activities and characterisation of the main scientific results

### 1. Insect diapause and cold tolerance

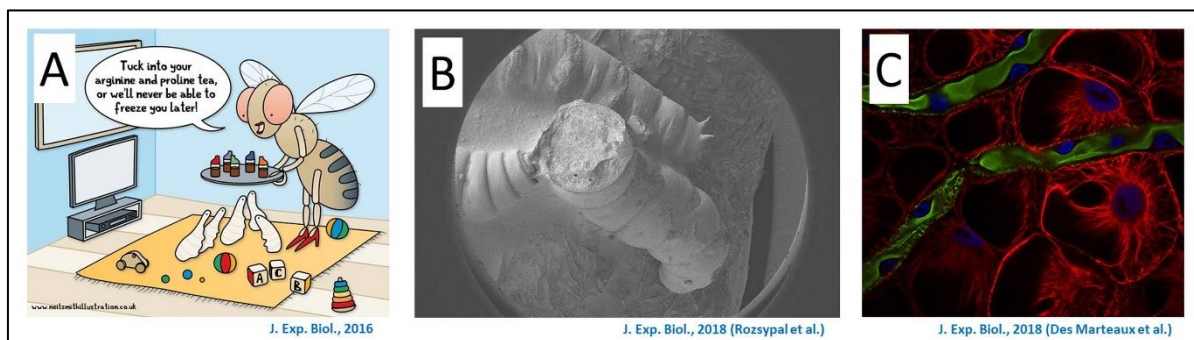
Insect diapause and cold tolerance are two broad and interlinked adaptive complexes that evolved in insects in response to environmental seasonality. In temperate habitats, these adaptations primarily assure that insects can safely overcome harsh conditions of winter season. We have been studying regulation of diapause in a model insect, drosophilid fly *Chymomyza costata*. Multiple environmental factors were found to cooperate during induction phase of diapause (Košťál et al., 2016, *Physiol. Entomol.*). Daily-rhythmic signals such as photoperiod and thermoperiod most reliably coded for seasonal time, and the photoperiodic signal had a dominating influence. Using RNAseq and custom microarrays, we characterized transcription profiles associated with photoperiodic diapause in *C. costata*. The short day photoperiod (long night) was associated to inhibition of 20-hydroxyecdysone signalling. Highly complex alteration of gene transcription was expressed already during the first extended night, within four hours after the change of the photoperiodic signal from long-day to short-day (Poupardin et al., 2015, *BMC Genomics*). The transcription profiles continued in gradual dynamics as insects traversed the diapause developmental program. We could readily distinguish distinct diapause developmental phases associated with induction/initiation, maintenance, cold acclimation, and termination by cold or by photoperiodic signal. Accordingly, each phase was characterized by a specific pattern of gene expression, supporting the physiological relevance of our original concept of diapause phasing (Košťál et al., 2017, *PNAS*). Metabolome dynamics described in the butterfly, *Pieris napi*, confirmed this concept (Lehmann et al., 2018, *J. Exp. Biol.*) and proved its usefulness as a general platform for the unification of diapause timing scales in diverse insect species encountering different ecological situations (Fig. 1).



**Figure 1:** Schematic representation of major results on regulation of diapause in *C. costata*. (A) Thermoperiodic response curve for induction of larval diapause. (B) The complexity of transcriptomic response to the first extended night after transfer to short-day conditions. (C) Clear separation and clustering of distinct transcriptomic profiles representing different phases of diapause development.

Cold tolerance typically develops in diapausing insects during seasonal decline of ambient temperatures. Similarly as in the case of diapause, the development of high cold tolerance was found to be based on deep reprogramming of the gene expression (Des Marteaux et al., 2019, *Proc. Royal Soc. B*). We focused on biochemical mechanisms associated with cold acclimation and found that the phospholipid composition of biological membranes is finely tuned to temperature conditions varying on a tropical to sub-polar gradient in 55 different species of *Drosophila* (Slotsbo et al., 2016, *Funct. Ecol.*). We focused on investigation into seasonal accumulation of the low molecular mass compounds and their influence on insect freeze tolerance. Broad

screening of 31 different amino compounds as food additives in the larval diet of *Drosophila melanogaster* revealed exceptional cryoprotective potential in proline and arginine (Košťál et al., 2016, J. Exp. Biol.). These two compounds are also exceptional in their ability to form supramolecular aggregates which probably bind partially unfolded proteins and inhibit their aggregation under increasing freeze dehydration. The cryoprotectants affect the temperatures of phase transition of body water and the relative fraction of internal ice that forms at a given subzero temperature. For some insects, such as the linden bug, *Pyrrhocoris apterus*, the seasonal decrease of relative ice fraction caused by accumulated cryoprotectants seems to be the crucial adaptation for survival after inoculative freezing (Rozsypal and Košťál, 2018, J. Insect Physiol.). In other insects, the colligative influence of cryoprotectants on ice fraction seems to play only a minor role, while different non-colligative effects on stability of protein structure, phase transitions in biological membranes, stimulation of glass transition, and integrity of tissues are likely more important (Rozsypal et al., 2018, J. Exp. Biol.; Toxopeus et al., 2019, Proc. Royal. Soc. B) (Fig. 2).



**Figure 2:** Pictures redrawn from three 'paper-highlights' that were published along with our original papers in the Journal of Experimental Biology. **(A)** Effect of diet augmentation by 31 different amino compounds on freeze tolerance in larvae of *D. melanogaster*. **(B)** Cryopreservation of larvae of *C. costata* (cryo-SEM); role of proline in ice fraction regulation and stimulation of glass transition. **(C)** Microscopic analysis of freezing induced injury at tissue level in *C. costata* (confocal microscopy); red staining, anti- $\alpha$ -tubulin antibody; green, phalloidin (filamentous actin); blue, DAPI (nuclear DNA).

The literature on mechanisms of insect cold tolerance has traditionally focused on adaptations preceding the cold stress (linked to cold acclimation) that are believed to prevent cold injury, *i.e.* the resistance mechanisms. We argued that the mechanisms that are induced after the stress might be equally important as they help to tolerate or repair cold injury (Košťál et al., 2019, J. Insect Physiol.). Using null mutants of *D. melanogaster* lacking all six copies of *Hsp70* gene, we proved that rapid expression of heat shock protein Hsp70 is required for successful repair of cold injury associated with acute and severe cold exposures (Štětina et al., 2015, PLoS One). Larvae of *D. melanogaster* exposed to different cold stress situations were found to respond by deeply altering their gene transcription profiles. The transcriptional activation of immune response pathways and antimicrobial peptides emerged as potentially important components of repair processes that are upregulated upon cold stress (Štětina et al., 2019, Insect Biochem. Mol. Biol.). Our study on recovery from cold stress in *C. costata* showed that lethal stress is associated with specific metabolic perturbations suggesting impaired mitochondrial function, and with a failure to up-regulate a set of 116 specific genes potentially linked to repair of cold injury (Štětina et al., 2018, Sci. Rep.).

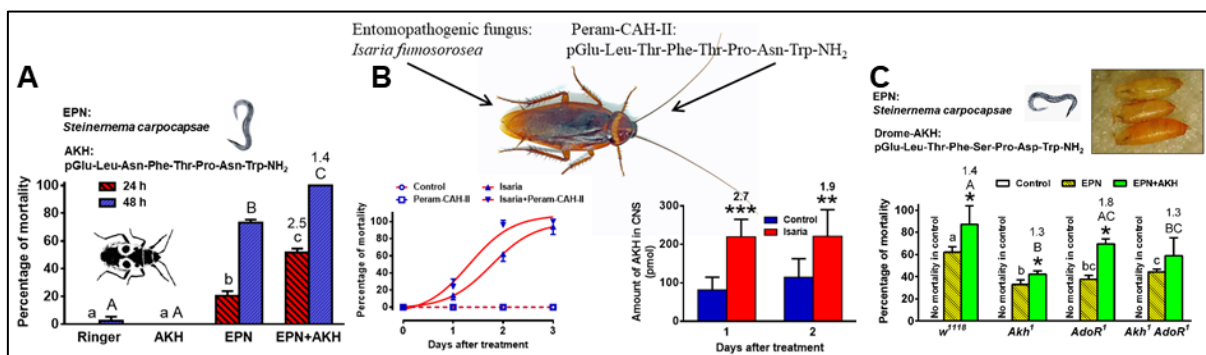


During last five years, we exploited our basic experience in the field of insect diapause cooperating in several projects that aimed to understanding select aspects of overwintering strategy in insect pests such as European corn borer (*Ostrinia nubilalis*), Colorado potato beetle (*Leptinotarsa decemlineata*), parasitoid wasp (*Nasonia vitripennis*), milkweed bug (*Oncopeltus fasciatus*), pistachio twig borer (*Kermania pistaciella*), false codling moth (*Thaumatotibia leucotreta*), and green-veined white butterfly (*Pieris napi*).

Long-term storage of insects at low temperature is another direction for transfer of basic knowledge on cold tolerance into practice. In this direction, we studied the application of fluctuating thermal regimes on storability of *D. melanogaster* larvae and adults (Colinet et al., 2016, Biochim. Biophys. Acta). Long-term storage has a potential to supplement, or even replace, tedious and expensive continuous rearing practices currently used in mass rearing facilities that produce insects for pest management purposes, or in large *Drosophila* centres that preserve valuable genetic lines and mutant strains. We described a temperature regime under which the larvae of *D. melanogaster* can be stored for up to two months. The development of larvae was arrested at the pre-wandering stage and, simultaneously, the rate of accumulation of indirect chill injuries slowed down (Košťál et al., 2016, Sci. Rep.).

## 2. Hormonal control of anti-stress reactions maintaining homeostasis

In insects, the biochemical and physiological defence reactions under unfavourable conditions are controlled predominantly by adipokinetic hormones (AKHs). Their principal function lies in the control of energy metabolism, nevertheless, AKHs, as pleiotropic hormones, exhibit substantially more activities, which boosts their main roles in energy metabolism. Accordingly, it is generally accepted that AKHs play a role in insect defence reactions against various pathogens, insecticides, and natural toxins (Kodrík et al., 2015, Int. J. Mol. Sci.; Plavšín et al., 2015, Comp. Biochem. Physiol. C). Thus, we monitored the role of AKH in the firebug *Pyrrhocoris apterus* adults infected by the entomopathogenic nematode (EPN) *Steinernema carpocapsae*. The EPN application increased *Akh* gene expression and AKH peptide level, and also nutrient amounts in the haemolymph. However, surprisingly, co-application of EPN and synthetic AKH enhanced firebug mortality about 2.5 times within 24 hours (Fig. 3) and

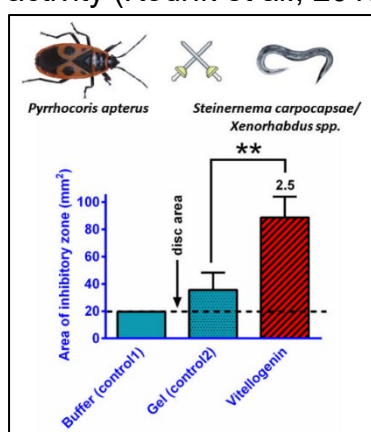


**Figure 3:** The effect of entomopathogens (*S. carpocapsae* and *I. fumosorosea*) and AKH treatments on mortality of (A) *P. apterus*, (B) *P. americana* and (C) *D. melanogaster*.

resulted in metabolism intensification (monitored by carbon dioxide production) (Ibrahim et al., 2017, Insect Physiol.). Accordingly, firebugs with reduced expression of AKH receptors (RNAi) showed a significantly lower mortality and lower general metabolism after EPN + AKH treatments. These results were verified on the cockroach

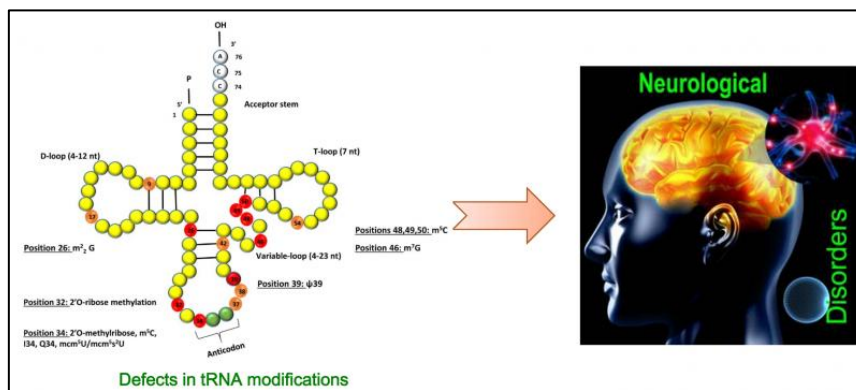
*Periplaneta americana* model infected with the entomopathogenic fungus *Isaria fumosorosea* (Gautam et al., 2020, Comp. Biochem. Physiol. C) (Fig. 3). We suppose that stimulation of metabolism by AKH plays a crucial role in the mechanism of this phenomenon. This effect can increase the turnover and thus efficacy of toxins produced by pathogens. Additionally, the AKH stimulated nutritional abundance in haemolymph could further enhance the growth and development of the pathogens. Those conclusions were supported on *Drosophila* model using loss-of-function mutants (Ibrahim et al., 2018, J. Insect Physiol.) (Fig. 3). We additionally showed that AKH and also adenosine (the first observation in insects) affect also antioxidative capacity (Zemanová et al., 2016, J. Insect Physiol.). However, it seems that more players are involved in the phenomenon (Ibrahim et al., 2018, J. Insect Physiol.).

Vitellogenins (Vgs), glycolipophosphoproteins mostly known and well-characterized as precursors of yolk proteins, are involved in the reproduction in the majority of oviparous animals. However, several recent studies suggested that Vgs play an important role also in other aspects of insect biology including defence immune-reactions. We monitored a role of Vg in the body of the firebug *P. apterus* during the infection elicited by two entomopathogenic organisms, the nematode *S. carpocapsae* and the fungus *I. fumosorosea*. Infection by the nematode significantly up-regulated Vg mRNA and Vg protein expressions in the male body. Remarkably, in females, the tendency was opposite: the infection significantly reduced both Vg mRNA and Vg protein expression levels in fat body and haemolymph, respectively. We speculate that infection of reproductive females reduces Vg expression to the level, which is still sufficient for defence, but insufficient for reproduction. This circumstance reduces energy expenditure and helps the individual to cope with the infection. Importantly, purified Vg significantly inhibited growth of *Xenorhabdus* spp., an entomotoxic bacteria isolated from *S. carpocapsae* (Fig. 4). However, the effect of Vg against *I. fumosorosea* was not so obvious. The fungus significantly stimulated Vg gene expression in males, however, did not change the Vg protein level. However, in females, both mRNA and protein Vg levels were significantly reduced after the fungal infection. The obtained data demonstrate that Vg is likely an important defence protein, possibly with a specific activity (Kodrík et al., 2019, J. Exp. Biol., featured).



**Figure 4:** Inhibiting effect of vitellogenin on the growth of entomotoxic *Xenorhabdus* spp. bacteria isolated from nematode *S. carpocapsae* tested by the disc diffusion method (mean  $\pm$  s.d.). Results evaluated 1 day after the vitellogenin application are expressed in areas of inhibiting zones. Statistically significant differences between the control and experimental groups at 1% level ( $n=5$ ) evaluated by Student's t-test are indicated by \*\*. The number above the bar represents fold-difference between the groups.

The endocrinological aspects of the stress response in insects include hormonal and neurotransmitter levels, and these parameters are changed significantly when an insect has to face a stress situation. The biogenic amines as is dopamine is considered to be one of the prominent element in this response. We found out that disruption of dopamine homeostasis has sexually dimorphic effects on senescence characteristics of *D. melanogaster* (Bednářová et al., 2017, Eur. J. Neurosci.). It has also been documented that perturbations in dopamine synthesis lead to discrete physiological effects and impact oxidative stress response in *Drosophila* (Hanna et al., 2015, J. Insect Physiol.). Moreover, we confirmed that AKH serves as “master regulator” of a number of processes in insects, especially those concerning stress responses and senescence characteristics (Bednářová et al., 2015, Comp. Biochem. Physiol. C; Bednářová et al. 2018, Front. Physiol). Furthermore, our team has focused on deciphering the links between dopamine homeostasis and its relationship to stress and recently also to neurodegenerative disorders (Fig. 5). We changed our strategy to involve looking at defects in tRNA modifications as a proximate cause for disruption of DA homeostasis and hence of Parkinsonian disorders (Bednářová et al., 2017, Front. Mol. Neurosci.).



**Figure 5:** Schematic representation of the secondary structure of tRNA with post-transcriptionally modified residues in light orange and red. The residues marked in red are of relevance to human neurological disorders.

### 3. Ecophysiology of insect pests and pest management

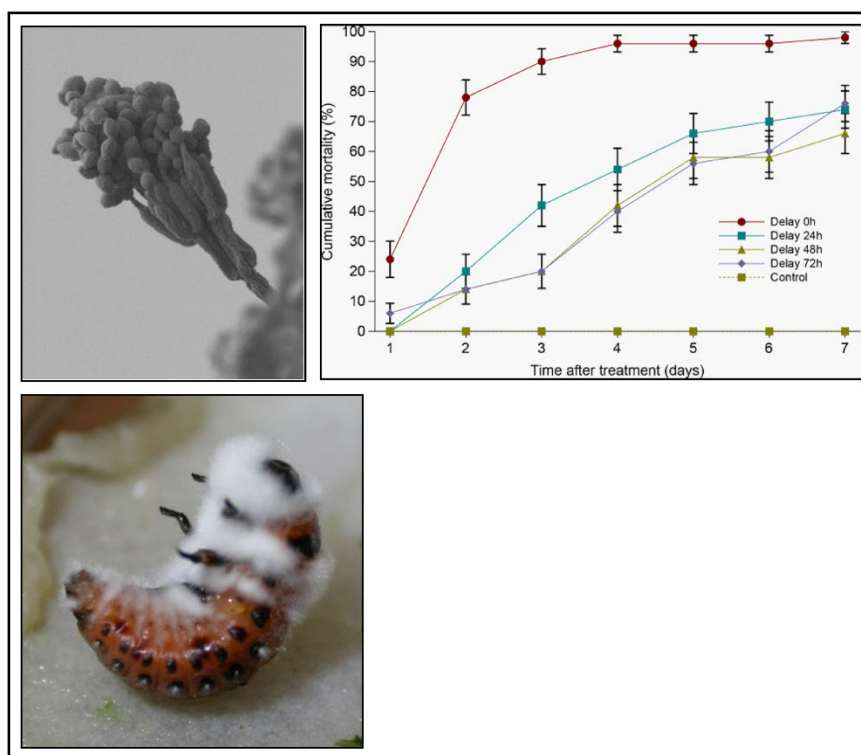
It is well-known that the bark beetles *Ips typographus* and *Ips duplicatus* are the most devastating pests of spruce and some other conifers in the Czech Republic. Therefore we concentrated on these two pests in close collaboration with the Forests of the Czech Republic, state enterprises, which contributed to fast implementation of the results to the methodology of forest protection against bark beetles as well as Czech legislative standards (ČSN 48 1000). We studied establishment of so called sister broods in *I. typographus*. Up to three sister broods (90–100% females reswarmed) were observed at 600–1100 m a.s.l. without a decrease in fecundity of the females. The number and intensity of sister broods increased due to climate change and rising temperature averages, which can be demonstrated on the comparison with historical data from 1950s (Davidková and Doležal, 2017, For. Ecol. Management). The effect of warming climate is obvious also on increasing ability of sub-adult stages of *I. typographus* to successfully overwinter. Our findings revealed that development slowly continues at low temperatures. Importantly, significant numbers of beetles were able

to complete development to adults during winter and contribute to damages the following spring (Štefková et al., 2017, Eur. J. Entomol.).

The double-spined spruce bark beetle, *Ips duplicatus*, represents a novel pest of spruce monocultures in EU. The information on its bionomy is scarce, which complicates proper management. In our experiments, development from egg to eclosion of adults shortened from 39.0 days at 15 °C to 10.3 days at 30 °C. Temperatures over 33 °C were lethal to all eggs, larvae and pupae, which explains the habitat selection of parental adults that infest shaded trees inside the canopy. The rapid development of *I. duplicatus* at 15 and 30 °C (10 and 4 days faster than *I. typographus*, respectively) may explain its recent outbreak dynamics and low effectivity of preventive control measures (Davídková and Doležal, 2019, Agric. For. Entomol.).

The implementation of genetically modified (GM) plants is a modern alternative to chemical insecticides. Within our study we examined the insect-resistant GM maize cultivars containing genes encoding Cry proteins - toxins from *Bacillus thuringiensis* (Bt). These toxins kill relatively narrow range of insect pests, and we verified their possible negative effect on a range of non-target invertebrate species under the field and laboratory conditions. In three consecutive years, we examined the maize, expressing the Cry3Bb1 toxin, its near-isogenic non-Bt hybrid and two non-Bt reference hybrids. Gained results confirmed that the species spectrum of non-target plant dwelling invertebrates changed during seasons and was affected by weather conditions, but not greatly by Bt toxin. (Svobodová et al., 2015, PLoS One). To verify if the Bt toxin is possible to transmit to the non-specific predators via the herbivorous pests, the laboratory experiment was performed. In this experiment, the GM maize producing several Cry proteins were exposed to the experimental arthropods. No acute lethal or sublethal effects on predators as well as on non-targeted invertebrate species were found (Svobodová et al., 2017, Proc. R. Soc. B). This results were confirmed by another study involving also various tillage regimes (Svobodová et al., 2018, Agric. Ecosyst. Environ.). Additionally, we have selected a group of Carabidae species as indicators for the detection of unintended environmental effects of GM crop (Skoková Habuštová et al., 2017, Toxins).

Next applied research was focused on biological control of pests using our patented strain CCM 8367 of entomopathogenic fungus *I. fumusorosea* (Prenerová et al., 2015, EPO patent, No. EP2313488). The most interesting results we obtained on the Colorado potato beetle *Leptinotarsa decemlineata*. Our strain caused higher mortality than the commercial strain Apopka and best results were obtained in simultaneous combination with entomopathogenic nematode *Steinernema feltiae* (Fig. 6). Interestingly, later application of *S. feltiae* had a negative effect on both the penetration rate and the development of nematodes inside a host (Hussein et al., 2016, PLoS One).

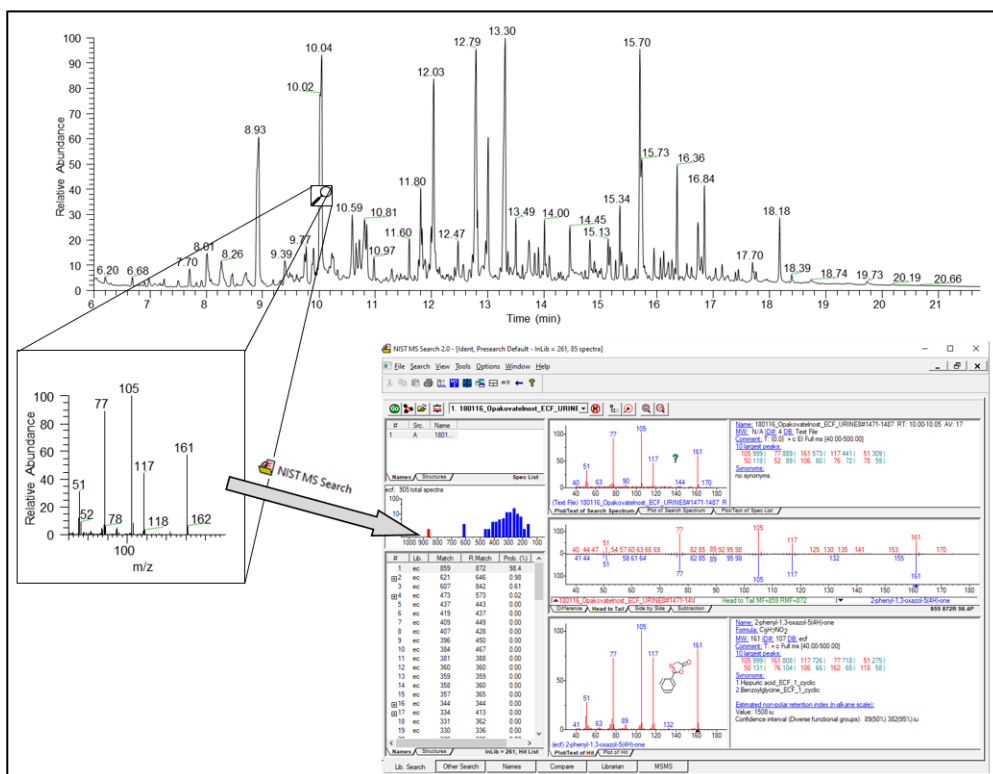


**Figure 6:** Left - conidiophore with conidiophores of *I. fumosorosea*. Middle - cumulative mortality of *L. decemlineata* last-instar larvae treated by *I. fumosorosea* CCM 8367 in combination with *S. feltiae*. Right - dead larva with mycosis.

#### 4. Implementation of the novel metabolomics infrastructure

Two major tasks for the Metabolomics group are to maintain and operate infrastructure for analytical metabolomics, and to develop and implement into research practice the novel methods for metabolite bioanalysis. With the financial support of the EU Fund for Regional Development and other providers, several top quality instruments were acquired. These include Orbitrap Q-Exactive high resolution MS, Agilent 7010C triple quadrupole MS, and Agilent 6475C triple quadrupole MS. The Metabolite Mapper platform – a novel tool for processing of global metabolomics datasets is being developed. This tool includes an internal metabolite library currently covering more than 2000 metabolites and, next, data visualizer that employs publicly available Kegg® platform enabling comprehensive metabolite identification and analysis of the metabolite networks.

We have developed a ToxiAuto® robotic workstation dedicated to automatic serial sample preparation for metabolomic profiling of ca 250 protic metabolites and xenobiotics (Fig. 7). Next, the MetaboAuto® platform represents an extension equipped with autosampler function allowing on-line, serial sample analysis. The sample preparation workstation with the trademarks ToxiAuto® and MetaboAuto® were registered by the Biology Centre and are now in the process of commercialization upon the license agreement with Pragolab s.r.o. Praha.





## Research activity and characterisation of the main scientific results

### Main research areas and results of the Laboratory of Temperate biodiversity:

**Applied conservation problems:** We studied the effect of wider landscape on faunas of protected areas (Bartoňová et al., 2016, *Ecography* 39, 456-464), the effect of grain size in farmland landscapes on butterflies and moths (Konvička et al., 2016, *J. Insect Conserv.* 20, 1113-1118; Novotný et al., 2015, *J. Appl. Entomol.* 139, 390-400). Using a multi-taxa study, we illustrated the role of farmland edges as biodiversity refuges (Šálek et al., 2015, *Basic Appl. Ecol.* 16, 714-725). To disclose traits and gradients associated with rarity and vulnerability and to facilitate for deeper understanding of ecological processes affecting moths, we generalised on life history traits analysis of Central European moths (Potocký et al., 2018, *Insect Conserv. Divers.* 11, 493-505). We broadened the scope of our interests to Mediterranean region, global biodiversity hot-spot still under-researched by insect conservation. We showed that forest encroachment homogenises butterfly communities of the Balkan Peninsula (Šlancarová et al., 2016, *Plos One* 11, e0152026), analysed butterfly fauna of Portuguese woodlands (Šlancarová 2015, *J. Insect Conserv.* 19, 823-836), and documented factors responsible for high papilionid diversity in Greek Thrace (Šlancarová et al., 2015, *J. Nat. His.* 49, 1825-1848). We also carried out detailed study of habitat requirements of the continental steppe relict *Proterebia afra* (Bartoňová et al., 2017, *J. Insect Conserv.* 21, 559-571). Extending our interest to larger scales, and international cooperation by team members, resulted in participation in new checklist of European butterflies (Wiemers et al., 2018, *Zookeys*, 811, 9-45 ). We also

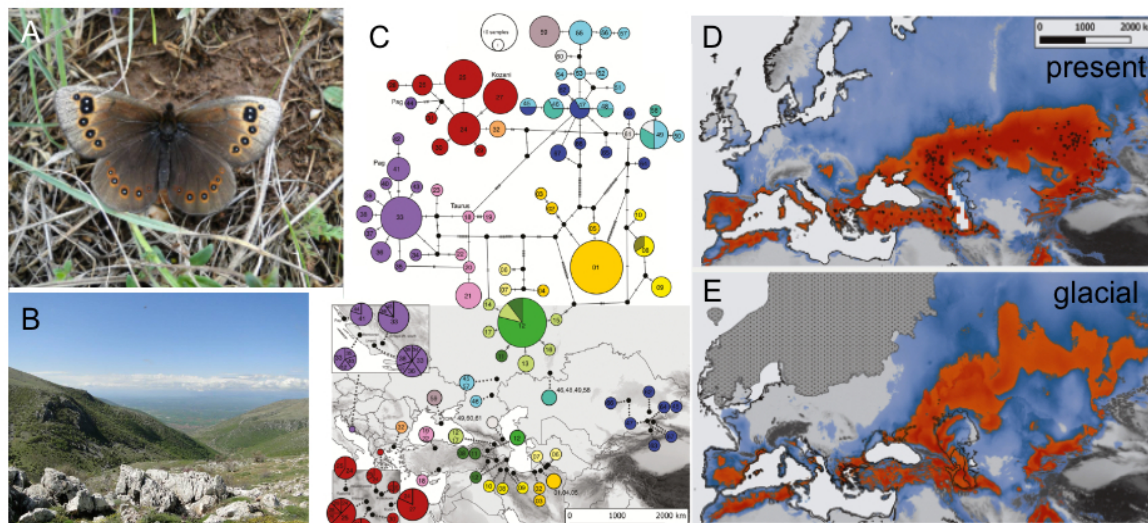


Fig. 1. a) *Proterebia afra* and b) its habitat in Greece; c) Mitochondrial phylogeographic patterns of *P. afra* in the Palaearctic steppes, TCS haplotype network of the 67 haplotypes detected; Species distribution models showing the climatically suitable areas for *Proterebia afra* (d) today and (e) the Last Glacial Maximum (~22,000 b. p.).

investigated the effect of climate change on biotic assemblages. Using a global species distribution database we showed that flight periods in temperate butterflies are not monotonously postponed towards North, but depend on their life strategies and various other factors (Faltýnek Fric et al., 2020, *Ecol. Let.* 23, 172-180).

**Ecology and conservation of European fauna in evolutionary contexts:** This topic represents the principal shift during the period. As part of an international team, we contributed to disclosing evolutionary history of cold-adapted butterfly genera *Erebia* (Pena et al., 2015, Biol. J. Linn. Soc. 116, 449-467) and *Oeneis* (Kleckova et al., 2015, Mol. Phyl. Evol. 92, 225-265). Detailed phylogeographies of the conservation priority species *Euphydryas aurinia* (Junker et al., 2016, Plos One 10, e0142282; Korb et al., 2016, Syst. Entomol. 41, 441-457). A pioneering work was studying the importance of quaternary climatic events for butterfly species across the entire Holarctic region (Eurasia + North America) (Marešová et al. 2019, Plos One 14, e0214483). Bartoňová et al. (2018, Biol. J. Linn. Soc. 125, 867-884; 202, Conserv. Genet. 21, 561-571) showed that alteration of glacial cycles influenced also fauna of arid steppes.

**Cold-adapted species of northern or mountain environments:** We participated in disclosing phylogeography pattern in the circumpolar *Colias palaeno*, associated with bog habitats in temperate region (Kramp et al., 2016, Biol. J. Linn. Soc. 119, 1068-1081). Using the model system of the subalpine butterfly *Erebia epiphron* in middle high mountains, we showed that warming of the last two decades caused earlier onset and longer duration of flight period but lower peak abundances (Konvička et al., 2016, Konvička, Eur. J. Entomol. 113, 295-301). Klečková et al. (2015, Eur. J. Entomol. 112, 114-119) presented the first quantitative analysis of biennial life cycle of *Erebia euryale* across Central European mountains. Several studies targeted ecophysiology of high-altitude insects overwintering, demonstrating high diversity of overwintering strategies in cold-adapted genera *Erebia* (Vrba et al., 2017, Eur. J. Entomol., 114, 470-480) and *Colias* (Vrba et al., 2017, CryoLetters 38, 330-338).

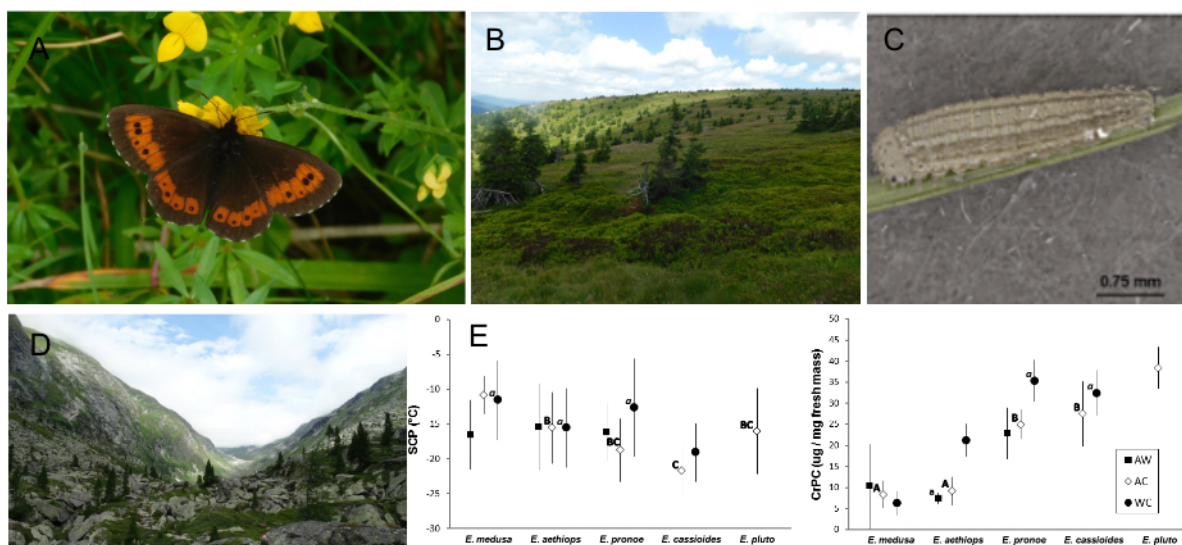


Fig. 2. a) Photograph of *Erebia euryale*; b) Jeseníky Mts. biotope; c) *Erebia aethiops* first instar larva with scale; d) Biotope in the Alps; e) Interspecific differences (means ± standard deviations) in the super cooling points (SCP – Plot A) and total cryoprotectant concentrations (CrPC – Plot B) in overwintering larvae of *Erebia* butterflies subjected to one of three treatments (AW – AutumnWarm, AC – AutumnCold, WC – WinterCold).

**Effects of trophic rewilding:** Current absence of entire trophic guilds, namely megaherbivores and megacarnivores, is a factor behind dependency of a many taxa on human-managed landscapes and habitats. With land use modernisation, large



portion of native biodiversity is losing even these essentially surrogate habitats. Return to preindustrial conditions in agriculture and forestry is impossible. But large stretches of land are becoming available, and regional returns of early Holocene, or even interglacial, ecosystem dynamics is becoming an option, at the very least for selected protected reserves (Vodičková et al. 2019, J. Nature Conserv, 52, 125755). Further understanding related to refaunation activities was gained by work with coprophagous beetles and flies. Buse et al. (2015, Biol. Conserv. 187, 112-119), in pan-European analysis, showed the importance of grazing continuity for coprophilous beetles richness. Sladecek et al. (2017, Plos One 12, e0170426; and 2017, Entomol. Sc. 20, 111-121) described spatiotemporal segregation in coprophilous insect assemblages, bringing evidence for habitat filtering structuring the communities.

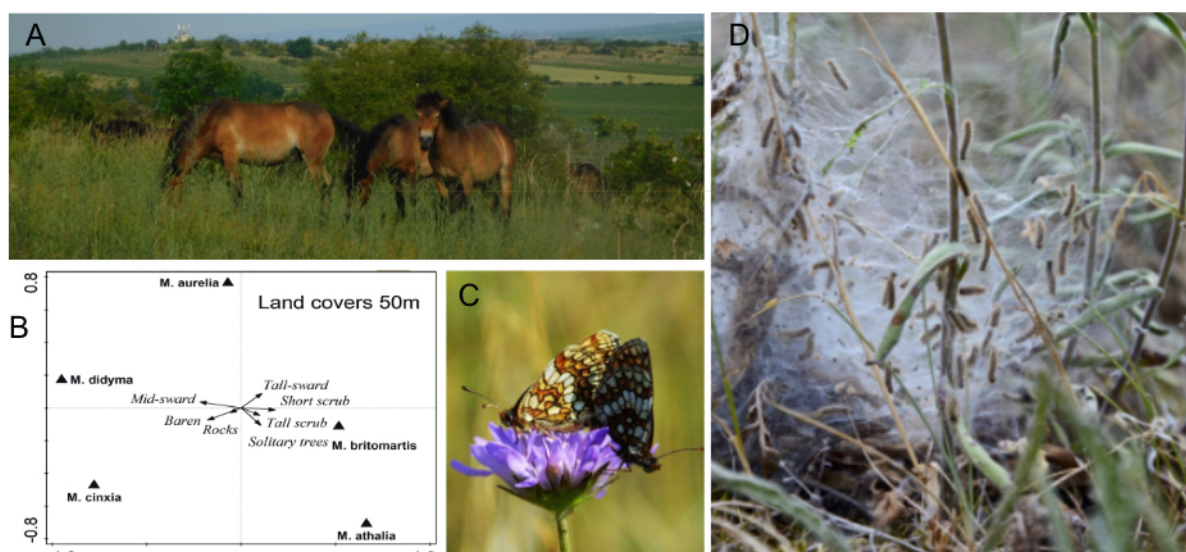


Fig. 3. a) Exmoor ponies grazing in the Podyjí National Park; b) RDA ordination biplots showing the effects of land covers on distribution of adult captures of five species of checkerspot butterflies, occurring at xeric grasslands of Podyjí National Park; c) Mating *Melitaea aurelia*; d) Nest of checkerspot butterflies larvae.

### Main research areas and results of the Laboratory of Woodland Ecology:

**Veteran trees and dynamics of woodland habitat:** Veteran trees are keystone structures sustaining biodiversity of wooded ecosystems. In our effort to understand dynamics and importance of this critical and highly threatened habitat structures, we inventoried ~ 12,000 veteran trees over 124 km<sup>2</sup> of floodplain woodlands in the SE tip of the Czech Republic (Miklín et al. 2015 J.Maps 13: 291-299). This data later served as base for delimitation of several protected areas by the Czech Ministry of Environment. And using current and historical aerial photos, we investigated how forest structure in 1930s affected current presence of the veteran trees and important saproxylic beetles associated with them (Miklín et al., 2018, Div. Dist. 24: 208–218). We also showed that veteran trees are important to other arthropod than just saproxylic beetles (Sebek et al., 2016, For. Ecol. and Management 380: 172-181). To allow for planning of conservation management of veteran trees, we investigated their age and growth patterns (Altman et al. 2016, For Ecol Management 380: 82-89). To shed light on the mechanisms behind the importance of veteran trees and the conditions enabling veteran tree specialists to exploit smaller trees, we investigated local patterns of tree

use by a typical veteran tree specialist, the great capricorn beetle (*Cerambyx cerdo*), at several localities where this beetle exploits oaks of large (~1.5 m), medium (~0.75 m) and small (~0.25 m) diameters (Platek et al., 2019, Eur. J. Entomol. 116: 64-74).

**Insects and ecological gradients:** Patterns of insect distribution along various gradients are a hot topic of current ecology. We participated on a study investigating patterns arthropod distribution in tropical forest in Panamá based on data from the large collaborative project IBISCA (Basset et al. 2015 PLoS ONE 10(12): e0144110). Using complementary data collected in Czechia, we investigated patterns of fine-scale vertical distribution of saproxylic beetles in temperate lowland and montane forests (Weiss et al., 2016 PLoS ONE 11: e0149506; Procházka et al., 2018, Ins. Cons. Div. 11: 534-544). We lead a study comparing patterns of vertical distribution of saproxylic beetles between tropical and temperate forest (Weiss et al. 2019 For. Ecol. and Management 444: 50-58). We also participated on a study investigating host specificity of phytophagous insects across the Globe (Forister et al., 2015, PNAS 112: 442-447).

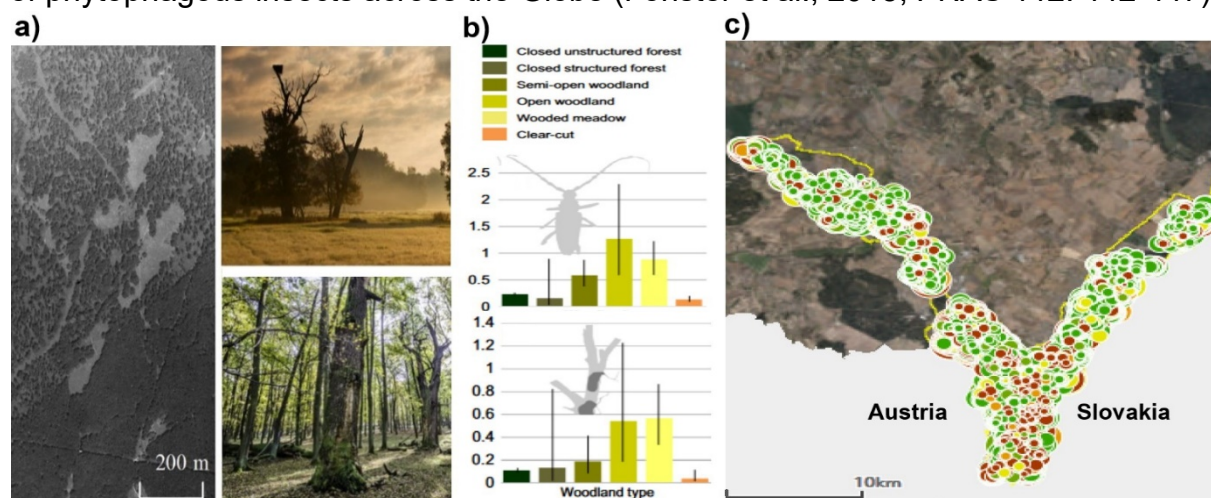


Fig. 4. Veteran trees, threatened beetles and forest history. a) Aerial picture of woodlands in the SE Czechia from 1936; examples of open and closed forest. b) Current density of trees with *Cerambyx cerdo* and hollowed trees in stands with closed canopy depend on canopy closure of the stands nearly century ago (1930s). c) Interactive map of veteran trees (~ 12,000 trees mapped)

**Conservation Management:** Absence of adequate disturbance regimes often threatens biodiversity in protected areas. We performed, or participated on several studies investigating multitaxa responses to various disturbance regimes in various habitats. In a large scale, manipulative experiment in core zone of a national park, we showed that number of threatened taxa benefits from active interventions aimed at decreasing forest canopy closure (Sebek et al. 2015, For. Ecol. Management 358, 80-89; Lanta et al., 2019, For. Ecol. Management 448 : 94-103). Using the multitaxa approach, we also showed how various disturbance regimes affect biodiversity in grasslands (Bonari et al., 2017, Agriculture, Ecosystems and Environment 246: 243-252) and postindustrial areas (Řehounková et al., 2016, Environmental Science and Pollution Research 23: 13745–13753; Tropek et al. 2016 Environmental Science and Pollution Research 23: 13653–13660).

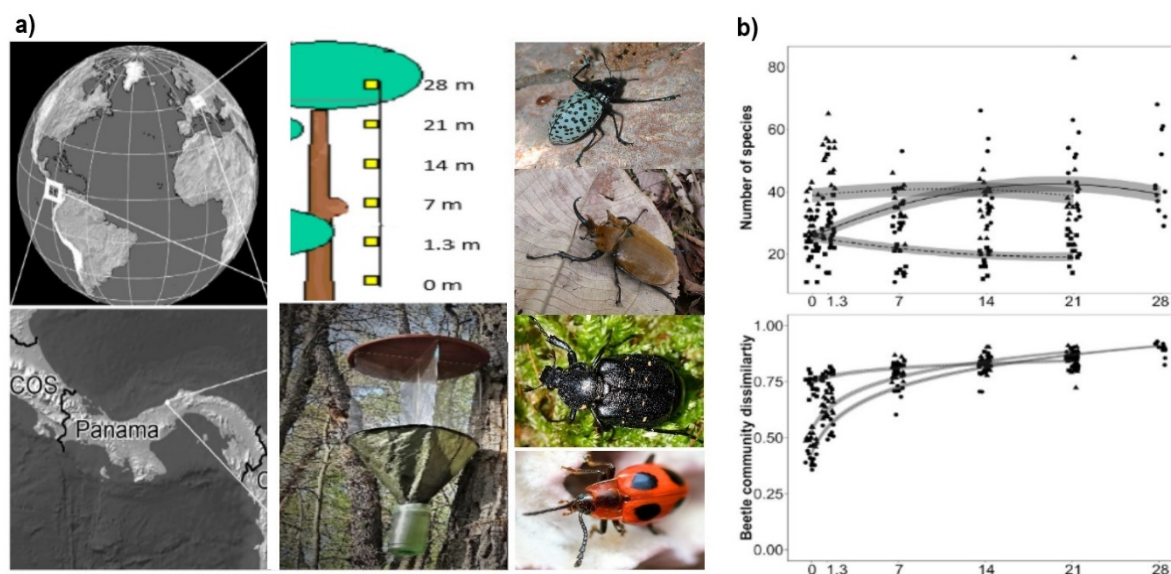


Fig. 5. Vertical stratification of saproxylic beetles in one tropical and two temperate forests. a) Location; schema of sampling design; flight intercept trap; examples of model organisms. b) Temperate montane forest were species poor in all heights, temperate lowland forest had nearly constant numbers of species, while species numbers were low near ground but increased rapidly towards upper canopy in the tropics.

**Biology and genetics of threatened species:** We started genetic studies of threatened saproxylic beetles. To allow for investigations of finer patterns, we developed microsatellite markers for some internationally protected and important model species, including the hermit beetle (*Osmoderma barnabita*) (Basiita et al., 2016, Cons. Genetics Res. 8: 85–87). We used the markers to investigate differentiation of the Alpine longhorn (*Rosalia alpina*) populations in the Central and SE Europe (Drag et al., 2015, Biol. J. Linnean Soc. 116: 911-925), and published whole range phylogeography (based on nuclear and mitochondrial markers) of this species (Drag et al. 2018, J. Biogeogr. 45: 2631–2644). We also used genetics to detect introduced and native populations of the great capricorn beetle (*Cerambyx cerdo*) (Drag & Cizek, 2015, Cons. Genetics 16: 267-276). Using radiotracking, we investigated movement patterns of the capricorn beetle (Drag & Cizek, 2018, J Ins Behaviour 31: 138–143). Further studies include habitat preferences of several threatened species including the highly threatened ground beetle *Carabus nitens* (Volf et al., 2018, J. Ins. Cons. 22: 321–328), the Alpine longhorn (*Rosalia alpina*) (Castro et al., 2019 Animal Biodiversity and Conservation 42.1: 59-63), the wrinkled bark beetle *Rhyssodes*



sulcatus (Kostanjšek et al., 2018, *Ins. Cons. Div.* 11: 545–553), and the violet click beetle (*Limoniscus violaceus*) (Gouix et al., 2015, *Ins. Cons. Div.* 8: 418-427). A collaborative effort resulted publishing list of beetles associated with primeval forests in Central Europe (Eckelt et al., 2018, *J. Ins. Cons.* 22: 15-28).

### Main research areas and results of the Laboratory of Aquatic Insects and Relic Ecosystems:

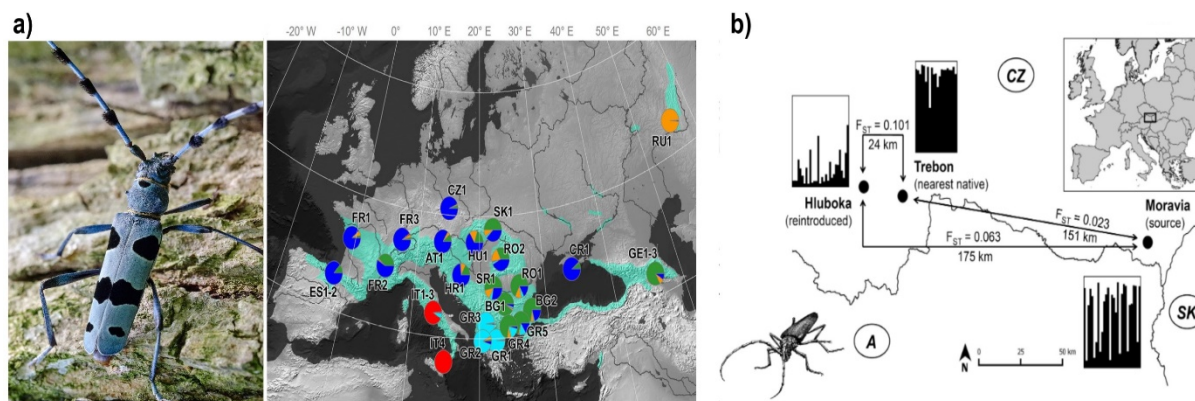


Fig. 6. a) Whole range phylogeography of *Rosalia alpina* showed highest genetic diversity of the species is associated with Southern Balkans; the pattern is typical for many organisms associated with broadleaf forests. b) A population of *Cerambyx cerdo* from Hluboka is substantially more related to distant populations from SE Moravia than to the population from nearby Trebon. This indicates recent translocation of individuals from Moravia to Hluboka, where the beetle went extinct in the mid 20<sup>th</sup> century.

**Impacts of climate change on aquatic insects and other ectotherms:** Our research has yielded several important results supported by empirical experiments and theoretical modelling. We published the first analysis of community-level effects of declining individual body sizes, which is a ubiquitous response to warming in ectotherms. This work showed that temperature-driven changes in body size can alter existing predictions on the effects of climate warming on food webs, and identified ecosystems and environmental conditions under which changes in body size have most profound effect on food web dynamics (Sentis et al. 2017, *Ecology Letters* 20, 852-862). Our novel combinations of experimental studies coupled with numerical modelling of predator-prey dynamics further demonstrated that (i) predator diversity and environmental change such as warming can substantially modify the strengths of trophic and nontrophic interactions (Sentis et al. 2017, *Global Change Biology* 23, 2629-2640) and (ii) acclimation to higher temperatures can substantially alter predator-prey dynamics and buffer against climate change (Sentis et al. 2015, *Global Change Biology* 21, 3290-3298). We also developed a novel method to quantify variation in temperature dependence of individual development across ontogeny and showed that the so-called developmental rate isomorphy concept is not as universal as previously thought (Boukal et al. 2015, *PLoS ONE* 10, e0129341). We used these findings together with an extensive literature review to develop a general framework linking biotic responses to climate change across individuals, populations and communities. In that study we outlined the main theoretical concepts used to characterize the effects of warming on insects and other ectotherms, provided examples of key theoretical and empirical studies, and outlined key questions and knowledge gaps for future research (Boukal et al. 2019, *Curr. Opin. Insect Sci.* 35, 88-95).

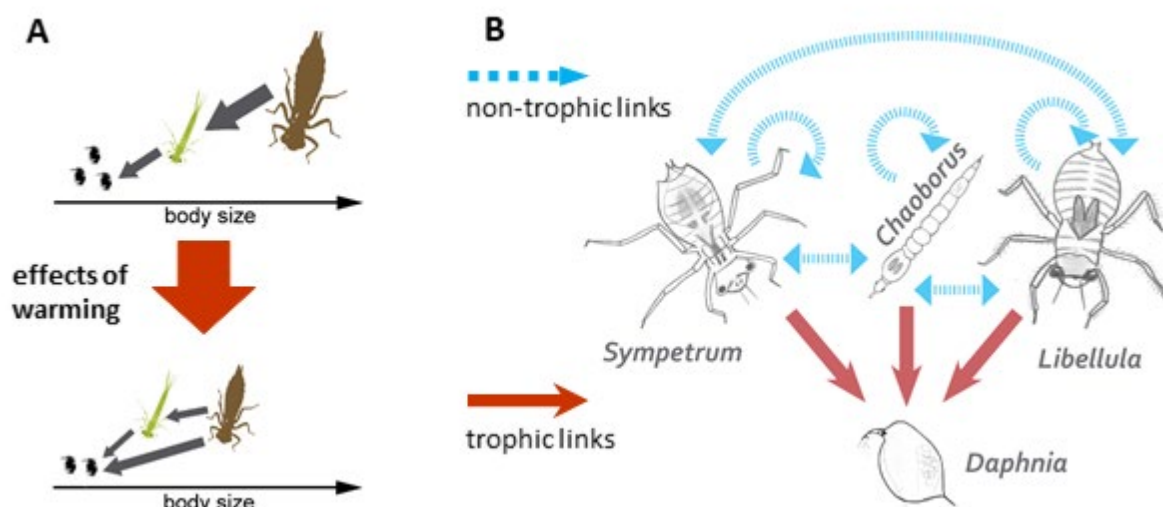


Fig. 7. A. We have used laboratory experiments and mathematical models to explore the effects of warming on individuals, species interactions and food web stability; B. Our experiments with combinations of different predatory aquatic insects provided novel insights into multiple predator effects on prey and on the dependence of trophic and non-trophic interactions on temperature.

**Trophic interactions in aquatic insects and beyond:** We compared existing methods to quantify multiple predator effects that arise when the combined effects of predators on their shared prey do not equal the sum of their individual effects, and used numerical simulations to provide guidance for future empirical studies trying to quantify multiple predator effects (Sentis & Boukal 2018, Scientific Reports 8, 11787). Using laboratory experiments, we also tested how common biotic and abiotic factors jointly modify the strengths of trophic interactions and energy transfer that underpin the structure of food webs. In a study based on odonate larvae as a model system, we showed that predation risk and habitat complexity can alter interaction strength and metabolic rates and both factors should be considered to better understand and predict the effects of environmentally driven variations on local populations and communities (Kolář et al. 2019, Freshwater Biology 64, 1480-1491).

**Research on fossil aquatic insects:** We provided important insights on the phylogeny of basal winged insects and to understanding of insect evolution (Sroka et al. 2015, J. Syst. Palaeontol. 13, 963-982). We also worked intensively on various fossils from the Cretaceous Burmese, Eocene Baltic and Miocene Dominican ambers. We discovered several new taxa including a new evolutionary lineage at the stem of infraorder Systellognatha and the first stonefly larva in the Burmese amber, thereby

contributing to the reconstruction of the Cretaceous palaeoenvironment (Sroka et al. 2018, PeerJ. 6, e5354).

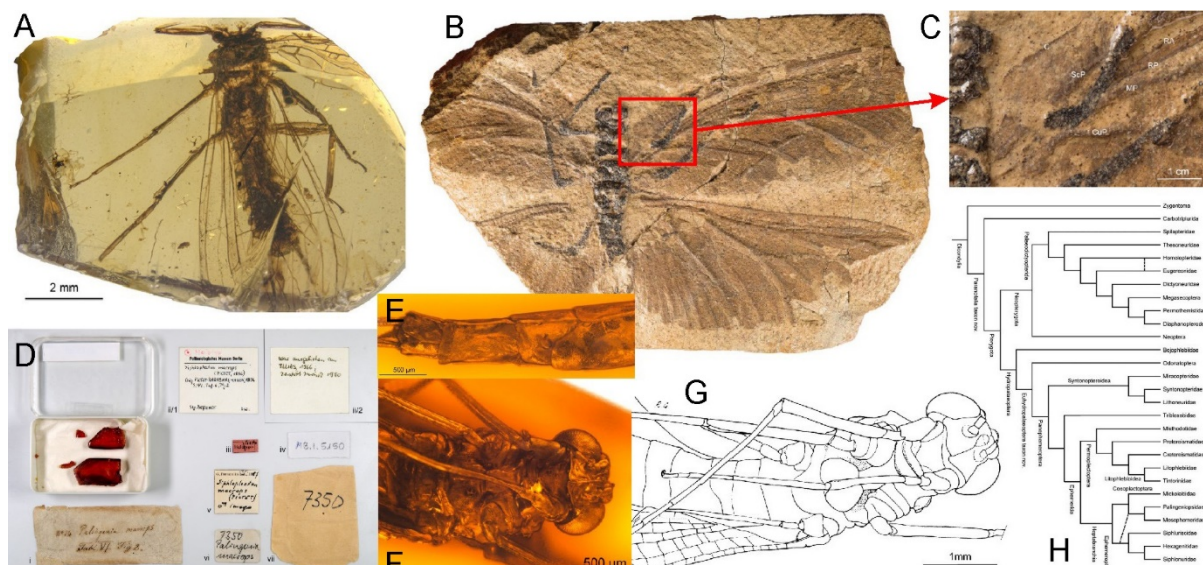


Fig. 8. A, *Lapisperla keithrichardsi*, one of the Cretaceous stoneflies described by our team; B, *Bojophlebia prokopi*, a keystone fossil from the Carboniferous, which we redescribed and used for elucidating evolution of early pterygotes; D-G, material and body structures of the mayfly genus *Siphloplecton* from the Eocene Baltic amber; H, phylogeny of Palaeopteropterous insects including fossil lineages.

**Research on recent aquatic insects:** We worked on inventories and taxonomy of mayflies, focusing predominantly on the Caucasus Mountains and surrounding area, as an important hotspot of biodiversity (e.g. Hrivniak et al. 2018, Zootaxa 4500, 195-221; Sroka et al. 2019, Zoosystematics and Evolution 95(1), 1-13; Sroka et al. 2019, ZooKeys 872, 101-126). These studies resulted in descriptions of new species and form a basis for a forthcoming broader phylogenetic research aimed at evolutionary processes involved in the formation of local unique mayfly fauna.

### Main research areas and results of the Laboratory of Entomopathogenic Nematodes:

**Nematode ecology with new qPCR-based methods:** We studied various questions of entomopathogenic nematode (EPN) ecology, distribution and interactions with other organisms using a novel approach of real-time qPCR. We showed competition for insect cadavers exists between entomopathogenic and free living nematodes (Campos-Herrera et al., 2015, Plant Soil 389, 237-255) and that some facultatively parasitic nematodes act as facultative kleptoparasites which can readily explain the low EPN numbers in field samples (Campos-Herrera et al., 2016, J. Invertebr. Pathol. 132, 216-227). We also developed a qPCR-based method for the detection of molluscoparasitic nematodes (MPN) in soil and host tissues. The method represents a new tool to unravel the ecology of nematode-slug complexes (Jaffuel et al., 2019, J. Invertebr. Pathol. 160, 18-25).



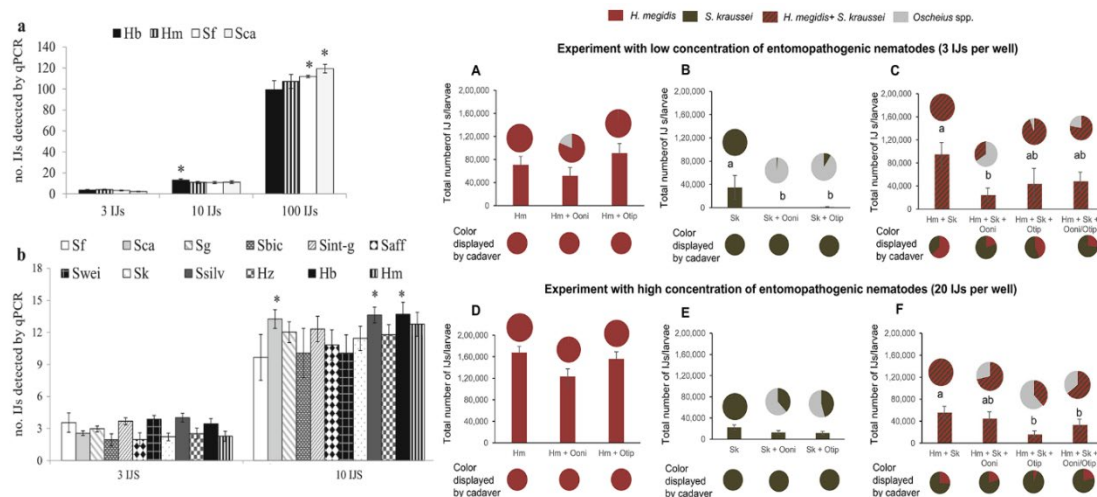


Fig. 9. a, b: Estimation of the number of infective juveniles (IJs) in soil samples using qPCR experiments. a Augmentation experiment with 3, 10, and 100 IJs of various EPN species. A-F: Competition experiments between entomopathogenic nematodes (EPNs) and *Oscheius* spp. Addition of 3 infective juvenile (A–C) and addition of 20 infective juvenile (D–F) (IJs) of either *H. megidis* (Hm) or/and *S. kraussei* (Sk) alone or in combination with *O. onirici* (Ooni) or/and *O. tipulae* (Otip). Pie graphs above each bar show the proportion of EPN and FLN over the total progeny per cadaver. Additionally, the putative identity of each of the cadaver defined by color (red, *H. megidis*; dark brown, *S. kraussei*) is shown in pie graphs below the corresponding treatment in axe “x”.

**Nematode diversity and phylogeny:** We found that the most important genetic marker in EPN systematics, the Internal Transcribed Spacer (ITS) displays larger variability within nematode individuals than between some established steinernematid

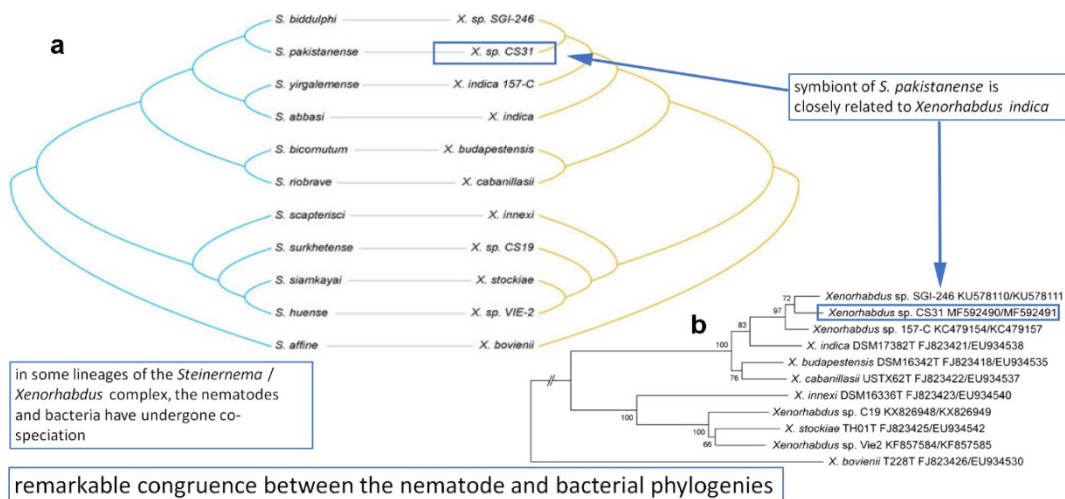


Fig. 10. Co-phylogeny of *Xenorhabdus* bacteria and their steinernematid hosts. a: Tanglegram showing correspondence between *Xenorhabdus* sp. CS31 and related *Xenorhabdus* spp. (right) and their nematode partners (left). Associated pairs are linked by a grey line. B. Phylogenetic relationships of *Xenorhabdus* sp. isolated from *Steinernema pakistanense* (CS31) with other closely related species of *Xenorhabdus*, based on analysis of *recA* and *gyrB* gene sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branch lengths indicate evolutionary distances and are expressed in units of number of base differences per site.

species. Our results thus significantly affected the systematics and phylogenetic reconstructions of steinernematid EPN (Půža et al., 2015, BioControl 60, 547-554).

Our studies in EPN and MPN systematics significantly contributed to our understanding of their diversity by discovering of 5 novel EPN species, and 5 MPN species, with important redescription of *Alloionema appendiculatum* (Nermut' et al., 2015, *Nematology* 17, 897-910). In our recent paper we documented co-cladogenesis of bacterial symbiont *Xenorhabdus indica* and its steinernematid nematode hosts (Bhat et al. 2019).

**Applied research:** We assessed the possibility of the use of entomopathogenic nematodes for the control of the bulb mite *Rhizoglyphus robinii* (Nermut' et al, 2019, *Biol. Control* 128, 102-110). In another study we investigated whether the slug parasitic nematode *A. appendiculatum* could be used in the control of noxious slugs *Arion* spp (Nermut' et al. 2019, *BioControl* 64,697–707). We also indentified the best of method of DNA extraction for taxonomic identification of early developmental stages of forensically important flies (Calliphoridae and Sarcophagidae). It was adopted for the routine work at the Department of forensics of the Institute of Criminalistics, Prague (Olekšáková et al., 2018, *Mitochondrial DNA Part A* 29 : 427-430).



## Research activity and characterisation of the main scientific results

Our research has progressed in the following directions, together providing a coherent focus on the ecology and evolution of interaction webs.

### [1] Elevation gradients as generators and hotspots of biological diversity

Elevation gradients are globally important biodiversity hotspots and important models to evaluate climate change. We proposed a new approach to explaining elevation biodiversity patterns, conjecturing that elevational range midpoints of species may be drawn towards a single midpoint attractor, and developed a Bayesian simulation model for that (Fig. 1A, Colwell et al., 2016, *Ecol. Lett.* 19, 1009–1022). Further, we have used globally available elevational data on geometrid moths to analyse ecological drivers behind their midpoint diversity maximum and identified plant productivity and habitat area as important (Fig. 1B, Beck et al., 2017, *Glob. Ecol. Biogeogr.* 26, 412–424). We studied evolutionary dynamics along elevation gradients, using cospeciation of figs and their fig wasp pollinators. Fig wasps accumulate genetic differences faster than their figs, breaking down their one-to-one matching, but genetic divergence of both partners eventually restores the one-to-one matching among the newly formed species (Fig. 1C, Souto-Vilaros et al., 2019, *Mol. Ecol.* 28, 3958–3976).

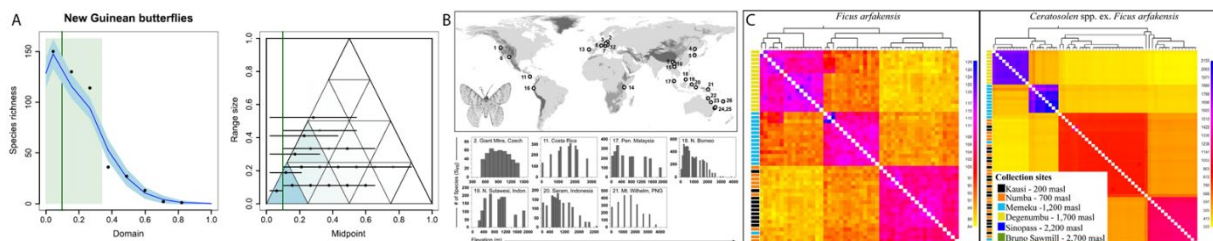


Fig. 1. The Bayesian midpoint attractor model applied to the New Guinea butterfly data set (A), the elevation diversity patterns for geometrid moths (B), and co-ancestry matrix for *Ficus arfakensis* and its fig-wasps along an elevation gradient (C).

## [2] Elevational trends in the ant-plant mutualistic networks

We found that ant communities are structured mainly by variance in tree size and nesting microhabitats in New Guinea rainforests, both in the lowlands (Klimes et al., 2015, *PLoS One* 10, e0117853; Klimes, 2017, In *Ant-plant interactions*, Ed. Oliveira, P.S. & Koptur S., pp. 26–51, Cambridge Univ. Press) and at high elevations (Mottl et al., 2019, *Ecosphere* 10, e02848).

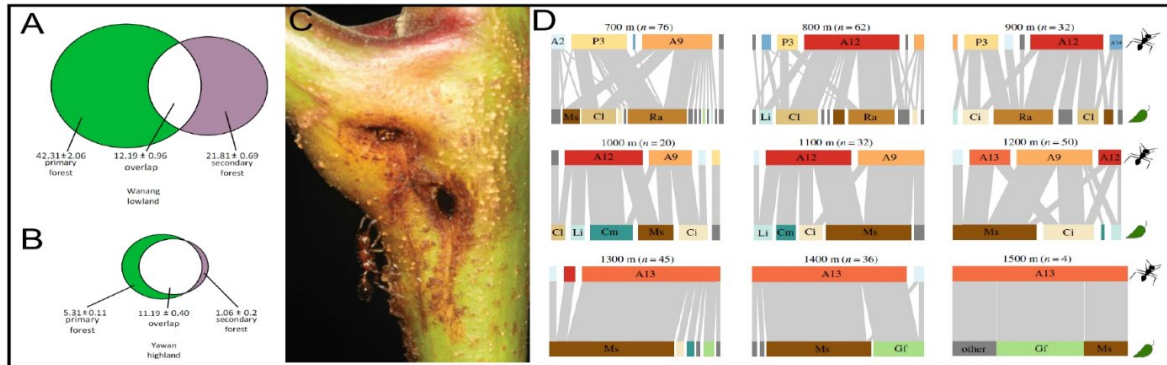


Fig. 2. Species composition of lowland (A) and montane (B) ant communities along successional gradient; Domatia (C, *Podomyrma* ants in *Chisocheiton* domatia) support ant-plant mutualistic networks varying in structure with elevation (D).

Lowland forests host much higher number of ant species and their composition varies between primary and secondary forests, while high elevation ant communities change surprisingly little in the course of ecological succession (Fig. 2A, B). Further, ant-plant mutualistic networks (Fig. 2C) were studied along an elevation transect in New Guinea. They exhibited decreasing number of plant and ant species and their interactions, as well as increasing network specialization with increasing elevation; plants suffered more herbivore damage and hosted less aggressive ants at higher elevations (Fig. 2D, Plowman et al., 2017, *Proc. R. Soc. B* 284, 20162564).

## [3] Bottom-up and top-down control in forest food webs

The relative importance of bottom-up and top-down control in food webs remains a key ecological problem. We contributed to a study documenting increasing predation from temperate to tropical ecosystems (Roslin et al., 2017, *Science*, 356, 742–744). We also showed that in the tropics, predation rates (Fig. 3, Sam et al., 2015, *Ecography*, 38, 293–300) as well as herbivory (Sam et al., 2020, *Biotropica*, 52, 263–276) reach their maximum in the lowlands, i.e. the most productive environment.

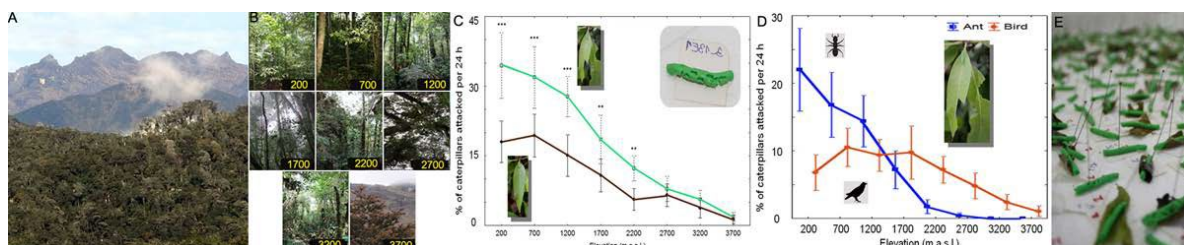


Fig. 3. Altitudinal gradient at Mt. Wilhelm (A) comprising rainforests from 200 to 3700 m asl. (B) with predation decreasing with altitude, and higher on damaged than intact leaves (C); ants were dominant predators in lowlands, birds at higher elevations (D), as measured by attack rate on artificial caterpillars (E).

The application of methyl-jasmonate (signalling herbivore attack) attracts birds to the plants; we have shown this effect for willows (Mrazova & Sam, 2018, *Arthrop.-Plant*

*Interact.*, 12, 1–8) and *Ficus* (Mrazova & Sam, 2019, *J. Trop. Ecol.*, 35, 157–164). We reviewed the current state of the research on the tri-trophic interactions (Mrazova et al., 2019, *Curr. Opin. Insect Sci.*, 32, 131–136). We also showed how the microbiome of tropical birds in New Guinea depends on their diet (Bodawatta et al., 2018, *Front. Microbiol.*, 9, 1830).

#### [4] Insect-plant interactions and evolution of chemical diversity in plants

We used our new protocols for sampling insects from forest canopies (Volf et al., 2019, *PLoS One* 14, e0222119) and demonstrated the importance of diffuse co-evolution of plants and their herbivores for shaping the evolution of plant defences in *Salix* and *Ficus* (Volf et al., 2015, *J. Anim. Ecol.* 84, 1123–1132; Volf et al., 2018, *Ecol. Lett.* 21, 83–92) as well as on diverse tropical vegetation (Fig. 4A, Segar et al., 2017, *Proc. R. Soc. B* 284, 20171803). The response to plant chemical defences differs between specialist and generalist herbivores, impacting evolutionary trajectories of individual defensive traits. In turn, plant defences shape herbivore diversity (Fig. 4B, Segar et al., 2019, *Curr. Opin. Insect Sci.* 32, 83–90; Volf et al., 2019, *Curr. Opin. Insect Sci.* 32, 91–97).

Knowing which changes in species interactions are a natural phase of cospeciation and which ones may endanger future diversity seems critical in a constantly changing world. We focused on insect-plant interactions and communities along elevational gradients as natural experiments to show that changing environmental factors can significantly contribute to the outcome of insect-plant interactions. We show that changes in abiotic conditions along elevational gradient in Papua New Guinea stimulate speciation in the keystone genus *Ficus* and that these effects cascade to other trophic levels, including highly specialized pollinators (Fig. 4C, Vilarós Souto et al., 2018, *J. Ecol.* 106, 2256–2273; Segar et al., 2017, *J. Evol. Biol.* 30, 512–523).

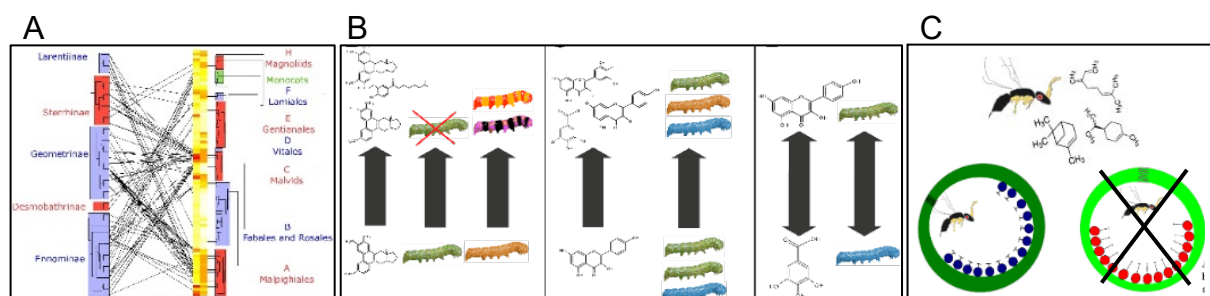


Fig. 4. Bipartite plant-insect phylogenies and chemical traits can be used to predict herbivore host ranges involved in diffuse coevolution with their hosts (A). The herbivore pressure impacts evolutionary trends in host plant chemistry, which in turn affects insect community structure (B): e.g., host specific volatile profiles affect host selection in fig wasps; high fitness costs of host selection errors affect wasp speciation (C).

#### [5] Ecology and evolution of Afrotropical pollination systems

Our studies of bird-pollinated plants revealed the eco-evolutionary background of the unique hovering behaviour of sunbirds during nectar foraging (Padyšáková & Janeček, 2016, *Biotropica* 71, 1380–1385). We examined physiological consequences of competition between sunbirds and bees for nectar (Padyšáková et al., 2018, *Oecologia* 183, 1111–1120). We also demonstrated a good ecological fit between sunbirds and



non-native *Heliconia*, despite their distinct evolutionary histories (Janeček et al. 2020, *Ecol. Evol.* 10, 1784–1795).

We characterized insect-pollinated systems, with focus on the role of plant traits on the interaction network specialisation (Fig. 5), including a key role of floral spur in temporal diversification of pollinators (Vlašánková et al., 2017, *New Phytol.* 215, 1574–1581), and the role of UV floral colour (Klomberg et al., 2019, *AoB Plants* 11, plz057) and nectar supply (Bartoš et al., 2015, *Arthropod-Plant Interact.* 9, 241–252) on pollinators.

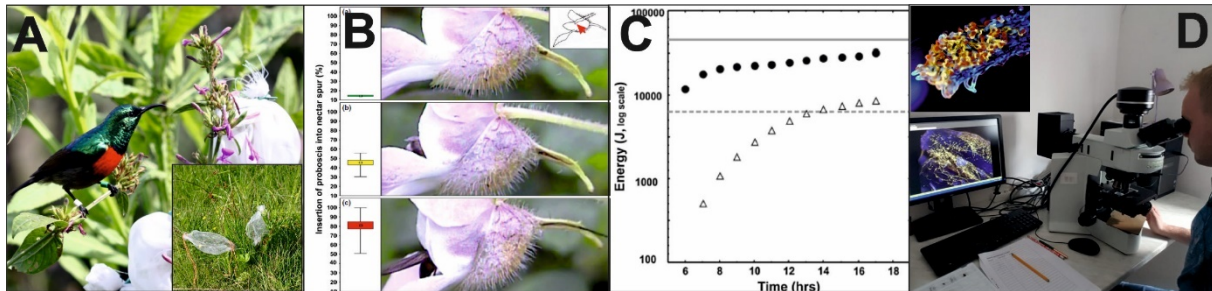


Fig. 5. Experimental enclosures of flowers (A); probosces of three main visitors of *Impatiens sakeriana* in its experimentally opened spur (B); cumulative energy intake of sunbirds and carpenter bees from nectar of *Hypoestes aristata* comparing to their daily needs (C); germinating pollen grains under fluorescence microscope (D).

## [6] Patterns in plant-pollinator networks along ecological gradients

We studied pollination networks along elevational gradients in Afrotropical and European mountains. On Mt. Cameroon, network specialisation decreased with elevation in (favourable) dry season, but not in (unfavourable) wet season (Fig. 6). We have also focused on the role of floral traits, and patterns in robbing behaviour at different elevations (Mertens et al., 2020, *Arthropod-Plant Interact.* 14, 215–226; Mertens et al., 2018, *Afr. J. Ecol.* 56, 146–152). In Czechia, we analysed changes of plant reproduction systems with habitat degradation (Bartoš et al., 2020, *Plant Biol.* 22, 120–128) and shown strong pollen limitation of specialised plants in degraded meadows (Bartoš et al., 2020, *Plants* 9, 640).

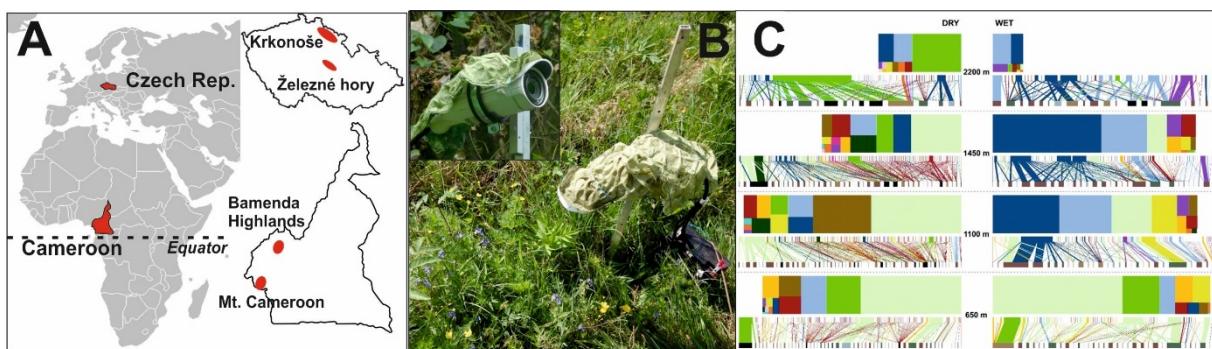


Fig. 6. Study areas for patterns of plant-pollinator interactions along ecological gradients (A), our camera systems record floral visitors (B), plant-pollinator interactions at four elevations and two seasons on Mount Cameroon (C).

## [7] Afrotropical butterflies and moths along ecological gradients

Lepidoptera communities on Mt. Cameroon showed seasonal elevational shifts of species and overall biodiversity patterns (Fig. 7, Maicher et al., 2020, *J. Biogeogr.* 47, 342–354) that could be erroneously attributed to climate change. In the lowland forests we described seasonal dynamics of lepidopteran communities (Maicher et al., 2018,

*Ecol. Evol.* 8, 12761–12772) We also described 19 new species of Lepidoptera (e.g. Ustjuzhanin et al., 2018, *Zookeys* 777, 119–139; Sáfián & Tropek, 2016, *Zootaxa* 4150, 123–132), and published on Lepidoptera phylogeography (Fric et al., 2019, *Syst. Entomol.* 44, 652–665), taxonomy (Przybyłowicz et al., 2019, *Zootaxa* 4674, 283–295), and biodiversity (Delabye et al., 2019, *Genome* 62, 96–107). We have raised the issue of insufficient protection for a key remnant of littoral forest ecosystems in Cameroon (Ferenc et al., 2018, *Afr. J. Ecol.* 56, 679–683) and are collaborating with local authorities on its conservation.

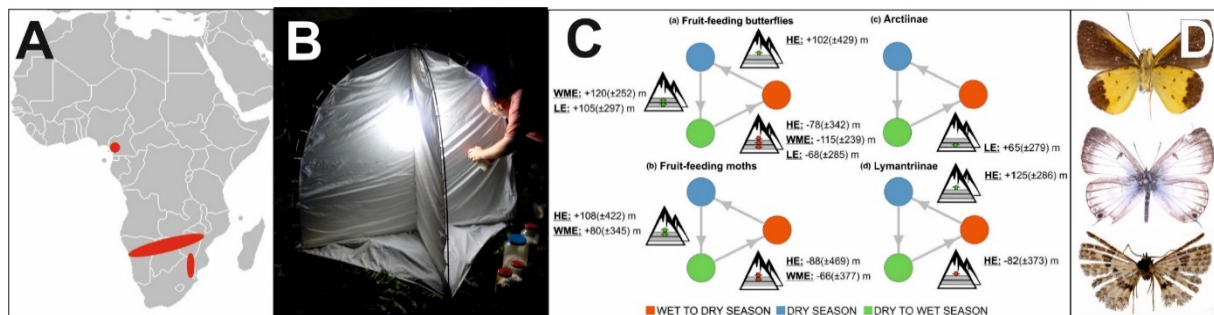


Fig. 7. Research areas for Lepidoptera (A); sampling of moths (B); seasonal shifts of butterflies and moths along elevation (C); newly described Lepidoptera species (D).

## [8] Plant-flower interaction webs in grasslands

We experimentally tested the effects of sequential removal of several plant species on the diversity of flower visitors, flower visitation rates, and plant-flower visitor network structure in grasslands. The total flower visitation decreased exponentially after removing flowers of 1–4 selected plants; responses at species level were mediated by plant functional traits (Biella et al., 2019, *Sci. Rep.* 9, 7376).

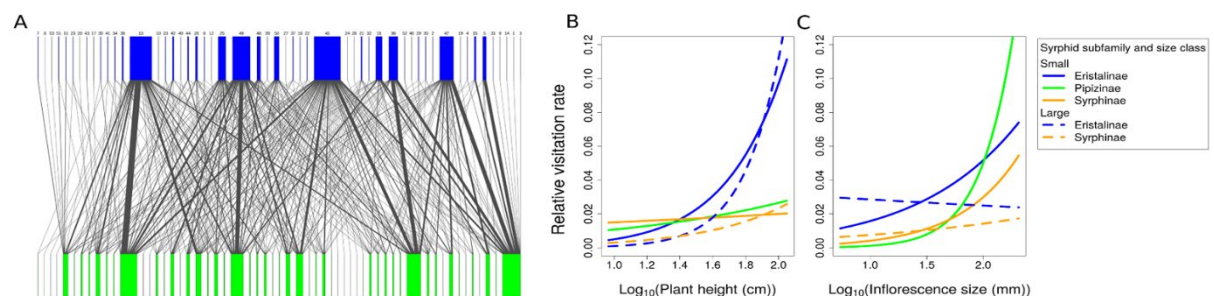


Fig. 8. Flower visitation network between plants (green) and hoverflies (blue) (A). Visitation depended on both plant and hoverfly size (B); plants with large inflorescences were preferred by small hoverflies, but not hoverflies with large body sizes (C).

The cumulative species and interaction extinctions increased faster with generalist plant loss than predicted by co-extinction models. These results highlight the high conservation value of abundant generalist plants as they sustain complex plant-pollinator interactions. Further, we experimented with the patch size of flowering plants for visitation by pollinators (Akter et al., 2017, *PLoS One*, 12, e0187976; Klecka et al., 2018, *PeerJ* 6, e4998). We also analysed in detail flower visitation patterns in hoverflies (*Syrphidae*) and documented how species traits shape the structure of their flower visitation networks (Fig. 8, Klecka et al., 2018, *PeerJ* 6, e6025). We have successfully implemented DNA metabarcoding in our lab to study multiple types of species interactions (Biella et al., 2019, *PLoS One* 14, e0224037).



### [9] Host-parasitoid food webs and climate change in the tropics

We studied tropical host (*Drosophila*) - parasitoid food webs in laboratory microcosms and found that parasitoids are at risk from rising temperatures so that their hosts might be released from parasitoid control due to climate change (Fig. 9C, Thierry et al., 2019, *Ecol. Entomol.*, 44, 581–592). We studied host-associated microbiomes and found that reduced parasitism at higher temperature cannot be explained by changes in the host microbiome (Fig. 9D). We have also published papers on herbivore distribution (Forister et al., 2015, *PNAS*, 112, 442–447) and protective symbiosis (Hrček et al., 2016, *J. Anim Ecol.* 85, 1605–1612).

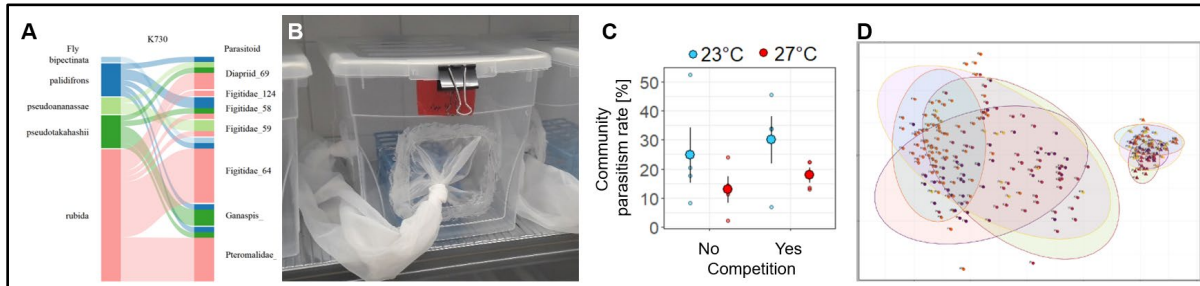


Fig. 9. *Drosophila*-parasitoid food web from Australian rainforest (A). Microcosms developed for long-term food web dynamics experiments (B). Parasitism rate is lower at elevated temperatures, suggesting host release with climate change (C). Microbiome of *Drosophila* is more complex in the field than laboratory lines (D).

### [10] Sampling and analytical methods for food webs along succession gradients

We have reviewed ecosystem-level experimental approaches to the study of food webs in forests (Fig. 10A, Fayle et al. 2015, *TREE* 30, 334–346), and developed a protocol for plot-based surveys of food webs (Fig. 10B, Volf et al. 2019, *PLoS One* 4, e0222119). Using these protocols, we have surveyed plant-caterpillar webs along a rainforest succession gradient, uncovering surprising stability of food web structure in the course of succession (Fig. 10C, Redmond et al., 2019, *Ecography* 42, 162–172). Our empirical work with food webs also led to the development of a new, phylogenetically based host specificity index that we applied on multiple herbivore guilds from rainforest ecosystems (Fig. 10D, Jorge et al., 2017, *Oecologia* 185, 551–559). We developed EcoVault, a database management software designed for ecologists, and freely available for the management of complex ecological data (<http://ecovault-dbms.org/>).

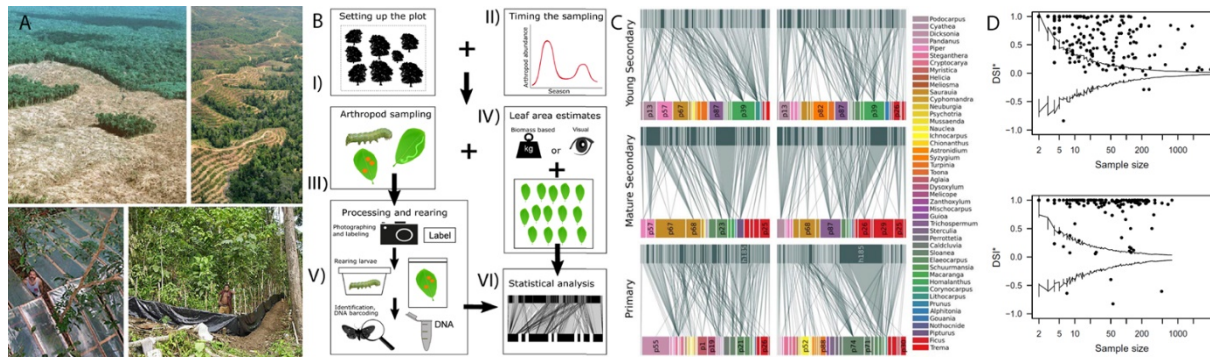


Fig. 10. Examples of “whole forest experiments” (A), the work-flow for the sampling of plant-herbivore interaction webs (B), the plant-caterpillar food web along a rainforest succession gradient (C) and examples of the new, phylogeny-based DSI\* host specificity index that shows random (within the wedge-like area defined by the lines) and non-random host plant selection for adult chewer (D, top) and leaf-tying caterpillar (D, bottom) communities.

### [11] Long-term dynamics of insect populations in the tropics

We studied how climate changes and anomalies (El Nino) affect insect communities and how these changes may lead to disruption in ecosystem functioning and trophic interactions in tropical forests. This insect abundance monitoring in the tropics has now 10-year time-series for focal insect taxa.

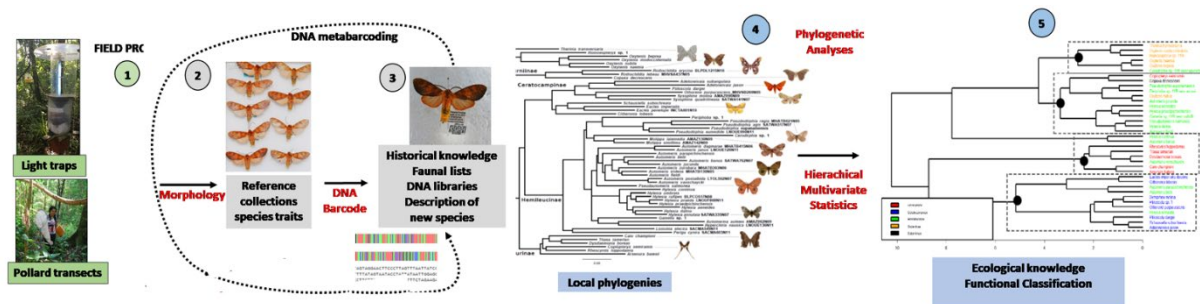


Fig. 11. Workflow of the monitoring scheme we use for long-term monitoring of population dynamics for common, poorly known and species-rich insect assemblages.

The insect sampling is combined with the study of their species traits and phylogenies (Fig. 11, Basset et al., 2017, *Ecol. Evol.* 7, 9991–10004). Our results did not confirm rapid decline in tropical insects in parallel to temperate zone decline and suggest the abundances of larger body-size species with good dispersal abilities may increase concomitantly with rising air temperature in the future. However, insect populations are facing threats from increasing frequency of climate anomalies (Basset & Lamarre, 2019, *Science*, 364, 1230–1231).

### [12] Impacts of anthropogenic habitat change on ant communities

Selective logging of rainforests and their conversion to oil palm plantations have divergent effects on canopy ants (Philip et al. 2018, *J. Trop. Biol. Cons.* 15, 139–154) and ground-dwelling ants (Gray et al. 2015, *J. Appl. Ecol.* 52, 31–40; Gray et al. 2016, *Biol. Cons.* 194, 176–183).

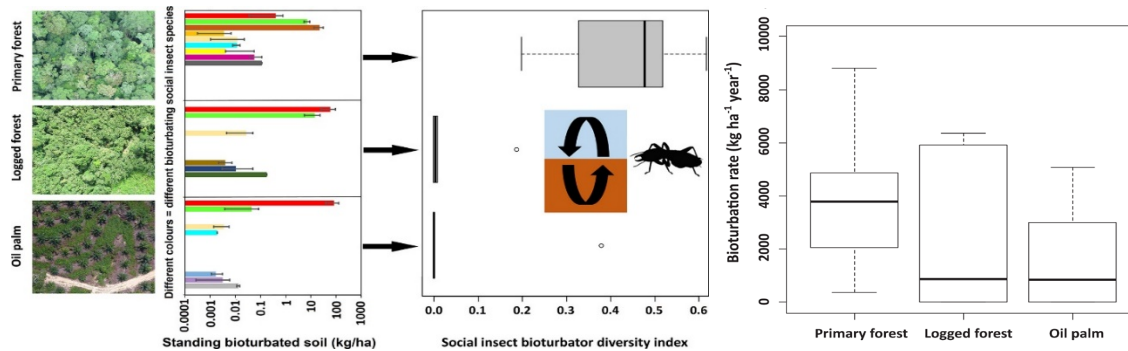


Fig. 12. Decrease in diversity of bioturbating invertebrates and bioturbation rate due to logging and conversion to oil palm plantation.

Even twice-logged forests supported similar numbers of canopy ant species as primary forests, while oil palm supported significantly fewer species. We found radical shifts in the contribution of invertebrates to ecosystem processes (litter decomposition, seed predation, invertebrate predation) between logged forests and oil palm plantations (Ewers et al. 2015, *Nature Comm.* 6, 6836). We also found reduced soil bioturbation by ants and termites when forests were logged and converted to oil palm (Fig. 12, Tuma et al., 2019, *Appl. Soil Ecol.*, 144, 123–133). In contrast, the relationship between ants and epiphytic ferns (nesting space for ants, protection from herbivory for ferns) is not affected by logging or conversion to oil palm (Fayle et al. 2015, *Oecologia* 178, 441–450). We contributed to a global analysis showing that ant communities were disproportionately vulnerable to anthropogenic habitat change in areas with low precipitation and high temperature (Gibb et al. 2015, *Proc. R. Soc. B*, 282, 20150418).

### [13] Applied ecology: Restoration of biodiversity at post-industrial sites

Our applied research focused on the restoration of European post-industrial sites, and their biodiversity and conservation value. We explored optimum restoration practice for biodiversity on fly ash deposits (Fig. 13, Tropek et al. 2016, *Environm. Sci. Pollut. Res.* 23, 13653–13660), coal spoil heaps (Tichanek & Tropek 2015, *J. Insect Conserv.* 19, 975–985; Kolar et al. 2017, *Ecol. Eng.* 99, 310–315; Moradi et al. 2018, *J. Environm. Manag.* 220, 1–7), and sand mines (Řehounková et al. 2016, *Environm. Sci. Pollut. Res.* 23, 13745–13753). We focused on endangered species colonising these habitats, e.g. critically endangered butterfly *Hipparchia semele* (Tropek et al. 2017, *Pol. J. Ecol.* 65: 385–399) and damselfly *Coenagrion ornatum* (Tropek & Tichanek 2016, *J. Insect Conserv.* 20, 701–710). Our results have been implemented in restoration strategies of companies and made into recommendations by the national conservation authorities.

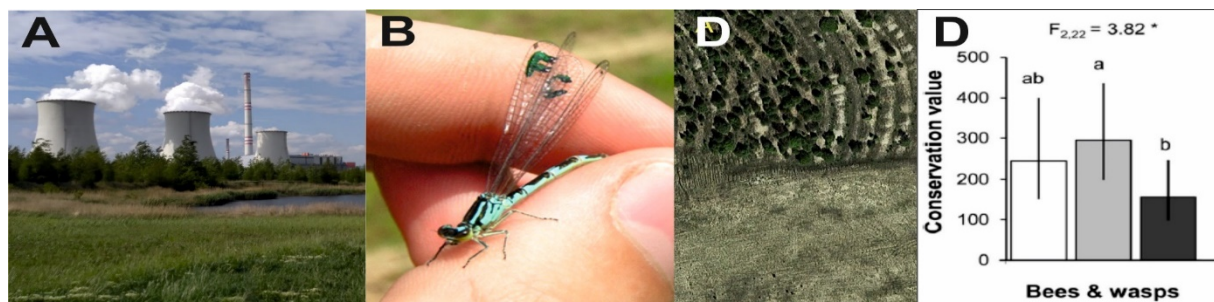


Fig. 13. Fly ash sedimentation used for study (A); a marked specimen of European-protected *Coenagrion ornatum* (B); restoration results visible even on aerial photos (C); comparison of insect communities in differently restored fly ash deposits (D).



#### [14] Evolutionary games with time constraints

We have developed a new approach to the Hawk-Dove and Prisoner's dilemma games that considers duration of interactions between individuals and makes new evolutionary predictions: for the Prisoner's dilemma, when interaction time between two cooperators is long enough and individuals are free to end interaction with the current partner, cooperation can evolve (Fig. 14A, Krivan and Cressman, 2017, *J. Theor. Biol.* 416, 199–207, Krivan et al., 2018, *J. Theor. Biol.*, 455, 232–248). These novel predictions change our way of thinking about evolution of aggressiveness and cooperation (Cressman & Krivan, 2019, *J. Theor. Biol.* 460, 262–273).

#### [15] Effects of adaptive behavior on the structure of plant-animal communities

Classic models (e.g., Lotka-Volterra) generally predict that exploiters destabilize plant coexistence (via apparent competition), but mutualists promote plant coexistence (via indirect facilitations). We explored how animal foraging adaptation modifies patterns of plant coexistence for exploiters/mutualists; we used a consumer-resource model assuming that adaptive pollinators play an ideal free distribution game. These feedbacks can lead to the extinction of the rare plant due to pollinator specialization, or to plant coexistence at alternative stable states when resource scarcity forces competing pollinators towards generalism (Fig. 14B, Revilla & Krivan 2016, *PLoS One*, 11, e0160076; 2018, *J. Theor. Biol.*, 440, 42–57).

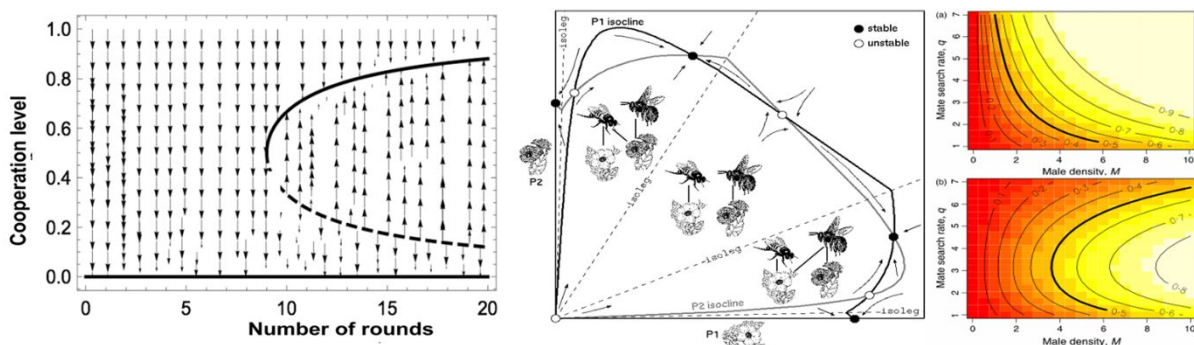


Fig 14. Evolution of cooperation as a function of the number of rounds the interaction is repeated; the lower line is the classic Prisoner's Dilemma prediction (L); two plants - two pollinators coexistence at alternative stable states when pollinator preferences for plants are adaptive (C); the mate-finding Allee effect, female mating probability for unlimited male polygyny as a function of male density and mate search rate in the absence (top R) and presence (bottom R) of the movement-viability trade-off.

#### [16] Ecology and evolution of the mate-finding Allee effect

Allee effect occurs when individual fitness declines as population size or density decrease. We determined the strength of Allee effect in response to the mate-finding process, such as degree of polygyny, mate search rate or within-population heterogeneity (Berec 2018, *Oikos*, 128, 972–983). We may also consider evolution of the Allee effect itself, dependent on frequently occurring trade-offs, such as an observation that faster movement may mean lower reproduction or higher mortality (Fig. 14C, Berec et al., 2018, *J. Anim. Ecol.*, 87, 24–35).

#### [17] Macroevolution, biogeography and phylogeography of Indo-Pacific ants

We integrated species trait data (habitat use) with the inference on ant dispersal across Indo-Pacific archipelagos (Matos-Maraví et al., 2018, *Mol. Ecol.* 20, 4090–4107) and evaluated how they relate to population genetic structure of ants (Janda et al., 2016,

*Inv. Syst.* 30, 28–40). We showed that shifts in habitat preferences predict ancient geographical range expansions and increased diversification in one of the earliest ant lineages that dispersed from Asia into Australia (Fig. 15, Matos-Maraví et al., 2018, *Mol. Phyl. Evol.* 123, 16–25). We also reconstructed evolution of myrmecophytic associations across multiple lineages of plants in Indo-Australia (Chomicki et al., 2017, *Proc. R. Soc. B*, 284, 1–9). A large dataset on ants from hundreds of islands allowed us to re-evaluate basic biogeographic patterns and effect of human introductions on South Pacific ant fauna (Economo et al., 2017, *J. Biogeogr.* 44, 1088–1097). We have also contributed to the development of diversification rate measurement methods (Matos-Maraví, 2016, *Evolution*, 70, 1638–1650).

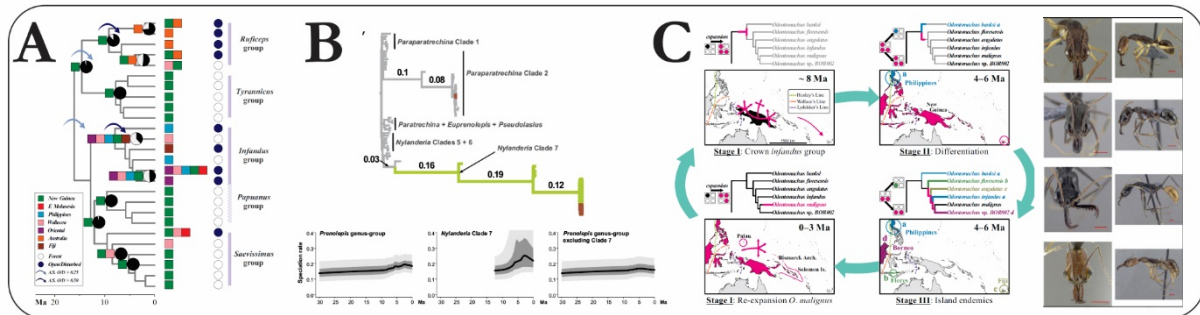


Fig. 15. A new trait-dependent dispersal model suggested that transitions from primary rainforests into marginal habitats enhanced dispersal capabilities of ants across Indo-Pacific archipelagos (A); Habitat preference is correlated with increased diversification in tropical ants (B), and our data support E.O. Wilson's taxon cycle concept, whereby habitat shifts can trigger dispersal and speciation across islands (C).



## Research activity and characterisation of the main scientific results

Twenty-six projects were in progress or inaugurated between 2015–2019, incorporating a wide range of research topics and funding boards (e.g., European Commission, Czech Science Foundation, Technology Agency of the Czech Republic (CR), Ministry of Education, Youth and Sport of the CR, National Science Foundation of the USA, Swedish Research Council, CAPES Brazil). Additional funding was also successfully acquired for ten grant-funded short-term studies and travel to foreign institutions (European Commission Horizon 2020: ASSEMBLE PLUS, SYNTHESYS, several mobility grants).

### 1. Summary of scientific outcomes

During five years (2015–2019), members of the team co-authored a total of **281 scientific articles published in journals with impact factors (IF)** including comprehensive papers of major impact in *Molecular Ecology and Evolution*, *PNAS*, *Clinical Microbiology Reviews*, *Current Biology*, *Molecular Ecology*, *Emerging Infectious Diseases*, *Nature Genetics*, *PLoS Pathogens*, and *Trends in Parasitology*. In addition, they co-authored several book chapters and **two monographs**. **The sum of IF increased** from 463.604 (2010–2014) **to 723.432** (2015–2019), i.e., **by more than 50%** (but LEM is newly included), and **the average IF increased from 1.99 (2010–2014) to 2.57 (2015–2019)**, with 30% of articles published in the first quartile journals.

### 2. Main results

The most important results achieved, and milestones reached during the period of 2015–2019 in the relevant research field are presented below. Additional data are summarised in the list of **29 selected outcomes** with brief annotations on their importance and the contribution made by team members.

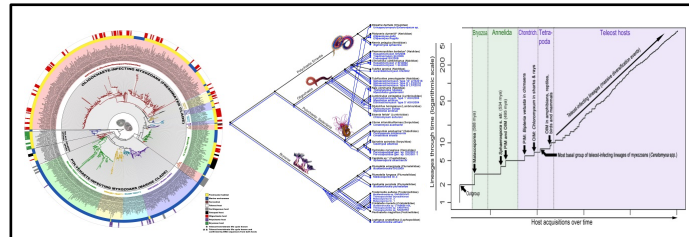
#### 2.1. Laboratory of Fish Protistology

##### 2.1.1. Biodiversity, phylogeny and evolution of myxozoans in fishes

Our research has considerably enriched the present knowledge of myxozoan biodiversity by adding more than 70 species and over 420 sequences of 18S rRNA gene, including a number of underexplored phylogenetic clades and host groups (e.g., Alama-Bermejo et al., 2016, *Parasit. Vector.* 9, 13, Bartošová-Sojková et al., 2015, *Parasitology* 142, 660–674 and Patra et al., 2018, *Parasit. Vector.* 11, 347). Myxozoan diversity and distribution were also elucidated with regard to ecological factors: In *Ceratomyxa* spp. from small clinid fish in intertidal rock pools, we demonstrated that parasite and fish host population structures overlap and how they are affected by oceanographic barriers and tidal water exchange (Bartošová-Sojková et al., 2018, *PLoS ONE* 13, e0194042). The influence of repatriation of the migratory allis shad on *de novo* establishment of myxozoan infections was investigated in the river Rhine (Wünnemann/Holzer et al., 2016, *Parasit. Vector.* 9, 505) and showed a slow invasion from genetically diverse sources, in contrast to fully established local strains, with river-specific signatures, which we found in allis shad-enzootic rivers.

Following evolutionary studies of myxozoans in ancient fish hosts (Kodádková et al., 2015, *Int. J. Parasitol.* 45, 269–276), we performed a large-scale study examining the history of adaptive radiations of myxozoans and their invertebrate and vertebrate hosts

by determining the degree of congruence between their phylogenies and by timing their emergence in relation to their hosts (Holzer et al., 2018, *Mol. Ecol.* 27, 1651–1666; Fig. 1). We demonstrated that the Myxozoa emerged almost 700 MYA and that bryozoans and annelids served as original hosts. Following invertebrate invasion, fish hosts were acquired multiple times, leading to parallel co-speciation patterns in all major lineages. The results transformed our understanding of the origins and evolution of parasitism in this group (26 citations in <2 years).



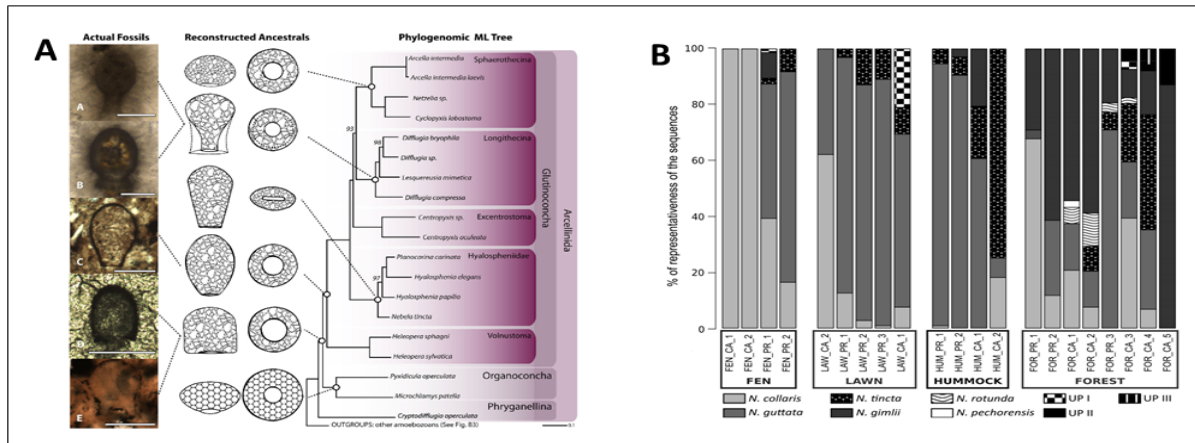
**Figure 1.** Co-evolution of myxozoans and their hosts: A – myxozoan phylogeny and mapped traits (environments, invertebrate and vertebrate hosts, host organ habitats), with the four major lineages defined by invertebrate host groups; B – overlap of myxozoan and invertebrate host phylogenies: parasite lineages (blue lines) mapped on to the host tree (black lines); C – lineages over time plot showing diversification of myxozoans once they had acquired fish hosts (purple squares), especially teleosts (large purple square).

### 2.1.2. Evolution, systematics and ecology of amoeboid organisms

In collaboration with Swiss and Brazilian partners, we focussed on a number of studies aimed at reconstructing amoebozoan phylogeny. We used 325 genes and morphotypes of 61 taxa to address major questions regarding diversification and evolution of this diverse supergroup (Kang et al., 2017, *Mol. Biol. Evol.* 34, 2258–2270). The last common ancestor had a multistate life cycle characterized by sex, flagella and sporocarps, which were lost in different lineages. We contributed to the understanding of macroevolutionary patterns in the Amoebozoa and exploring convergent and homologous morphological and genomic character-states that shaped this supergroup (80 citations in <3 years).

In the earliest amoebozoan lineages, arcellinid testate amoebae, we conducted phylogenetic analysis, based on 250 genes, to resolve their deepest nodes (Lahr et al., 2019, *Curr. Biol.* 29, 991–1001). Surprisingly, the morphological reconstruction of the ancestral shell shapes showed remarkable similarity with Neoproterozoic vase-shaped microfossils (730 MYA) (Fig. 2A). This demonstrates that the major lineages of testate amoebae were already diversified before the Sturtian glaciation (720 MYA).

Furthermore, environmental DNA studies revealed unexpected pseudocryptic diversity within the arcellinid genus *Nebela* (Singer et al., 2018, *Ecology* 99, 904–914). A survey of the distribution of these organisms across the microniches of two peatbogs showed that each form had diverging ecological optima, suggesting resource partitioning and environmental filtering drive community composition (see Fig. 2B). We also explored how climate and geographical processes are linked to major diversification events in *Hyalosphenia papilio*, where patterns of biodiversity are correlated with the history of habitat expansions (Singer et al., 2019, *Mol. Ecol.* 28, 3089–3100; 14 citations in 1 year).



**Figure 2.** Evolution of the Arcellinida: A – ancestral state reconstruction and neoproterozoic fossils. Five rediscovered neoproterozoic fossils (left), reconstruction of ancestral morphology, organic and agglutinated shells (middle), and phylogenetic tree obtained from the analysis of 250 genes (right); B – community profiles of the *Nebela collaris* complex extracted from *Sphagnum* samples from two peatlands in the Swiss Jura. Each bar plot represents the relative ratio of clone sequences corrected by the cell volume.

### 2.1.3. Parasites threatening amphibian biodiversity

The myxozoan *Cystodiscus axonis* known previously from Australian frogs was discovered in two South American caecilians (Apoda). Its occurrence in caecilians indicates a previously unknown myxozoan capacity for infecting highly divergent amphibian hosts. However, our data strongly imply infection in captivity, prior to sale, and indicate that the exotic pet trade may pose a threat to caecilians (Hartigan et al., 2016, *Int. J. Parasitol.* 46, 375–381).

We discovered the diversity of perkinsid-like protists causing mass mortalities of amphibians in the USA (Chambouvet et al., 2015, *PNAS* 112, E4743–E4751). More recently, we focussed on another devastating pathogen of amphibians, *Batrachochytrium dendrobatidis*, a chytrid fungus. We showed that this pathogen has distinct phylogenetic lineages, which vary in geographical extent and virulence, and we developed a genotyping method to understand their distribution and interaction (Byrne et al., 2019, *PNAS* 116, 20382–20387). This will allow us to better track pathways of disease spread in this system and link specific pathogen lineages to outbreaks in wild populations.

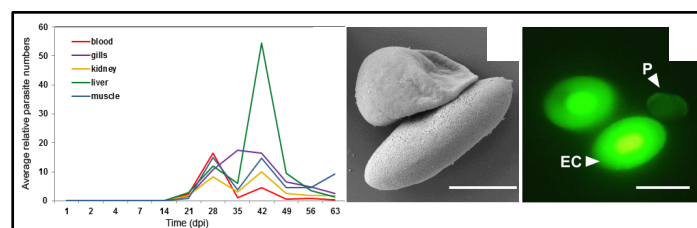
### 2.1.4. Host-parasite interactions and fish immune responses

We established the world's first *in vivo* and *in vitro* model for myxozoan proliferation research, *Sphaerospora molnari* in common carp, allowing us to perform targeted infections under controlled laboratory conditions.

We elucidated motility mechanisms in myxozoans and their relevance in regard to host invasion, exploitation and host immune evasion. *Sphaerospora molnari* has a motility mode which is unique amongst eukaryotes. Using specific inhibitors *in vitro*, we demonstrated that it reduces attachment and lysis of parasites by host immune cells (Hartigan et al., 2016, *Sci. Rep.* 6, 39093). In *Ceratonova shasta*, we investigated changes in morphology and motility gene expression during host invasion, migration and proliferation of virulent *versus* non-virulent strains, and identified rapid parasite multiplication, fast bleb-based migration and strong adhesion as important virulence mechanisms (Alama-Bermejo et al., 2019, *Microorganisms* 7, 397).

The kinetics of the presporogonic development of *S. molnari* was characterised in parallel to analysing the kinetics of host responses (Korytář et al., 2019, *Parasit. Vector.* 12, 208). Following an initial covert infection period, we observed parasite multiplication associated with a massive lymphocyte response, specific antibody production and a parasite-mediated switch to an anti-inflammatory response. Investigations into *S. molnari*-carp interactions also revealed previously unknown mechanisms of host erythrocyte exploitation, leading to hemolytic anemia (Korytář et al., 2020, *Parasit. Immunol.* – doi.org/10.1111/pim.12683).

Finally, we addressed host-parasite interaction in epicellular apicomplexans in fish, based on molecular and ultrastructural data (Bartošová-Sojková et al., 2015, *PLoS Path.* 11, e1005080), and contributed to the analyses of a number of parasite's enzymes and interactive functional proteins in other parasites (Sojka et al., 2016, *Trends Parasitol.* 32, 708–732; Urbanová et al., 2018, *Comp. Immunol.* 79, 86–94).



**Figure 3.** *Sphaerospora molnari* in common carp. A – kinetics of development and localisation in different fish organs (qPCR); B – parasite (top) gnawing on erythrocyte; C – protein transfer from erythrocyte to parasite as demonstrated by CSFE staining.

## 2.2. Laboratory of Helminthology

### 2.2.1. Planetary Biodiversity Inventory (2008–2017): Global diversity of tapeworms

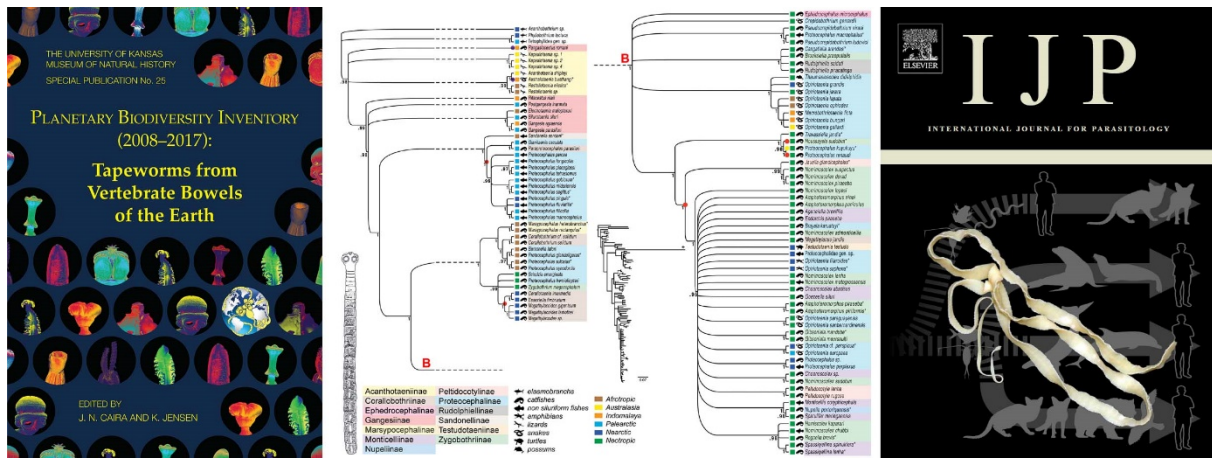
For 10 years, an international team of this project funded by the National Science Foundation has worked to discover and describe tapeworms from major groups of vertebrates across the globe. This project has also included substantial formalised databasing (Global Cestode Database – <https://tapeworms.uconn.edu/>), training and outreach activities. In a final monograph (Caira and Jensen, Eds., 2017 – see below Participation in large collaborations), an account has been provided of the results of collective efforts to discover, describe and explore the distributions, host associations and phylogenetic relationships of tapeworms across the globe (Fig. 4). The results of the team working mainly on cestodes of bony fish and herptiles, coordinated by T. Scholz, have been summarised in 11 chapters co-authored by J. Brabec, R. Kuchta and T. Scholz.

### 2.2.2. New phylogenetic hypotheses on major tapeworm orders (Cestoda)

The above-mentioned NSF-PBI project made it possible to assess the interrelationships of tapeworms of three major orders. Using a phylogenetic framework based on four genes (*ssrDNA*, *lsrDNA*, *rmlL* and *COI*), new phylogenetic hypotheses were proposed for the Bothriocephalidea, parasites of bony fishes (Brabec et al., 2015, *Int. J. Parasitol.* 45, 761–771), and the Diphyllbothriidea, which includes the principal agents of widespread food-borne diseases (diphyllbothriosis with 20 million people infected and potentially lethal sparganosis with about 2,000 cases). In the latter case, data indicate that humans have been acquired as accidental hosts at least four times (Waeschenbach et al., 2017, *Int. J. Parasitol.* 47, 831–843). We also analysed the largest dataset of proteocephalideans to date comprising more than 100 species (30



new) from 54 genera (80%) and all subfamilies (Fig. 4). The Old World origin of proteocephalideans in lizards in the tropics or in catfishes was confirmed, with a more recent expansion in South America (de Chambrier et al., 2015, *ZooKeys* 500, 25–59).



**Figure 4.** Covers of a monograph on the global diversity of tapeworms (left) and an article on the phylogeny of broad tapeworms (Diphyllobothriidea; right), and the phylogenetic tree of the Proteocephalidea (middle).

### 2.2.3. Parasites of freshwater fish in Africa – diversity, ecology and research methods

Based on long-term research, including numerous field trips to Africa and collaboration with researchers from Belgium and South Africa, a monograph on the parasites of freshwater fishes in Africa was published (Fig. 5). It describes methods to be used in studies on fish parasites and provides the first comprehensive list of all known freshwater fish parasites in Africa, with keys to all genera and representative illustrations for every genus (Scholz et al., Eds., 2018, *A Guide to the Parasites of African Freshwater Fishes*, ABC Taxa, 18, 422 pp.). In addition, the first monograph on the nematodes of freshwater fishes in Africa was published (Moravec, F., 2019, *Parasitic Nematodes of Freshwater Fishes of Africa*. Academia, Prague, 406 pp.).

### 2.2.4. Diversity and phylogenetic relationships of helminths in the Neotropical region

Numerous multidisciplinary studies have been carried out on all major groups of helminth parasites in the Neotropical region, based on the long-term involvement of team members in research and training in South America (Argentina, Brazil and Peru) and Mexico. Forty-seven new species were described, and 11 new genera proposed; 24 species and 7 genera were re-described in a total of 34 papers. New molecular data on 73 taxa were provided. Among other initiatives, the first molecular phylogenetic study on monogeneans from Neotropical catfishes was carried out to reconstruct their evolutionary history based on partial sequences of the *18S*rDNA of 25 species. The most intriguing result is revealing non-monophyly of Neotropical dactylogyrids from siluriforms, even though they share similar morphology and parasitise the same fish hosts (Mendoza-Palmero et al., 2015, *Parasit. Vector* 8, 164).

### 2.2.5. Species boundaries, diversity, ultrastructure and interrelationships of tapeworms (Cestoda)

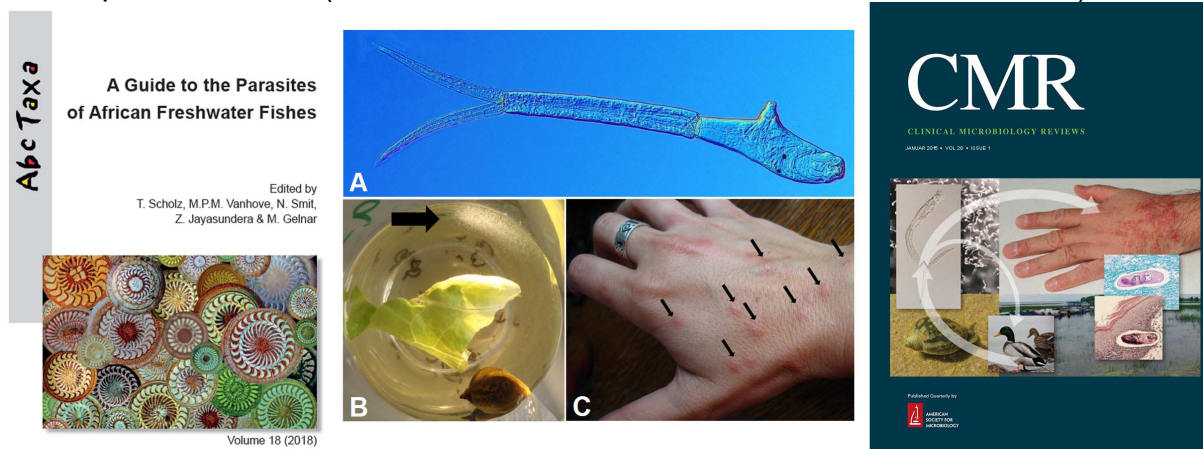
A multidisciplinary approach to the systematics of tapeworms and intensive international collaboration made it possible to define species boundaries in several groups of cestodes, unravelling hidden species diversity in some groups. New morphological, ultrastructural, biological and molecular data enabled us to assess



more reliably the interrelationships of a wide spectrum of tapeworms of fishes and reptiles throughout the world. These studies included the first global “digest”, i.e., detailed analysis of diversity, host associations and distribution, of 461 species of fish (Scholz and Kuchta, 2017, *Vie Millieu* 67, 43–58).

#### 2.2.6. Integrative taxonomy approaches to trematode diversity and ecology

The application of an integrative approach facilitated the elucidation of the diversity of larval and adult trematodes throughout the world, including the Arctic, where diversity of trematodes is largely unexplored. Molecular characterisation of 120 isolates from northern Norway revealed an unexpectedly high diversity (24 species or species-level genetic lineages and 16 putative new species) unique for these ecosystems, which contradicts the generally accepted dogma that the Arctic ecosystems are depauperate and trophic links short (Soldánová et al., 2017, *Int. J. Parasitol.* 47, 327–345).



**Figure 5.** Covers of a monograph on the parasites of African freshwater fishes (left) and a review article on cercarial dermatitis (right), and the causative agent of cercarial dermatitis, *Trichobilharzia* spp. (A), its intermediate snail host (B) and a human hand with exanthema (C) (middle).

#### 2.2.7. Global diversity of nematodes parasitising teleost fish

Extensive taxonomic studies on parasitic nematodes from freshwater and marine fishes in different geographical regions, carried out mainly by F. Moravec, resulted in the discovery of a large number of previously unknown species and the clarification of many existing taxonomic problems. Special attention was paid to an important group, the tissue-dwelling philometrid nematodes. A total of 28 new species of philometrids including three new genera were established and a high degree of host specificity in gonad-infecting species revealed. An additional 44 new species of fish nematodes including three new genera were described.

#### 2.2.8. Fish- and water-borne helminthoses as emerging human diseases

New data on several human-infecting species of broad tapeworms (Diphyllbothriidea) have been accumulated, including the first detection of larvae of *Dibothriocephalus nihonkaiensis* in Pacific salmon from North America (Kuchta et al., 2017, *Emerg. Inf. Dis.* 23, 351–353), *D. latus* in salmonids from South America (Patagonia) (Kuchta et al., 2019, *Emerg. Inf. Dis.* 25, 2156–2157) and a comprehensive survey of human cases caused by the Pacific broad tapeworm *A. pacificus* (Kuchta et al., 2015, *Emerg. Inf. Dis.* 21, 1697–1703). A-Book chapters and several reviews on this topic have also been published.

#### 2.2.9. Invasive fish parasites

Our research focused mainly on one of the most successful invasive parasites, the Asian fish tapeworm (AFT; *Schyzocotyle acheilognathi*). We have increased updated the number of reported hosts of the AFT to 312 fish species and 11 non-fish hosts, which is quite unusual among helminths. It has spread to almost all continents; its broad environmental tolerance (biotic and abiotic) is a key factor in its successful dispersal (Kuchta et al., 2018, *Trends Parasitol.* 34, 511–523).

#### 2.2.10. High-throughput Sequencing and mitogenomics

As a key innovation, we started applying High-throughput Sequencing (HTS) technology in studies of non-model aquatic helminths to provide a foundation for large-scale analyses. We characterised novel complete mitochondrial genomes and nuclear rRNA operons of two closely related species of diplostomid trematodes, important pathogens causing eye fluke disease in cultured fish. Using the new data, we provided the first genome-wide estimation of the phylogenetic relationships of the order Diplostomida (Brabec et al., 2015, *Parasit. Vector.* 8, 336). We also published the first complex survey of the diversification of the AFT based on samples at multiple locations across continents and discovered distinct lineages through comparisons of mitochondrial genomes (Brabec et al., 2016, *Int. J. Parasitol.* 46, 555–562). We participated in a broad comparative study of 81 genomes of parasitic helminths published in *Nature Genetics* (Coghlan et al., 2019, *Nature Gen.* 51, 163–174)

### 2.3. Laboratory of Electron Microscopy

#### 2.3.1. Service in the field of biological electron microscopy & development of equipment and technologies

The modernisation of our best transmission electron microscope – TEM JEOL 2001F equipped by the field emission cathode – was performed in 2019. The new direct camera K2 (Gatan), cryo-attachment and phase plate technology were added to equip the microscope for cryo-applications, such as cryo-electron tomography or single-particle analysis of vitreous ice-embedded samples. The upgrade of TEM JEOL 2001F resulted from collaboration with a newly created multidisciplinary research centre “Makrokomplex” at the Faculty of the Science, University of South Bohemia. This group is focussed on studying the mechanisms of large macromolecular assemblies. In this research, TEM with the highest resolution performed on vitrified specimens plays an irreplaceable role.

In 2019, we installed a new scanning electron microscope equipped for 3D reconstructions. The high-resolution SEM Apreo VS (Thermo Fisher Scientific) is able to work at accelerating voltage below 5 kV using BSE imaging. The microscope is designed for 3D reconstructions using serial block face method, a microarray tomography. Both methods expand possibilities for volume microscopy of biological samples in size ranging from a few cubic micrometres to millimetres in the Laboratory of EM. The purchase of HR SEM was financed in large part from the project “Modernisation and support of research activities of the national infrastructure for biological and medical imaging Czech-Biolmaging” supported by the European Development Fund.

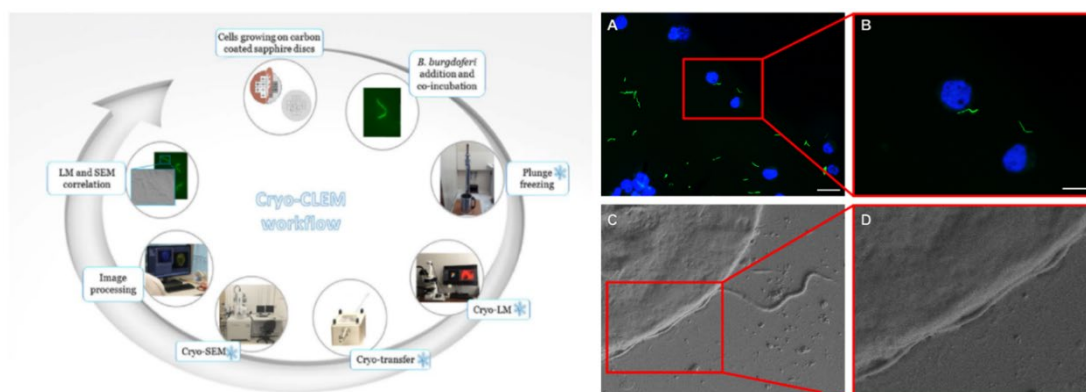
A critical step in electron microscopy of biological samples is specimen preparation. Therefore, the relevant equipment for this was also modernised. In 2019, new instruments for freezing biological specimens at high pressure (HPF Ice Leica

Microsystems) and immersion in liquid nitrogen (plunge freezer Leica Microsystems) were installed. Also, the equipment necessary for cutting ultrathin sections was strengthened by the purchase of a new ultramicrotome UCT7 (Leica Microsystems).

### 2.3.2. Collaborative research in the field of electron microscopy of biological objects

Seven projects were investigated in the LEM and 59 papers were published. In the consortium “Electron Microscopy” funded by the Technology Agency of the Czech Republic, we closely collaborated with companies producing electron microscopes (Thermo Fischer Scientific – TFS, DeLong Instruments, Crytur). Our task was to find sample preparation procedures for newly developed techniques and to demonstrate their advantages in suitable applications. We developed new methods of multiple immunolabelling for SEM working in the STEM mode. This method allows a separate visualisation of both sides of the ultrathin sections that can be used for immunolabelling. As a result, four different molecules of interest can be detected simultaneously using conjugates of gold nanoparticles with two diameters as markers (Nebesářová et al., 2016, *Nanomed. Nanotechnol., Biol. Med.*, 12, 105–108).

In collaboration with TFS, we established a protocol for the preparation of standard specimens of mouse brain tissues for Serial Block Face SEM. The protocol is based on the procedure published by Derrinck (2016), which has been further improved and modified. At present, the standard samples produced by our lab are supplied to TFS and used to check the functionality of SEM equipped with SBF. The standard sample, together with the protocol, is included in the delivery documentation of each SBF SEM that TFS sells.



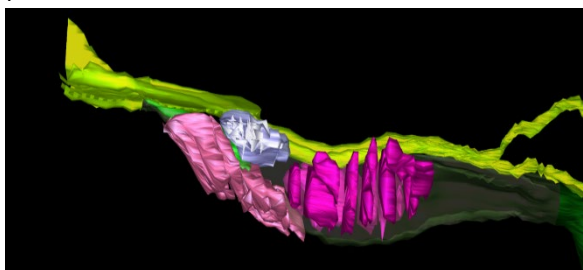
**Figure 6.** Work-flow of the CLEM approach used for visualizing interactions of *Borrelia burgdorferi* expressed GFP with neuroblastoma cells.

In the evaluated period, we examined the correlative approach in selected applications. We took a unique opportunity to use, as one of the first laboratories in the world, a cryo-fluorescence microscope in combination with cryo SEM. This new correlative approach was exploited to study preparation-artefact-free interactions of GFP expressing *Borrelia* with host cells *in vitro* (Strnad et al., 2015, *Sci. Rep.* 5, 18029), or their morphological changes under physiological stress (Vancová et al., 2017, *Front. Microbiol.* 8, 596).

Electron tomography was used for 3D visualisation of neural cells infected with tick-borne encephalitis virus and revealed changes in the organisation of the rough endoplasmic reticulum and the formation of its virus-induced sub-compartments (Bílý et al., 2015, *Sci. Reports*, 5, 10745). We also contributed to the characterisation of Zika virus infection in primary human astrocytes employing electron tomography (Stefanik

et al., 2018, *BMC Neurosci.*, 19, 5). The combination of immunogold labelling of cryosections with electron tomography allowed us to localise two cytoplasmic proteins responsible for the unusual movement of the myxozoan *Sphaerospora molnari* and created a new cell motility model (Hartigan et al., 2016, *Sci. Rep.* 6, 39093). Electron tomography was also used for studying the ultrastructure of the sperm cell of basal fish tapeworms (Cestoda: Caryophyllidea) (Matoušková et al., *Sci. Rep.* 9, 12991; Matoušková et al., 2018, *Parasitol. Res.* 117, 3091–3102).

LEM is considered as a leader in the Czech Republic in SEM of biological specimens. This position led to many successful international collaborations, where we exploited our knowledge with specimen preparation (Sytnyk et al., 2017, *Nat. Comm.* 8, 91) and the capability to observe vitrified specimens at cryo conditions (Monikh et al., 2019, *Environ. Sci. Technol.* 53, 946–953; Monikh et al., 2019, *Sci. Total Environ.* 660, 1283–1293). In recent years, we have focussed on the introduction of 3D techniques for SEM that have expanded our ability to visualise organisms up to several  $\mu\text{m}$  in size. The model of the tick *Ixodes ricinus* mouthparts and associated structures during blood meal acquisition and salivation represents the first result (Vancová et al., 2020, *Sci. Rep.* 10, 165 – Fig. 7).



**Figure 7.** 3D model of the feeding apparatus of the nymph of *Ixodes ricinus* created using the modified microarray method.

### 2.3.3. Education in the area of biological electron microscopy including on-site training for methods of specimen preparation and use of equipment

Staff of LEM have been teaching courses in electron microscopy for biologists at the Faculty of Science at the University of South Bohemia and recently for students of the cross-border program Biological Chemistry with the Johannes Kepler University in Linz.

LEM is internationally recognised for organising prestigious international European Molecular Biology Organisation (EMBO) courses on advanced methods of electron microscopy for cell biology. In the evaluation period, the course was held twice (2016 and 2019) and in both cases rated as excellent by students and teachers. The regular organisation of these practical EMBO courses illustrates the high methodological and technical level achieved by LEM and its staff.

### **Practical and applied outcomes**

*Protistology* established several new diagnostic methods for myxozoans in common carp and successfully developed an *in vivo* challenge system to test the effects of immunostimulants and potentially parasitocidal substances (prototype/functional sample #0488261, 2018) for research into dietary food additions against myxozoans. The laboratory also had two commercial contracts (2014–2015 and 2016–2018) with Skretting, Norway (NUTRECO company), the largest world producer of fish and shrimp

feed, focussing on *in vivo* trials in *Paramoeba perurans*, which causes Amoebic Gill Disease in Atlantic salmon. The laboratory assisted in the development of the commercial diet *Protec Gill*, currently one of their best-selling functional diet products. The lab also produced a new methodology for alternative ecosystem management, certified by the Czech Ministry of Environment.

Since 2016, LEM has been involved in a distributed national research infrastructure Czech-BioImaging (<https://www.czech-bioimaging.cz/>), which provides open access to a wide range of imaging technologies and expertise to all scientists in the Czech Republic and abroad by a coordinated logistic approach.



## Research activity and characterisation of the main scientific results

(underlined contributions fall into the evaluated period)

### Chromerids and evolution of parasitism in Apicomplexa

We have substantially participated on the discovery of **chromerids** *Chromera velia* (*C. velia* was elected as the Alga of the Year 2020 by the German Society for Plant Sciences (DBG) <https://www.dbg-phykologie.de/en/alga-of-the-year/alga-of-the-year-2020>) and *Vitrella brassicaformis* (Moore and Oborník et al., 2008, *Nature* 451, 959-963), complex algae isolated from Australian stony corals. We have performed various studies on their **phylogeny, morphology, ultrastructure and life cycles** (Oborník et al., 2011, *Protist* 162, 115-130; Oborník et al., 2012, *Protist* 163, 306-323; Füssy et al., 2017, *Protist* 168, 80-91; Vazač et al., 2018, *Protist* 169, 53-63), their plastid (Janouškovec et al., 2010, *PNAS* 107, 10949-10954), mitochondrial (Flegontov et al., 2015, *Mol. Biol. Evol.* 32, 1115-1131) and nuclear **genomes** (Woo et al., 2015, *eLife* 4, e06974). We showed that the respiratory chain of *Chromera velia*, a phototrophic relative of parasitic apicomplexans, lacks complexes I and III, making it a uniquely reduced aerobic mitochondrion. In *Chromera*, putative lactate: cytochrome c oxidoreductases are predicted to transfer electrons from lactate to cytochrome c, rendering complex III unnecessary. The mitochondrial genome of *Chromera* has the smallest known protein-coding capacity of all mitochondria, encoding just *cox1* and *cox3* on heterogeneous linear molecules. In contrast, another photosynthetic relative of apicomplexans, *Vitrella brassicaformis*, retains the same set of genes as apicomplexans and dinoflagellates (*cox1*, *cox3*, and *cob*) (Flegontov et al., 2015, *Mol. Biol. Evol.* 32, 1115-1131). We provided evidence for the long-disputed evolutionary link between **apicomplexan and dinoflagellate plastids** (Janouškovec et al., 2010, *PNAS* 107, 10949-10954) and suggested possible tertiary origin of chromerid plastids within the stramenopiles (Ševčíková and Horák et al., 2015, *Sci. Rep.* 5, 10134; Sobotka et al., 2017, *Sci. Rep.* 7, 13214). We also contributed to the description of putative **novel lineages** and species of **apicomplexans** infecting invertebrates (Janouškovec et al., 2012, *Curr. Biol.* 22, R518-R519; Janouškovec et al., 2013, *ISME J* 7, 1-4; Rueckert & Horák, 2017, *PLoS ONE* 12: e0187430).

We showed the drastic reduction of protein subunits involved in the architecture of **photosystems in chromerids**, which appeared to use the most reduced set of photosystem proteins in phototrophs (Sobotka et al., 2017, *Sci. Rep.* 7, 13214), despite their highly efficient photosynthesis. We predicted using modified, carefully selected predictors (ASAFind, TargetT) **organellar proteomes** of chromerids (Füssy et al., 2019, *Genome Biol. Evol.* 11, 1765-1779). We developed the protocol for **isolation of organelles** from *C. velia* (Sharaf et al., 2019, *Planta* 250, 1731-1741) usable for further experimental work, for example, to identify membraneous proteins in *C. velia* organelles, which could be found by predictions of targeting signals.

We have contributed to **genomic sequencing** studies in **chromerids** (Woo et al., 2015, *eLife* 4, e06974). We showed that apicomplexan parasites share a high number of genes with their phototrophic ancestor and modified the genes for the parasitic lifestyle.

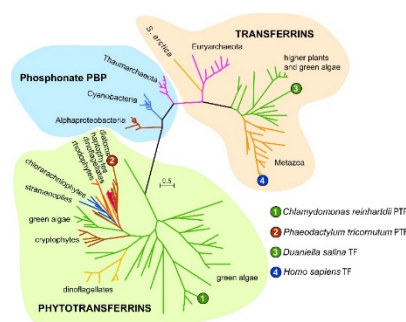
We have also significantly contributed to our understanding of the evolution and diversity of Apicomplexa-related lineages by molecular characterization of several lineages closely related to the Apicomplexa – *Piridium* and *Platyproteum*. Here we

have demonstrated that **“Apicomplexa-like parasites” have evolved several times independently** (Mathur et al., 2019, *Curr. Biol.* 29, 2936-2941). We have also analyzed the worldwide distribution of these lineages in short-read sequencing amplicons and identified several novel clades of Apicomplexa-related lineages (Mathur et al., 2018, *Environ. Microbiol.* 20, 2824-2833).

## Evolution of metabolic pathways

We also focused on the **evolution of metabolic pathways** (mainly the heme pathway) in pioneering studies showing their evolutionary mosaic character (the pathway is composed of enzymes of various evolutionary origins) (Oborník and Green, 2005, *Mol. Biol. Evol.* 22, 2343-2353; Jiroutová et al., 2007, *J. Mol. Evol.* 65, 496-511; Kořený and Oborník, 2011, *Genome Biol. Evol.* 3, 359-364). We also discovered the **non-canonical heme pathway** in chromerids (Kořený et al., 2011, *Plant Cell* 23, 3454-3462) and followed the work with analysis of the pathway in algae with complex plastids. We published a paper about heme biosynthesis in the cryptophyte *Guillardia theta*, chlorarachniophyte *Bigeloviella natans*, green dinoflagellate *Lepidodinium chlorophorum* and dinotoms *Durinskia baltica*, *Glenodinium foliaceum*, and *Kryptoperidinium foliaceum*. *B. natans* appears to contain two separate heme pathways analogous to those found in *Euglena gracilis* (Kořený and Oborník, 2011, *Genome Biol. Evol.* 3, 359-364); one is predicted to be mitochondrial-cytosolic, while the second is putatively plastid-located. In the remaining algae, only plastid-type tetrapyrrole synthesis is present, with a single remnant of the mitochondrial-cytosolic pathway, a ferrochelatase of *G. theta* putatively located in the mitochondrion. The green dinoflagellate contains a single pathway composed of mostly rhodophyte-origin enzymes, and the dinotoms hold two heme pathways of apparently plastidial origin. We suggest that heme pathway enzymes in *B. natans* and *L. chlorophorum* share predominant origin in rhodophytes. It implies the ancient presence of a rhodophyte-derived plastid in the chlorarachniophyte alga, analogous to the green dinoflagellate, or an exceptionally massive horizontal gene transfer (Cihlář et al., 2016, *PLoS ONE* 11, e0166338).

We summarized our work on the evolution of the heme pathway in photosynthetic eukaryotes in the invited review/book chapter (Cihlář et al., 2018, *Adv. Bot. Res.* 90, 273-309).



**Figure 1.** Evolution of transferrins and phytotransferrins from an ancestral phosphonate periplasmic binding protein.

We contributed to the breakthrough discovery of **phytotransferrins** in diatoms (ISIP2A protein), a novel group of iron-binding proteins essential for the uptake of the picomolar labile iron in the ocean (McQuaid et al., 2018, *Nature* 555, 203-209).

Phytotransferrins share a deep ancient common origin with transferrins; however, their ancestral protein (Periplasmic Binding Protein, PBP) did not bind the iron; the iron-specific binding site has evolved in both groups independently. When the ISIP2A gene was disrupted in the diatom *P. tricornutum*, the phenotype was successfully rescued by the human transferrin, despite their very low sequence similarity. The follow-up study focused on another mechanism of iron uptake in diatoms, the **reduction-dependent siderophore assimilation** (Coale et al., 2019, *PNAS* 116, 23609-23617).

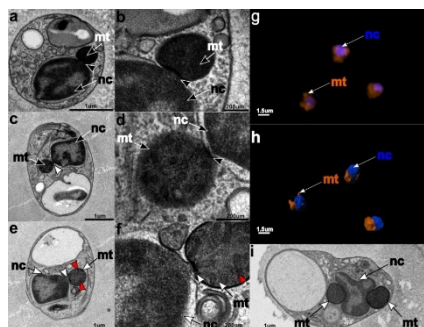
We participated in the discovery of the **urea pathway (OUC) in diatoms** (Allen et al., 2011, *Nature* 473, 203-209). The pathway had been known only in animals before we found it in diatoms. However, diatoms do not use it to get rid of superfluous nitrogen but more likely to sufficiently manage the nitrogen in the N-poor marine environment as we showed in the follow-up study describing the evolution and regulation of **nitrogen flux in diatoms** (Smith et al., 2019, *Nat. Commun.* 10, 4552). The article shows the location of the nitrogen-associated metabolic pathways in plastid and mitochondria, based on the availability of nitrates in diatoms. We performed an evolutionary analysis of all involved genes and specified the contributions of a plastid endosymbiont and the host cell to the nitrogen-associated gene repertoire in diatoms.

We participated in the discovery of the biosynthetic pathway used by marine bloom-forming diatoms for the **synthesis of the neurotoxin domoic acid** (Brunson et al., 2018, *Science* 361, 1356-1358). This toxin causes various symptoms in humans (and mammals and birds), including the irreversible loss of short-time memory and even death. The description of the genetic bases of the synthesis allows the detection of the danger of poisoning from the water samples in advance.

Based on transcriptomic studies, we revealed the roles of **detoxification systems** in response to mercury in *C. velia* (Sharaf et al., 2019, *Biomolecules* 9, 647). We also characterized aminoacyl-tRNA synthetases in chromerids and showed their complex evolutionary history (Sharaf et al., 2019, *Genes* 10, 582).

### Non-photosynthetic stramenopiles

We discovered novel bicosoecid (Stramenopila, SAR group), the marine non-photosynthetic flagellate living in association with algae, and formally classified it as a new genus and species *Cafileria marina* (Jirsová et al., 2019, *Microorganisms* 7, 240). We sequenced its mitochondrial genome and showed an unprecedented association (conjunction) between the nucleus and mitochondria in this bacterivorous micro-eukaryotic flagellate (Figure 2).



**Figure 2.** The intimate association between the mitochondrion and nucleus in the bicosoecid *Cafileria marina* (Stramenopila) as seen by the transmission electron microscopy (a–f, i) and confocal microscopy (g,h). nc–nucleus; mt–mitochondrion

## Genomics, transcriptomics, evolution, and ecology of euglenozoans

We participated in the groundbreaking analysis of the **global eukaryotic plankton**, based on the *Tara* Oceans metabarcoding dataset, for the first time showing the diversity, abundance, and distribution of major planktonic lineages in the global ocean (de Vargas et al., 2015, *Science* 348, 1261605). The results brought into the scientific spotlight so far overlooked group of euglenozoans – **diplonemids**. In the follow-up study, we showed that diplonemids are **the most diverse** and fifth most abundant planktonic eukaryotes (Flegontova et al., 2016, *Curr. Biol.* 26, 3060–3065). Compared to diplonemids, **kinetoplastids** (one of the best-studied and most-diverse land protists) are much less prominent in the oceans, represented mostly by ***Neobodo*** and ***Rhynchomonas*** (Flegontova et al., 2018, *Env. Microbiol.* 20, 878–889). In this study, we also uncovered novel putative kinetoplastid parasites, including their hosts.

Given the abundance and diversity, diplonemids are one of the essential heterotrophic flagellates of the global ocean. However, only several diplonemid species have been described so far. We contributed to the description of five novel diplonemid genera and eight new species (Tashyreva et al., 2018, *Protist* 169, 158-179; Prokopchuk et al., 2019, *Protist* 170, 259-282). We also contributed to uncovering the widespread presence of bacterial endosymbionts of diplonemids. Although at least two independent symbiont acquisitions are currently described in diplonemids (Tashyreva et al., 2018, *mBio* 9, e02447-17; Prokopchuk et al., 2019, *Protist* 170, 259-282), they share many common features suggesting a firm control from the host system (George et al., 2020, *Curr. Biol.* 30, 925-933.e3).

*Euglena longa*, a close relative of the photosynthetic alga *Euglena gracilis*, possesses a non-photosynthetic plastid. The *E. longa* plastid genome has retained a gene for the large subunit of the enzyme RuBisCO (*rbcl*). We demonstrated an extreme divergence of the *E. longa* RBCL protein sequence compared to its photosynthetic homologs, suggesting a possible functional shift upon the loss of photosynthesis (Záhonová et al., 2016, *PLoS ONE* 11, e0158790). Our analysis of *E. longa* transcriptomic data confirmed the absence of genes for the photosynthetic machinery but provided candidate plastid-localized proteins bearing typical N-terminal bipartite topogenic signals (BTSS). Further comparative analyses enabled us to unveil salient aspects of the basic euglenophyte plastid infrastructure. Strikingly, no homologs of any key component of the TOC/TIC system and the plastid division apparatus are discernible in euglenophytes, and the machinery for intraplastidial protein targeting has been simplified. Our study documents a substantial remodeling of the euglenophyte plastid compared to its green algal progenitor (Záhonová et al., 2016, *Sci. Rep.* 8, 17012). The follow-up manuscript (Füßy et al., 2019, *bioRxiv* doi.org/10.1101/765255) has not been published yet and is available only as a preprint.

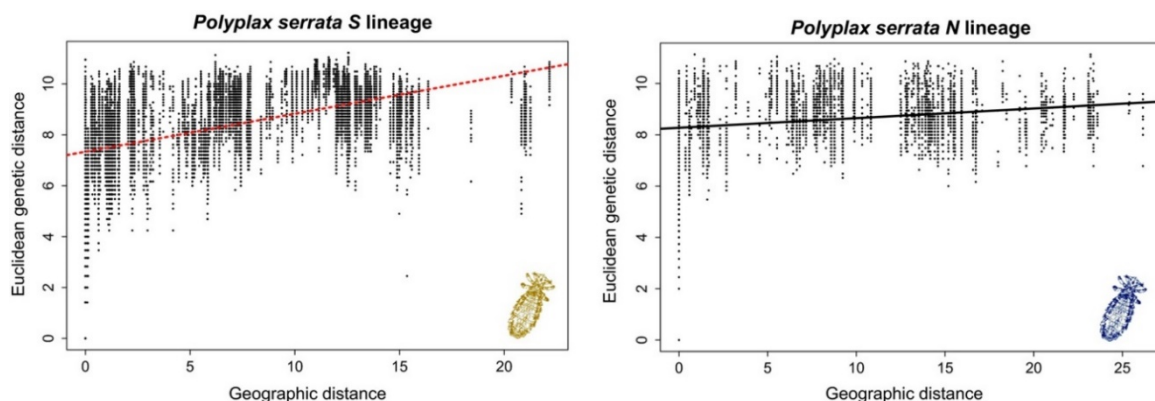
## Evolution of host specificity and adaptation of parasites

In our research of population genetic diversity related to the **evolution of host specificity and adaptation** of parasites we have focused on several models, studying **processes of co-divergence** in lice, coccidia and their rodent hosts (Martinů et al., 2018, *Ecol. Evol.* 8, 10008–10022; Mácová et al., 2018, *Mol. Phylogenet. Evol.* 127, 179–189), in birds and their ectoparasites (Martinů et al., 2015, *Int. J. Parasitol.* 45, 63-73), in tapeworms and fish (Jirsová et al. 2017, *PLoS ONE* 12: e0175286.), and most recently in dicyemid parasites of cephalopods (Drábková et al., 2019, *Sci. Rep.* 9, 14300). In most of the studied systems, both the **host specificity and geography**



**interfered** in forming the genetic diversity of parasite populations. However, we also found some unexpected patterns. For example, in *Polyplax* lice, we found a case of the **cryptic hybrid zone**, which was not reflected by any divide in the population structure of the host, *Apodemus flavicollis* mouse (Martinů et al., 2018, *Ecol. Evol.* 8, 10008–10022). Now we continue to study this topic using whole-genome re-sequencing approaches. We also found **support for the Nadler's rule**, predicting that multi-host parasite species should show more genetically rich and less structured populations due to their larger effective population sizes (Fig. 3). We also studied the **diversity of immunogenetic loci** in vertebrates as a **response to habitat size** (Vlček et al., 2016, *Mol. Ecol.* 25 4757-4772) or historical biogeography (Morger et al., 2015, *Biol. J. Linn. Soc.* 116, 124133).

The research of population structure in parasites provided us with analytic tools to investigate geographic origin and **routes of invasion** in several parasitic organisms, such as carp tapeworms (Bazsalovicsová et al., 2018, *Parasitol. Int.* 67, 366-374) and giant fluke (Bazsalovicsová et al., 2015 *Parasites & Vectors* 8, 288; Juhásová et al., 2016, *Parasites & Vectors* 9, 547).



**Figure 3.** Proof of the Nadler's rule. Correlation between Euclidean genetic distances and geographic distances for pairs of *Polyplax serrata* louse individuals is significant for a host-specific lineage (dashed red line, yellow louse logo on the left). In contrast, the correlation is non-significant for a non-specific lineage found on at least two rodent hosts (solid black line, blue louse logo). Pairwise distances were calculated using multilocus genotypes obtained for 400+ louse specimens collected across Europe.

### Evolution of symbiotic bacteria in insects

Research in insect-bacterial symbiosis aimed to answer questions related to the **origin of endosymbiotic bacteria** (Nováková et al., 2015, *Appl. Environ. Microbiol.* 81: 6189-6199), levels of **horizontal gene transfer** (Říhová et al., 2017, *Genome Biol. Evol.* 9, 2946-2957), **degeneration of metabolic pathways** related to endosymbiosis (Nováková et al., 2016, *Stand. Genom. Sci.* 11, 72), or **microbiome compositions** in blood-sucking insects (Nováková et al., 2017, *Front. Microbiol.* 8, 526; Rodríguez-Ruano et al., 2018, *Front. Microbiol.* 9, 1167; Song et al., 2019, *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374, 1777). The intricate process of endosymbiosis was demonstrated in the study sequencing genomes of mealybug (*Planococcus citri*) and its two symbiotic bacteria. Here it was shown that the two endosymbionts live inside its host's cells nested in each other, thus creating a **“Russian doll-like” arrangement** of symbiosis (Husník & McCutcheon, 2016, *PNAS* 113, E5416-24).

## Phylogenomics of protists

Using state-of-the-art **phylogenomic** methods, we have investigated the **phylogeny of the Ciliata** (Lynn and Kolisko, 2017, *Int. J. Syst. Evol. Micr.* 67, 3676-3682; Lynn et al., 2018, *Protist* 169, 180-189). We have also investigated the evolution of anaerobic lineages of ciliates and their reduced mitochondria, where we have proposed that **transition from facultative to obligatory anaerobiosis might have been driven by bacterial symbionts** (Rotterová et al., 2020, *Curr. Biol.* 30, 2037-2050). We have also studied the evolution of symbiosis between ciliates and their bacterial symbionts and identified the **evolutionary forces driving the genome reduction** of the symbionts (Boscaro et al., 2018, *Nat. Ecol. Evol.* 2, 750-750).

We have explored the phylogenomics and evolution of diverse excavate lineages. We have significantly contributed to resolving the phylogenetic position of taxon *Malawimonas* using several advanced phylogenomic techniques, including removal of fast-evolving taxa and fast-evolving sites (Heiss et al., 2018, *Roy. Soc. Open Sci.* 5). We have also identified **Tim17/22/23 translocase** in the mitosome of *Giardia intestinalis*, which appeared to be one of the last “big mysteries” of mitosomes (Pyrihova et al., 2018; *Genome Biol. Evol.* 10, 2813-2822). Moreover, we have participated in several studies of oxymonads, which are generally unculturable and, therefore, we have used techniques of **single-cell transcriptomics and genomics** (Vacek et al., 2018, *Mol. Biol. Evol.* 35, 2712-2718; Treitli et al., 2019, *PNAS* 116, 19675-19684). We have also participated in the description of **seven new species of parabasalids** (Boscaro et al., 2017, *Int. J. Syst. Evol. Micr.* 67, 3570-3575; del Campo et al., 2017, *Sci. Rep.* 7, 16349).

We have contributed to phylogenomic and comparative analyses of novel lineages of microbial eukaryotes that branch deeply within the Holozoa, shaping our understanding of the **evolution of multicellular animals** (Hehenberger et al., 2017, *Curr. Biol.* 27, 2043-2050). We have also participated in several genomic and single-cell genomic studies of microbial eukaryotes (Gentekaki et al., 2017, *PLoS Biol.* 15; Strassert et al., 2018, *ISME J.* 12, 304-308). As a side project, we have also participated in comparative analyses of cnidarian parasites (Alama-Bermejo et al., 2020, *Genome Biol. Evol.*, evaa109.)

We have also participated in several efforts for the scientific community, including the design and release of software for **identification and removal of cross-contamination** from next-generation sequencing datasets (github.com/kolecko007/WinstonCleaner) and used this software to clean-up 667 transcriptomic dataset of microbial eukaryotes sequenced by the Gordon and Betty Moore Foundation (iMicrobe.us). We have also taken part in design and software development for EukRef, which aims at the creation and annotation of reference databases for analyses of environmental amplicon data ([www.eukref.org](http://www.eukref.org); del Campo et al., 2018, *PLoS Biol.* 16).

## Review articles and book chapters

We have substantially contributed to the knowledge in the field in numerous **review and opinion articles** (Oborník et al., 2009, *Int. J. Parasitol.*, 39, 1-12; Kořený et al., 2010, *Int. J. Parasitol.* 40, 149-156; Oborník and Lukeš, 2013, *Int. Rev. Cell. Mol. Biol.* 306, 333-369; Kořený et al., 2013, *PLoS Pathog.* 9, e1003088; Oborník and Lukeš, 2015, *Ann. Rev. Microbiol.* 69, 129-144; Oborník M, 2018, *Trends Parasitol.* 34, 923-925; Oborník M, 2019, *Biomolecules* 9, 266; Oborník M, 2019, *Microbial Cell* 6, 134-

141; Husník & McCutcheon, 2018, *Nat. Rev. Microbiol.* 16, 67-79.; Lukeš J & Husník, 2018, *Curr. Biol.* 28, R712–R715; Roumbedakis et al., 2019, *Front. Physiol.* 9, 1573) and **book chapters** (Votýpka et al., 2017, *Handbook of Protists*, pp. 567-624; Füssy and Oborník, 2017, *Evolutionary Biology: Self/Nonself Evolution, Species and Complex Traits Evolution, Methods and Concepts*, pp. 217-236; Füssy and Oborník, 2017, *Adv. Bot. Res.* 84, 187-218; Füssy and Oborník, 2018, *Methods in Molecular Biology* 1829, 17-35; Cihlář et al., 2019, *Adv. Bot. Res.* 90, 273-309)

## Research activity and characterisation of the main scientific results

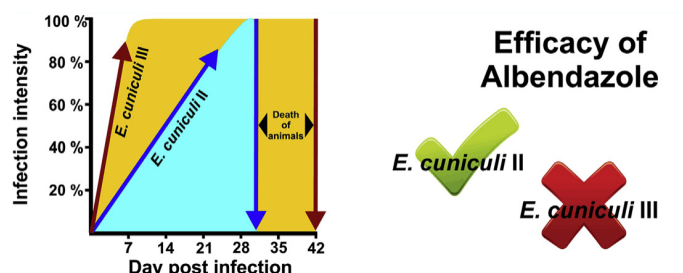
The most important results of the team are divided into the relevant research fields presented below:

### A) Course of infection and antimicrosporidial treatment

We quantified microsporidial burden in individual organs of murine hosts during the course of microsporidiosis using qPCR methods and compared the parasitic load of two genotypes of *Encephalitozoon cuniculi*, namely genotype II and III. The infection caused by *E. cuniculi* III had a rapid onset with massive dissemination of the parasite in the host body and albendazole treatment had limited efficacy with very weak effects in both immunocompetent BALB/c mice and immunodeficient SCID mice when compared to the *E. cuniculi* genotype II (Kotková et al., 2017, Exp. Parasitol. 182, 16-21). Moreover, similar results were reported in a comparison of the course of infection in immunocompetent C57Bl/6 mice and CD4<sup>-/-</sup> and CD8<sup>-/-</sup> mice (Sak et al., 2017, Parasite Immunol. 39, e12496). However, despite the burden of *E. cuniculi* genotype III reaching up to 10<sup>12</sup> spores per one gram of tissue, the immunodeficient mice were able to control and survive the microsporidiosis caused by *E. cuniculi* genotype III for a longer period without any clinical signs of infection (Kotková et al., 2018, Exp. Parasitol. 192, 93-97). These findings showed that different courses of infection and responses to treatment depend not only on the immunological status of the host, but also on the genotype of microsporidia (Fig. 1).

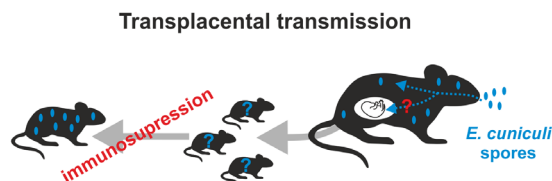
We also evaluated antimicrosporidial activities of the methanolic extracts of *Archidendron fagifolium*, *Diospyros sumatrana*, *Shorea sumatrana* and *Piper betle* that are widely used in traditional medicine to cure digestive tract disorders in immunocompetent BALB/c mice. *Diospyros sumatrana* reduced shedding of spores of *E. cuniculi* by 88% compared to untreated controls. Moreover, the total microsporidia burden per one g of tissue in *D. sumatrana* extract-treated groups was reduced by 87% if compared to untreated controls, which is similar to the effect of the standard drug, albendazole (Sak et al., 2017, Exp. Parasitol. 181, 94-101).

Finally, we provided the evidence of transplacental transmission of *E. cuniculi* in mice under experimental conditions. Microsporidial infections were detected in cubs from mothers in both acute and chronic infection (Kotková et al., 2018, Exp. Parasitol. 193, 51-57; Fig. 2).



**Figure 1.** The course of infection caused by *Encephalitozoon cuniculi* genotype II and III and Efficacy of Albendazole treatment.



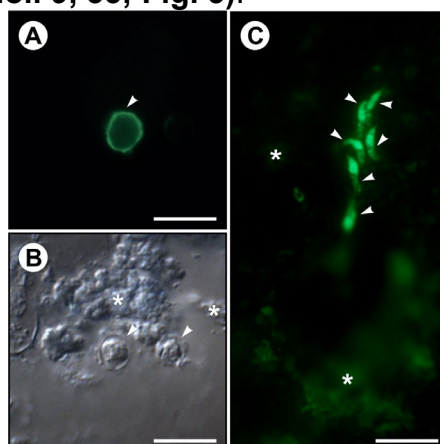


**Figure 2.** Transplacental transmission of *E. cuniculi* in mice.

## B) Human microsporidiosis and cryptosporidiosis

We described microsporidial infections caused by *Enterocytozoon bieneusi* and *Encephalitozoon cuniculi* i) in the urinary tract of HIV/AIDS patients under cART therapy (Wesołowska et al., 2019, *Ann. Parasitol.* **65**, 77-81); ii) in the intestinal and urinary tracts of renal transplant recipients (Kicia et al., 2016, *Clin. Microbiol. Infect.* **22**, 462.e5-9); iii) during infections of respiratory tract of immunosuppressed renal transplant recipients (Kicia et al., 2019, *Int. J. Infect. Dis.* **79**, 21-25); and, iv) in patients after hematopoietic stem cell transplantation (Kicia et al., 2018, *Int. J. Infect. Dis.* **77**, 26-28). We also described the connection between *E. cuniculi* presence and periprosthetic osteolysis after primary hip arthroplasty leading to implant loosening and urgent arthroplastic revision (Kicia et al., 2018, *Clin. Infect. Dis.* **67**, 1228-1234).

We described the first report of human *Cryptosporidium meleagridis* infection in Poland (Wesołowska et al., 2016, *Ann. Parasitol.* **62**, 239-241) and investigated the potential linkage between *C. meleagridis* infection and human colorectal cancer based on malignant tissue collected from patients with colorectal cancer (Kopacz et al., 2019, *Font. Cell. Infect. Microbiol.* **9**, 35; Fig. 3).



**Figure 3.** Microscopic examination of homogenized colonic tissue from a patient with adenocarcinoma and *Cryptosporidium meleagridis* infection. (A) *Cryptosporidium* oocyst labeled with mouse monoclonal antibody binds to oocyst wall. (B) *Cryptosporidium* oocysts (arrowheads) under differential interference contrast (C) Six merozoites (arrowheads) labeled with anti-*Cryptosporidium* sporozoite polyclonal antibody. Bar = 10 μm.

## C) *Cryptosporidium* spp. and microsporidia in small mammals

The occurrence and prevalence of *Cryptosporidium* spp. and/or microsporidia of the genera *Encephalitozoon* and *Enterocytozoon*, respectively, in voles, tree and ground squirrels, *Apodemus* mice, hedgehogs, bats, fur animals, dogs, cats and foxes in Europe and North America were studied using molecular tools. Unlike in microsporidia, the high diversity of *Cryptosporidium* spp. was detected.

We found that tree and ground squirrels with overlapping ranges host different *Cryptosporidium* species and genotypes. Further, *Cryptosporidium* genotypes associated with ground squirrel species are adapted to the transmission in arid conditions (Stenger et al. 2015, Infect. Genet. Evol. 36, 287-293). The *Cryptosporidium* skunk genotype and possibly *C. ubiquitum* XIIb were introduced to Europe with eastern grey squirrels (Prediger et al., 2017, Eur. J. Protistol. 61, 64-75). Two novel genotypes, namely *Cryptosporidium* bat genotype III and IV, were identified in bats in North America and Europe, respectively (Kváč et al., 2015, Parasitol. Res. 114, 3917-3921). Similar to squirrels, we detected that voles and *Apodemus* mice do not share *Cryptosporidium* spp. and they are parasitized with high number of host-specific species and genotypes when compared to other rodents (Čondlová et al., 2018, Eur. J. Protistol. 63, 1-12; Čondlová et al., 2019, Eur. J. Protistol. 69, 1-13; Horčíčková et al., 2019, Parasitology 146, 220-233; Myšková et al., 2019, Parasitol. Res. 118, 3409-3418; Stenger et al., 2015, Infect. Genet. Evol. 36, 287-293; Kváč et al., 2018, Eur. J. Protistol. 63, 96-104).

Although wild rodents do not represent major reservoirs of human pathogenic *Cryptosporidium* spp. (Stenger et al., 2018, Parasitology. 145, 326-334), they can be a source of microsporidia infecting humans (Perec-Matysiak et al., 2019, Eur. J. Protistol. 69, 14-19; Perec-Matysiak et al., 2015, Vet. Parasitol. 214, 242-246).

On contrary, dogs, cats, hedgehogs and fur animals (including American mink and long-tailed chinchilla) represent a reservoir of both human pathogenic *Cryptosporidium* spp. and microsporidia (Kváč et al., 2017, Folia Parasitol. 64, 034; Piekarska et al., 2017, Vet. Parasitol. 246, 108-111; Hofmannová et al., 2016, Eur. J. Protistol. 56, 15-20; Kellnerová et al., 2017, Eur. J. Protistol. 59, 108-113; Fig. 4).



**Figure 4.** Prevalence and source of *Cryptosporidium* infection in the Czech Republic.

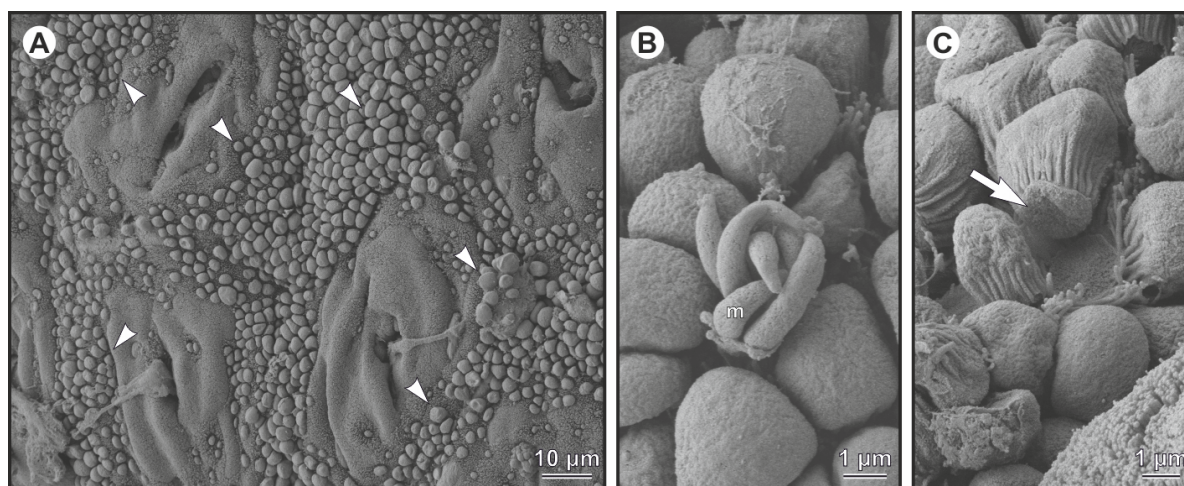
#### D) *Cryptosporidium* spp. in birds

Hundreds of birds belonging to the orders Accipitriformes, Anseriformes, Columbiformes, Galliformes, Gruiformes, Falconiformes, Psittaciformes, Passeriformes, Strigiformes and Struthioniformes from North America, Africa and Europe were tested for the presence of *Cryptosporidium* infection (Laatamna et al., 2017, Folia Parasitol. 64, 018; Holubová et al., 2019, Eur. J. Protistol. 69, 70-87). We found a *Cryptosporidium* avian genotype VI, host specific for North American red-winged blackbirds (Chelladurai et al., 2016, Parasitol. Res. 115, 1901-1906). Biological properties were studied in detail in numerous isolates (see E). Host- and

age-specificity of *Cryptosporidium avium* was also assessed (Holubová et al., 2018, *Exp. Parasitol.* 191, 62-65).

### E) Description of new species of *Cryptosporidium*

We described several new species of the genus *Cryptosporidium*, greatly increasing the knowledge of diversity and biology of *Cryptosporidium*. The size of oocysts and other developmental stages, host specificity, course of infection, localization of infection in the host, pathogenicity and phylogenetical analyses were conducted for each of following new species: *C. avium* sp. n. (Holubová et al., 2016, *Parasitol. Res.* 115, 2243-2251), *C. testudinis* sp. n. (Ježková et al., 2016, *Folia Parasitol.* 63, 035), *C. proliferans* sp. n. (Kváč et al., 2016, *PloS One* 11, e0147090), *C. occultus* sp. n. (Kváč et al., 2018, *Eur. J. Protistol.* 63, 96-104; Fig. 5), *C. apodemí* sp. n. and *C. ditrichi* sp. n. (Čondlová et al., 2018, *Eur. J. Protistol.* 63, 1-12), *C. microti* sp. n. and *C. alticolis* sp. n. (Horčíčková et al., 2019, *Parasitology* 146, 220-233), *C. proventriculi* sp. n. (Holubová et al., 2019, *Eur. J. Protistol.* 69, 70-87). Additionally, we provided previously unreported data on *C. ducismarci* to confirm its validity (Ježková et al., 2016, *Folia Parasitol.* 63, 035).



**Figure 5.** Scanning electron photomicrograph of the colon epithelium of *Rattus norvegicus* infected 5,000 oocysts of *Cryptosporidium occultus* sp. n. (A) Colon epithelium covered by developmental stages of *C. occultus* sp. n. (arrowhead). (B) Ruptured parasitophorous sac releasing Type I merozoites (m). (C) Site of parasitophorous sac interaction with the host cell (arrow).

### F) Food as potential source of microsporidial infection

Our studies focused on the occurrence and prevalence of *Encephalitozoon cuniculi* in raw cow's milk and pork meat, respectively, and the evaluation of the effects of different food processing procedures (e.g., milk pasteurization treatments and sausage fermentation) on the *E. cuniculi* infectivity. We found that *E. cuniculi* is occasionally present in cow's milk and pork meat, and that pasteurization treatment at 72 °C for 15 s or 85 °C for 5 s as well as standard sausage fermentation process, respectively, do not hamper the viability of *E. cuniculi* spores. Thus, prepared food represents a potential source of *E. cuniculi* infection in humans (Kváč et al., 2016, *Foodborne Pathog. Dis.* 13, 77-79; Sak et al., 2019, *Foodborne Pathog. Dis.* 16, 394-398).

### G) Ecology of primate parasites

Our team has a long history of the research on parasite taxa in human and non-human primates, to elucidate their diversity and risk of transmission among various hosts. Wild chimpanzees and other African great apes live in regions where African sleeping sickness is endemic; yet, little is known about their trypanosome infections given the difficulty in obtaining blood samples. We established a protocol allowing us to detect trypanosomes from faeces and using this assay we revealed the presence of trypanosomes of the *T. brucei* group in the faecal samples of wild chimpanzees, providing strong evidence that these infections were caused by *T. b. rhodesiense* and/or *T. b. gambiense* (Votýpka et al., 2015, *Int. J. Parasitol. Parasites Wildl.* **4**, 277-282).

Recently it was demonstrated that African great apes can host *Leishmania* parasites. This finding resulted in a strong negative reaction from leishmania experts without a clear resolution following subsequent study and discussion. We analysed faecal samples of wild lowland gorillas and central chimpanzees collected from the same area in Cameroon as in the original study. We did not detect any leishmania parasites in analysed faeces; however, we revealed an unexpected diversity of free-living bodonids and parasitic trypanosomatids other than *Leishmania* (Votýpka et al., 2018, *Int. J. Parasitol. Parasites Wildl.* **7**, 322-325). We have been invited to write a chapter about trypanosome, leishmania and filarial infections in non-human primates for the book *Neglected Diseases in Monkeys*.

Syngamid strongylids of the genus *Mammomonogamus* are among the least known nematodes with zoonotic potential. Eggs of *Mammomonogamus* sp. are frequently found in faeces of African forest elephants and western lowland gorillas in the Dzanga Sangha Protected Areas (DSPA) in the Central African Republic. Two haplotypes were found in both gorillas and elephants suggesting the potential for sharing *Mammomonogamus* nematodes between phylogenetically distant hosts. Assignment of the parasite to *M. loxodontis* was proposed (Červená et al., 2017, *Parasitology* **144**, 1016-1025). We also assessed the diversity of *Mammomonogamus* in all large African herbivores (Červená et al., 2018, *Parasitol. Res.* **117**, 1013-1024).

In DSPA, we studied ecology and transmission of malaria infections in lowland gorillas and humans, and observed switching between two different *Plasmodium* spp. Younger animals were more susceptible to malaria infections. We confirmed the presence of the human malaria parasite *Plasmodium ovale wallikeri* in both gorillas and humans (Mapua et al, 2015, *Parasitology*. **142**, 890-900; Mapua et al., 2018, *Emerg. Infect. Dis.* **24**, 1581-1583). In a follow-up study, we found significant differences in the prevalence of malaria parasites among free ranging chimpanzee populations living in different habitats (Mapua et al., 2016, *Malar. J.* **15**, 423). Given that little is known about the mechanisms underlying the presence of *Plasmodium* DNA in ape faeces, our study assessed if strongylid infections have an impact on detection of *Plasmodium* DNA in faeces and concluded that bleeding caused by strongylid nematodes of the genus *Necator* cannot explain the presence of *Plasmodium* DNA in ape faeces (Mapua et al., 2017, *Malar. J.* **16**, 175).

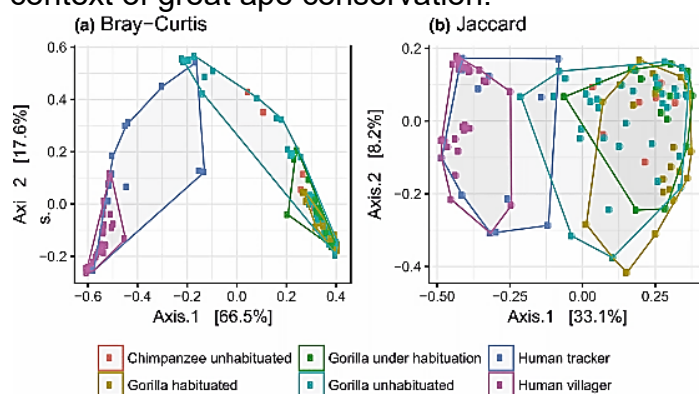
Although habitat sharing between hosts contributes to parasite transmission in primates [see also part H and first report of infection with a gorilla *Necator* species (*N. gorillae*) other than *N. americanus* in humans (Kalousová et al., 2016, *Parasit. Vector.* **75**, 9) and report of *Schistosoma mansoni* from great apes in Gabon (Červená et al., 2016, *Am. J. Trop. Med. Hyg.* **95**, 849-851), for particular parasite taxa we



reported that sequences obtained from humans differ from those in the non-human primates (e.g., *Strongyloides*: Hasegawa et al., 2015, *Parasitol. Int.* **65**, 367-370 or trichomonads: Čepička et al. 2020, *Parasitology* **147**, 225-230) and ecology and spatial overlap among primates in the tropical forest ecosystem does not have always lead to parasite exchange among even closely related hosts.

#### H) Metabarcoding approach for strongylid and *Entamoeba* communities in primates

We designed and tested a high-throughput sequencing approach for strain-level identification of gastrointestinal strongylids and *Entamoeba* spp. in primates using ITS-2 and 18S rDNA metabarcoding at the MiSeq Illumina platform (Pafčo et al., 2018, *Sci. Rep.* **8**, 5933; Vlčková et al., 2018, *Int. J. Parasitol.* **48**, 519-530). We implemented this approach to study strongylid and *Entamoeba* communities of sympatric primates in DSPA (Central African Republic) and the Dja Faunal Reserve (Cameroon). Strongylid communities of DSPA hunter-gatherers employed as trackers in non-human primate research were more similar to those of gorillas when compared to their families, who spent more time in villages. The occurrence of the human-specific strongylids in DSPA gorillas did not increase with direct contact between gorillas and humans. Rather, our results indicate that the degree of habitat sharing between hosts, together with mode of parasite transmission, is the most important factor for parasite spillover among primate species (Pafčo et al., 2019, *Mol. Ecol.* **28**, 4786-4797; Fig. 6) and supports our previous findings that habituation (resulting in close contact) may not necessarily pose a greater risk of protist and helminth infections in gorilla groups (Pafčo et al., 2017, *Parasitol. Res.* **116**, 3401-3410). In the Dja primates we detected 36 *Entamoeba* haplotypes belonging to six haplotype clusters, containing haplotypes possessing high and low host specificity. Most of the detected haplotypes belonged to commensal *Entamoeba*, but pathogenic species (*Entamoeba histolytica* and *Entamoeba nuttalli*) were also detected. We observed that some *Entamoeba* haplotypes are shared between humans and other primates, indicating zoonotic potential. Our findings are important not only for understanding the epidemiology of amoeba and strongylid infections in humans in rural African localities, but also in the context of great ape conservation.



**Figure 6.** PCoA for human and NHP samples. Bray-Curtis and Jaccard dissimilarities accounting for relative abundance and prevalence of ITS-2haplotypes. Ordination space occupied by different sample categories is indicated by convex Hull polygons.

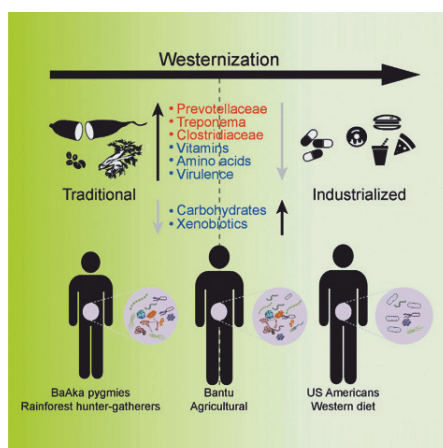
#### I) Fractal analyses and parasite infections

Objectively measuring the effects of parasitism on animal health is challenging, especially in field settings. Analyses of behavioural organization are increasingly used to help identify animals in a pathological or otherwise challenged state. These analyses rely on fractal analysis of behaviour as a diagnostic tool for detection of infected animals. We investigated fractal patterns in the behavioural activity of domestic sheep

and wild chimpanzees in relation to strongylid infections. The complexity of chimpanzee feeding sequences and the intensity of infection with strongylid nematodes were positively related: individuals with more intense infections exhibited more stochastic feeding sequences (**Burgunder et al., 2017, Anim. Behav. 129, 257-268**). Sheep treated with anthelmintics exhibited a higher complexity in their activity sequences than parasitized sheep, suggesting that organizational patterns of behaviour change with gastrointestinal parasite infection (**Burgunder et al., 2018, Appl. Anim. Behav. Sci. 205, 44-53**). Our work provides evidence for the potential utility of fractal methods in behavioural welfare and health monitoring.

## **J) Primate gut microbiome**

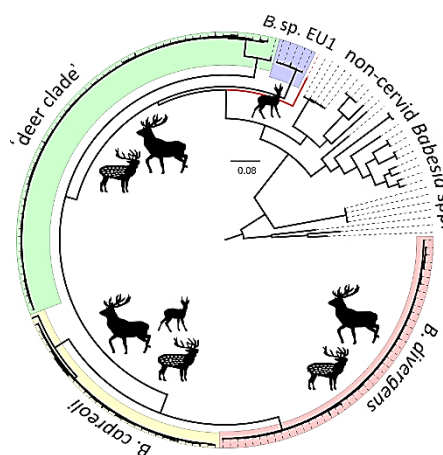
Our team has been involved in research on sympatric human and non-human primate microbiomes, including traditional human societies in DSPA, Central African Republic. This work pre-dates recent increased interest in microbiome studies among the global scientific community. We characterized faecal bacterial communities and, for the first time, gut metabolomes of four wild lowland gorilla groups and determined how these microbial communities respond to the host's external environment (**Gomez et al., 2015, Mol. Ecol. 24, 2551-2565**). By studying microbiomes of western and eastern gorillas we showed differences and convergence of diet-microbe, co-metabolic fingerprints in two geographically isolated primate species, under specific dietary stimuli. We suggest that dietary constraints triggered during their adaptive radiations were potential factors behind the species-specific microbiome patterns observed in primates today (**Gomez et al., 2016, ISME J. 10, 514-526**). We also explored the impact of host traits, parasite infections, stress and antibiotic treatment on microbiomes of wild lowland gorillas (**Pafčo et al., 2019, Am. J. Phys. Anthropol. 169, 575-585**; **Vičková et al., 2018, Front. Microbiol. 9, 1202**; **Vičková et al., 2018, Microbiology – SGM 164, 40-44**; **Vičková et al., 2016, Microb. Ecol. 72, 943-954**). The microbiomes of hunter-gatherers and agriculturalists inhabiting DSPA also reflected dietary gradients (e.g. traditional subsistence strategies, agriculture and industrialization) which have triggered loss of traditional microbes and increased carbohydrate and xenobiotic metabolism in humans (**Gomez et al., 2016, Cell Rep. 14, 2142-2153; Fig. 7**). We also showed that the taxonomic composition of the human gut microbiome exhibits increased compositional plasticity and revealed unexpected similarities between African monkeys that rely on eclectic foraging and human populations engaged in non-industrial subsistence patterns; these similarities transcend host phylogenetic constraints (**Gomez et al., 2019, mSphere. 4, e00271-19**).



**Figure 7.** Gut microbiome of coexisting BaAka pygmies and Bantu reflects gradients of traditional subsistence patterns.

### K) Ecology and distribution of vector-borne diseases of wild and domestic animals

We studied the diversity, vectors and host specificity of selected tick-borne (*Babesia*, *Theileria* and *Cytauxzoon*) and insect-borne (*Dirofilaria*) pathogens, both in temperate Europe and tropical Africa. Novel findings of pathogens emerging in Europe (i.e., *Babesia canis* and *Hepatozoon canis*) are noteworthy as they represent the first reports from the territory of the Czech Republic (e.g., **Modrý et al., 2017, Vector-Borne Zoonot. 17, 66-72**; **Mitková et al., 2017, Vet. Med. 62, 138-146**; **Mitková et al., 2017, Parasit. Vector. 10, 1-11**; **Hrazdilová et al., 2019, Infect. Gen. Evol. 77, 104060**; **Fig. 8**). These lines of research resulted in a series of joint publications on tick-borne bacterial and viral diseases (e.g., **Jaarsma et al., 2019, Parasit. Vector. 12, 328**; **Nziza et al. 2019, Med. Vet. Entomol. 33, 177-184**; **Salát et al., 2017, Emerg. Infect. Dis. 12, 2065-2067**).



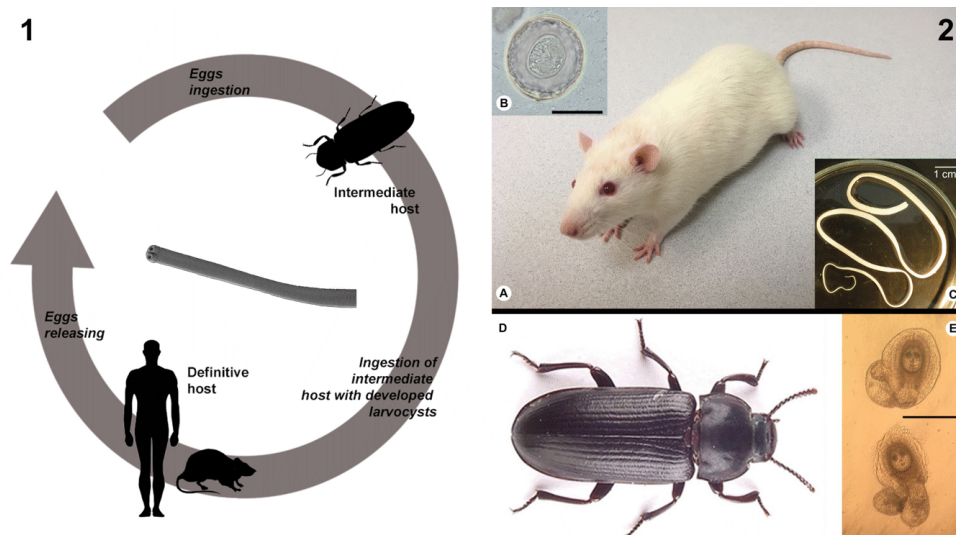
**Figure 8.** Phylogenetic analysis of cox1 demonstrating surprising presence of undescribed new species of *Babesia* in European cervids.

### L) *Angiostrongylus cantonensis*: molecular diversity, ecology and life cycle.

This is a new research line addressing the ongoing global invasion of a zoonotic parasitic nematode and its emergence in Europe. Our team is the only laboratory in the EU maintaining an experimental strain of *Angiostrongylus cantonensis*. Recently, a diversity of mitochondrial genomes of *A. cantonensis* was described (**Červená et al. 2019, Parasit. Vector. 12, 241**).

### M) Effect of the colonization by a benign tapeworm on healthy and diseased state

Helminths are known to have a strong influence on the host immune system, being able to activate/manipulate anti-inflammatory mechanisms. This trait is used to investigate their effects on the suppression of inflammatory processes in the human body. Recently, a novel therapeutic strategy, known as helminth therapy, has been developed using experimental models to test the efficacy of several dozen different helminths (Sobotková et al., 2018, *Trend Parasitol.* **35**, 501-515). One of the most promising helminths in these studies is the benign tapeworm *Hymenolepis diminuta*. Thus, we chose this species for our research. In addition to its ability to modulate the immune system, data also suggest that the therapeutic effects of this helminth partially result from shifts in the intestinal microbiom induced by worm colonization. We have revealed that *H. diminuta* has the ability to modulate the immune system in healthy hosts (Parfrey et al., 2017, *PLoS ONE* **12**, e182205) and to suppress gut inflammation in the patent period of colonization (Jirků-Pomajbíková et al., 2018, *Parasitology*. **145**, 1324-1335). In contrast, the effects of *H. diminuta* on the composition of the intestinal bacterial component are minor and are especially observable at the level of beta-diversity (Parfrey et al., 2017, *PLoS ONE* **12**, e182205; Jirků-Pomajbíková et al., 2018, *Parasitology*. **145**, 1324-1335). Finally, we found high genetic uniformity of the laboratory isolates of *H. diminuta* tested for helminth therapy (Řežábková et al., 2018, *Parasitol. Int.* **71**, 121-125; Fig. 9).



**Figure 9.** The life cycle of *Hymenolepis diminuta* under laboratory conditions.

### N) Role of the commensal protist in the health of the host

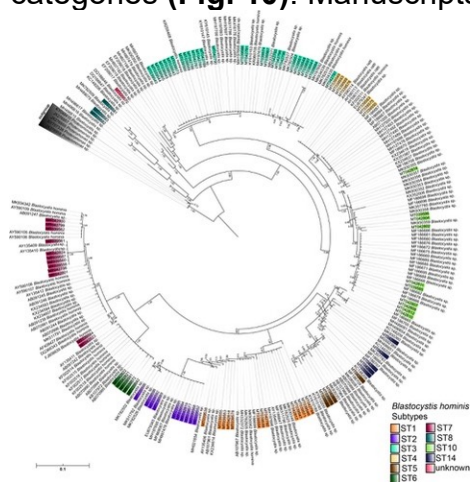
Although current research points to the fact that intestinal commensal protists can have a positive impact on human health (similar to intestinal bacteria or worms), they are still a neglected component of the gut ecosystem. Until recently, *Blastocystis* was often associated with the clinical manifestation of inflammatory bowel diseases, but accumulating evidence suggests that it is a commensal organism with predominance in healthy individuals. Nevertheless, *in vivo* experimental studies are still lacking to reveal the specific impact of *Blastocystis* on the health of the host – the immune system and the gut microbiome, as well as to decipher the causes of various clinical results. For *in vivo* studies on *Blastocystis*, we evaluated a rat model for the maintenance of human-derived *Blastocystis* sp. and assessed the success and longevity of colonization (Růžková et al., 2018, *Exp. Parasitol.* **191**, 55-61). Subsequently, we



tested its effect on gut inflammation in rats at different lengths of exposure to colonization (after 3 and 13 weeks of colonization). Our results revealed a reduction in intestinal inflammation during long-term colonization. We further found that the composition of the bacterial microbiom, but not its richness, changes after *Blastocystis* colonization.

### O) Epidemiological study focused on the occurrence of intestinal protists in the modern society

Intestinal protists are a common part of the intestinal ecosystem, and recent evidence suggests they play a key role in human health (Lukeš et al., 2015, PLoS Pathogens 11, e2005039). In addition to experimental studies, it is necessary to obtain information about their epidemiology not only in populations with a traditional lifestyle, but also in industrialized populations. We monitored the prevalence and diversity of several gut protists in an industrialized population. So far, we have obtained a total of 320 human samples from various regions of the Czech Republic and 170 samples of their animals. We also monitored various factors that could affect the occurrence of these protists in humans, specifically (i) lifestyle (urban vs. rural), (ii) contact with animals (we collected 170 samples of animals in which volunteers are in close contact), (iii) traveling, (iv) age category and (vi) gender. We have reported prevalence of 24% for *Blastocystis* sp., 16% for *Dientamoeba fragilis* and 3% for *Giardia intestinalis*. In the case of *Blastocystis*, we detected eight different subtypes in both humans and animals, and its higher prevalence in travellers outside Europe, subjects with more frequent contact with animals and in higher age categories (Fig. 10). Manuscripts are under review.



**Figure 10.** *Blastocystis* subtypes detected in human and animals in the Czech Republic.

## Research activity and characterisation of the main scientific results

### Comments on bibliographic performance of the team

The team members authored or co-authored **110 papers** in journals with impact factor:

- **45 papers** (41%) with the first author from our team.
- **65 papers** (59%) with the first author not affiliated with our team.
- Out of 110 papers, **78 papers** (71%) were co-authored by colleagues from abroad (see part "*Cooperation within international research area*").
- There is an increasing trend in the number of papers published in the top journals over the evaluating period. For instance, in 2015, there was a single paper published in the first AIS (Article Influence Score) decile, while in 2019 there were already eight (for details see the bibliographic profile of the team).
- **Out of the total 110 papers, 24 and 39 papers** were published in journals pertaining to the first AIS decile ( $Q^*$ ) and quartile ( $Q_1$ ), respectively. These include the core journals of microbial ecology and limnology, as exemplified below:

ISME Journal (**11x**,  $Q^*$ ), Microbiome (**5x**,  $Q^*$ ), Science Advances (**1x**,  $Q^*$ ), Nature Microbiology (**1x**,  $Q^*$ ), Limnology and Oceanography (**3x**,  $Q^*$ ), Environmental Microbiology (**6x**,  $Q_1$ ), Science of the Total Environment (**3x**,  $Q_1$ ), Freshwater Biology (**2x**,  $Q_1$ ); Aquatic Sciences (**3x**,  $Q_1$ ), mBio (**1x**,  $Q_1$ ).

### Projects Awarded

In the period 2015–2019, the team members coordinated a total of **14 research projects** at the position of principal investigators (PI, **12x**) or co-investigators (Co-PI, **2x**). Eight projects were already completed, six projects started in 2019, thus giving reasonable grounds for near-future team funding and our main proposed research directions listed in the "*Activity plan*".

The majority of funding (13 projects) came from a national source - Czech Science Foundation (CSF), one project was supported by the Swiss National Science Foundation (SNSF), giving a total of **2.51 million EUR** (~65 mils. CZK), which accounted for ~70% of the total funding of the team. **The most significant projects are as follows:**

- 2013–2017: Unveiling life strategies of selected groups of planktonic betaproteobacteria in relationship to carbon flow to higher trophic levels (Reg. code CSF 13-00243S), **239 thous. EUR**, **PI: Šimek K**
- 2015–2017: Phytoplankton responses to environmental forcing, **245 thous. EUR**, **PI: Znachor P**
- 2015–2017: Factors regulating the phototrophic activity of freshwater community of Betaproteobacteria (Reg. code CSF 15-12197S), **213 thous. EUR**, **PI: Kasalický V**
- 2016–2018: Bioactive cyanobacterial lipopeptides: genome mining, detection, and structure-activity relationships (Reg. code CSF 16-09381S), **194 thous. EUR**, **Co-PI: Mareš J**
- 2017–2019: Fishponds as models for exploring plankton diversity and dynamics of hypertrophic shallow lakes (Reg. code CSF 17-09310S), **141 thous. EUR**, **Co-PI: Nedoma J**

- 2017–2019: Unveiling life strategies of uncultivated viruses in freshwater environments using metagenomics (Reg. code CSF 17-04828S), **378 thous. EUR**, **PI: Ghai R**

## Characterisation of the main results and research lines

### 1. Ecology of bacterivorous and omnivorous flagellates and ciliates

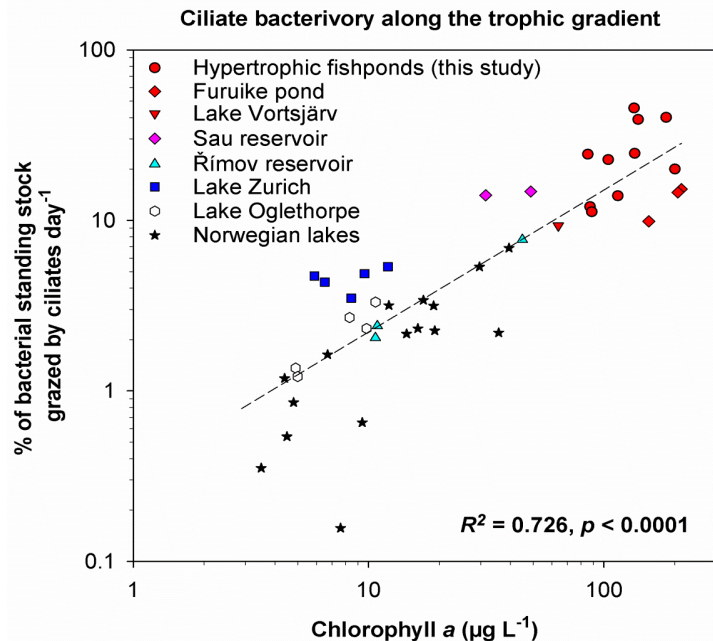
The current "sequencing era" has brought about an increasing amount of environmental studies on the importance of protists and their diversity in various aquatic ecosystems based on next-generation sequencing results (**Bock et al.**, 2018, *Front Microbiol* 9, 1290). This approach alone, however, offers only a limited possibility to conduct hypothesis-driven research on ecological traits of particular protistan taxa. Consequently, our knowledge of their ecology, feeding modes and survival strategies *in situ* is still in its infancy. To fill this knowledge gap, we based our research on exploiting a broad suite of **single-cell microscopy and molecular approaches**, that allow the assessment of highly complex microbial communities, or specific predator-prey interactions at a high taxonomic resolution.

Our research strategy combines Catalysed Reporter Deposition Fluorescence In Situ Hybridization (**CARD-FISH**) **approaches targeting both pro- and eukaryotic organisms** (including the design of novel FISH-probes) with a wide variety of other ecological approaches, such as **tracer techniques** (fluorescently labelled bacteria used in grazing experiments, **Šimek et al.**, 2017, *J Eukaryot Microbiol* 64, 336–348; **Šimek et al.**, 2019, *Limnol Oceanogr* 64, 2295–2309; **Šimek et al.**, 2019, *JOVE-J VIS EXP* 151, e59903), or **targeted experimental manipulations** with natural microbial communities (**Grujčić et al.**, 2015, *Appl Environ Microbiol* 81, 4993–5002; **Grujčić et al.**, 2018, *ISME J* 12, 1668–1681; **Šimek et al.**, 2018, *Limnol Oceanogr* 63, 484–502; **Piwoz et al.**, 2018, *ISME J* 12, 2640–2654). Such advanced methodological combinations provide ecologically meaningful data on life strategies, ecophysiological traits, and feeding modes of major protistan groups in the plankton, or in highly specific environments such as microbial communities in the trap fluid of carnivorous plants (**Šimek et al.**, 2017, *J Eukaryot Microbiol* 64, 336–348; **Pitsch et al.**, 2017, *J Eukaryot Microbiol* 64, 322–335). This approach facilitated important new findings concerning the trophic role of specific pelagic protists through simultaneous analyses of their food vacuole contents and taxonomic affiliation.

Despite the fact that shallow lakes are the most abundant lentic waterbodies in the global landscape, the knowledge of microbial components in hypertrophic ponds remains scarce. We quantified **bacterial production and total protistan bacterivory** in ten shallow hypertrophic fishponds in South Bohemia (**Šimek et al.**, 2019, *Limnol Oceanogr* 64, 2295–2309). On average, ciliate bacterivory was even more important than that of heterotrophic flagellates, accounting for 56% and 44% of total protistan grazing, respectively. Thus, the original view of a minor role of ciliate bacterivory in pelagic systems requires considerable revision. Moreover, general ecological theories assume that specialised bacterivorous ciliates should be a prominent ciliate group in hypertrophic aquatic environments that are high in bacterial numbers and biomass. However, our findings show that bacterivorous Peritrichia (genera *Vorticella*, *Epistylis*) and Scuticociliata contributed only moderately to total ciliate bacterivory. Unexpectedly, highly abundant omnivorous *Halteria/ Pelagohalteria* (Stichotrichia) and, to a lesser extent, also omnivorous *Rimostrombidium* spp. (Oligotrichia), contributed significantly more (mean 71%) to total ciliate bacterivory than typical

bacterivorous taxa. This suggests that unselective omnivorous grazers, which feed on a broader size spectrum from bacteria to small algae, may have a considerable competitive advantage in hypertrophic environments rich in small particles. Besides, a meta-analysis of available literature data (Šimek et al., 2019, *Limnol Oceanogr* 64, 2295–2309) showed that the role of ciliate bacterivory increases significantly, relative to flagellate bacterivory, along a trophic gradient towards hypertrophic habitats (see Fig. 1).

**Figure 1:** The metaanalysis of the relationship between chlorophyll *a* concentrations (as a proxy of the trophic status of lakes) and proportion of bacterial standing stock grazed daily by ciliates; shown as log-log linear regression (a dashed line). The data from fishponds were analysed together with the available literature data from various lakes along the trophic gradient from oligotrophy to extreme hypertrophy. Note that all hypertrophic systems are plotted as red symbols.



## 2. Metagenomics of microbial and viral communities

Already at the turn of the century, with the increasing use of sequence-based approaches to studying natural environments, it turned out that the cultivated diversity was far outstripped by the diversity in the habitats, so much so that only 1% of taxa present were estimated to be cultured. We have taken advantage of the continuous increase in sequencing throughput to tackle the uncultured microbial majority using **metagenomics**. For the last few years, we have focused on two sites in particular: the Řimov reservoir and Jiřická pond (both have been studied for many years) as model systems for a comprehensive metagenomic characterisation of microbes and their viruses. We now have a long-term data series for the Řimov reservoir, encompassing more than three years (monthly sampling, both epilimnion and hypolimnion) and 1.5 years worth of high-frequency sampling (every three weeks) at Jiřická pond. We have been successful in **describing multiple novel groups of microbes**. For example, using metagenomic approaches, we showed that sediment/soil Planctomycetes transitioned to aquatic environments, where they gave rise to new freshwater-specific clades (Andrei et al., 2019, *ISME J* 13, 1056–1071).

We also examined the **evolutionary history and lifestyles** of freshwater and marine Chloroflexi. In the deep sea, the SAR202 group is one of the most successful and widespread microbes. We showed that among the reasons for the success of this clade is the ability to metabolise multiple organosulfur compounds – thus elucidating their

major role in sulfur turnover in the dark water column (**Mehrshad** et al., 2018, ISME J 12, 655–668). We also demonstrated that freshwater habitats (lakes and rivers) harbour the most phylogenetically diverse community of pelagic Chloroflexi representatives that reside both in the epi- and hypolimnion (**Mehrshad** et al., 2018, Microbiome 6, 176).

In collaboration with others, we have also looked into the diversity of the poorly studied freshwater representatives of the phylum Verrucomicrobia. Some were small-celled, cosmopolitan heterotrophs, with a preference for carbohydrates (xylan, chitin or cellulose) while others were larger, with a higher number of transporters, polysaccharide degrading pathways and in general more diverse strategies for nutrient uptake (**Cabello-Yeves** et al., 2017, Front Microbiol 8, 2131). Freshwaters also host a diverse assortment of Cyanobacteria. While the colonial and filamentous representatives have so far attracted most of the attention, freshwater **picrocyanobacteria** remain less studied. Using a metagenomic assembly approach, we discovered two novel and nearly identical *Synechococcus* sp. genomes from two geographically distant freshwater reservoirs Tous (Spain) and Lake Lanier (USA) that were abundant at both sites (**Cabello-Yeves**, et al., 2017, Front Microbiol 8, 1151).

We have considerable expertise in the **recovery of viral genomes** from metagenomes. We described the first complete viral genomes using metagenomic fosmid libraries by sequencing 6,000 fosmids obtained from the deep-sea. We found multiple genomes that could be found globally in the bathypelagic habitat (**Mizuno** et al., 2016, mBio 7, e00805–16). Applying similar methods to the freshwater habitat, we focused on recovering phages infecting Actinobacteria that are the most abundant microbes in inland waters. We described the first phages from these cosmopolitan microbes. Surprisingly, we found an Actinobacterial phage encoding the ADP-ribosylating component of the widespread AB family of bacterial toxins (for example, clostridial toxin) that target eukaryotes. We posit that the presence of this toxin reflects a 'trojan horse' strategy, providing protection at the population level to the abundant host microbes against eukaryotic predators (**Ghai** et al., 2017, ISME J 11, 304–308). In a much larger follow-up study on freshwater phages, we recovered complete genomes of >2000 phages, including small "miniphages" and large "megaphages" infecting iconic freshwater prokaryotic lineages. We described many phages encoding genes that likely afford protection to their host from reactive oxygen species (ROS) in the aquatic environment and the oxidative burst in protist phagolysosomes (phage-mediated ROS defence). On an ecological note, we observed that phage and host abundances corresponded closely, with distinct populations displaying preferential distributions in different seasons and depths, closely mimicking overall stratification and mixis (**Kavagutti** et al., 2019, Microbiome 7, 135).

In collaboration with other groups, we have also examined **microbial diversity in brackish sediments**. It has been recently shown that Asgardarchaeota are the closest lineages to eukaryotes. We showed that Heimdallarchaeia (the closest lineage to eukaryotes to date) are likely to be aerobes living in a microoxic niche. We also provided evidence of mixotrophy within Asgardarchaeota; and describe a previously unknown family of rhodopsins encoded within the recovered genomes (**Bulzu** et al., 2019, Nature Microbiol 4, 1129–1137). Our other collaborations on sediment microbiota involve hypersaline soda brines that harbour extremely diverse microbial communities compared to neutral brines of similar salinities. We were part of a series of publications on the microbial diversity in soda-lakes lakes from the Kulunda Steppe (Altai, Russia) covering a salinity range from 170 to 400 g/L (**Vavourakis** et al., 2016,



Front Microbiol 7, 211; **Vavourakis** et al., 2018, Microbiome 6, 168; **Vavourakis** et al., 2019, BMC Biol 17, 69). In total, we reconstructed nearly 2000 metagenome-assembled genomes (MAGs) spanning more than 45 phyla. We discovered the first extremophilic members of the Candidate Phyla Radiation (CPR), novel lineages of colourless sulfur-oxidising bacteria (SOB), anoxygenic purple sulfur bacteria, heterotrophic SOB, and highly active litho-autotrophic sulfate reducers. Surprisingly, we discovered the potential for carbon fixation in a new species of phototrophic *Gemmatimonadetes*, and elemental sulfur/sulfite reduction in the "*Candidatus* Woesearchaeota." Only a few environmental metagenomics studies have targeted sediment microbial communities and never to this extent.

We also had occasional collaborations with groups working on **rhodopsins**, proton pumps commonly found in microbial metabolic pathways in various aquatic habitats. We took part in several studies indicating that heliorhodopsins (the latest discovered rhodopsins) are absent in Gram-negative bacteria (**Flores-Urbe** et al., 2019, Environ Microbiol Rep 11, 419–424) or solving the structure of novel inward H<sup>+</sup> pumping xenorhodopsins (**Shevchenko** et al., 2017, Sci Adv 3, e1603187). Some team members also had collaborations from their past laboratories, and these could be brought to completion (**Andrei** et al., 2017, Sci Rep 7, 6150; **Andrei** et al., 2017, Front Microbiol 8, 372; **Carda-Dieguez** et al., 2017, Microbiome 5, 162; **Al-Qahtani** et al., 2017, OncoTarget 8, 105115–105125).

### 3. Cultivation and eco-genomics of abundant planktonic freshwater microbes

The most predominant planktonic prokaryotes in lakes are notoriously hard to cultivate as they are adapted to low nutrient conditions that prevent growth in conventional media. We successfully **cultivated some of the most abundant planktonic microbes** by employing high-throughput dilution-to-extinction isolation in liquid media mimicking natural conditions and a modified filtration-acclimatisation protocol for isolating *Limnohabitans* spp. (**Salcher** and Šimek, 2016, Aquat Microb Ecol 77, 183–196). So far, we isolated and currently maintain >700 prokaryotic strains from various European lakes and performed whole-genome sequencing of >150 strains. Apart from that, we have built a large culture collection of freshwater *Synechococcus*-like picocyanobacteria counting >300 living strains from various freshwater habitats of different trophic states, which are now being genome-sequenced and studied in more detail.

**Cultivation, lab experiments, in situ quantification, and state-of-the-art comparative genomics** are combined to shed light on metabolic properties, biogeographic and microdiversification patterns and the evolutionary history of freshwater microbes. One focus was put on **microbes with reduced (streamlined, i.e. <1.5 Mbp) genomes**. We isolated >90 strains of two new *Candidatus* genera of methylotrophic bacteria that are abundant in the water column ('*Ca. Methylopumilus*' spp. and '*Ca. Methylosemipumilus*' spp.) and sequenced the complete genomes of 41 strains (**Salcher** et al., 2015, ISME J 9, 2442–2453; **Salcher** et al., 2019, ISME J 13, 2764–2777). Through comparative genomics, we could track their evolutionary history from sediment microbes with medium-sized genomes via an intermediate ('*Ca. Methylosemipumilus*') to the most genome-streamlined '*Ca. Methylopumilus*' (1.3 Mbp genome size).

The transition from freshwater sediment to the relatively oligotrophic open water was accompanied by progressive gene loss and adaptive gains (**Salcher** et al., 2019, ISME J 13, 2764–2777).

Moreover, we putatively described a novel order of Actinobacteria, '**Ca. Nanopelagicales**' (formerly known as *acl*-Actinobacteria), the most abundant pelagic freshwater bacteria that were 'hunted' by several research teams for years. We transiently isolated and genome-sequenced 16 strains that showed remarkable microdiversity patterns (**Neuenschwander** et al., 2018, ISME J 12, 185–198; Editor's choice as top 20 articles of the ISME Journal in 2018). While being practically identical on the level of 16S rRNA, we could distinguish two new genera ('*Ca. Nanopelagicus*' and '*Ca. Planktophila*') and nine new species with a surprising degree of inter- and intraspecific diversity in metabolic pathways, especially of carbohydrate transport and metabolism. Genome reduction in these bacteria (1.2–1.3 Mbp genome size) have led to auxotrophy for various vitamins, amino acids and reduced sulphur sources, thus creating dependencies to co-occurring organisms.

Our research on **freshwater *Limnohabitans***, building on the worldwide largest collection of strains from the genus (>100 strains), allowed for a broad variety of specific studies focused on their diversity and ecophysiology. We have sequenced 31 strains and demonstrated a photoheterotrophic lifestyle for several *Limnohabitans* spp. through the discovery of photosynthetic gene clusters (**Kasalický** et al., 2018, Appl Environ Microbiol 84, e02116–17). Several strains were used for in-situ and laboratory experiments that documented their high importance in the microbial food web (**Grujčić** et al., 2015, Appl Environ Microbiol 81, 4993–5002; **Grujčić** et al., 2018, ISME J 12, 1668–1681; **Salcher** et al., 2016, FEMS Microbiol Ecol 92, fiv156; **Šimek** et al., 2018, Limnol Oceanogr 63, 484–502) and strain-specific utilisation of carbon sources (**Horňák** et al., 2017, Environ Microbiol 19, 4519–4535; **Shabarova** et al., 2017, Environ Microbiol 19, 1296–1309).

In cooperation with the research team of M. Hahn (Mondsee, Austria), we used series of transplantation experiments followed by comparative genomics to confirm the ecological isolation level of *Polynucleobacter* strains difficult to distinguish by classical methods (**Hahn** et al., 2016, ISME J 10, 1642–1655; **Hoetzing** et al., 2017, Appl Environ Microbiol 83, e02266–16). In collaboration with C. Callieri (Verbania, Italy), we focused on genomic characteristics of several *Synechococcus* strains isolated from the mesopelagic anoxic water column of the Black Sea (**Callieri** et al., 2019, ISME J 13, 1676–1687). These remarkable cyanobacteria could survive anoxic/dark conditions for several months and were able to switch back to photosynthesis when re-exposed to light. We also assisted other teams in genome analysis of isolated microbes, e.g. *Halonotius* sp. from saline soil (**Durán-Viseras** et al., 2019, Front Microbiol 10, 1928), and *Herminimonas arsenitoxidans* (**Koh** et al., 2017, Sci Rep 7, 15007).

#### 4. Freshwater prokaryote diversity and ecophysiology

Freshwater environments are inhabited by hundreds of prokaryotic taxa of still largely unknown **diversity and metabolic capabilities**. In the last years, we focused our research effort on an investigation of the Římov reservoir (**Jezberová** et al., 2017, Appl Environ Microbiol 83, e01530–17; **Shabarova** et al., 2017, Environ Microbiol 19, 1296–1309; **Kasalický** et al., 2018, Appl Environ Microbiol 84, e02116–17), Lake Zurich

(**Shabarova** et al., 2017, *Environ Microbiol* 19, 1296–1309), Lake Maggiore (**Coci** et al., 2015, *Archaea*, 590434), alpine and subalpine lakes in Austria and Switzerland (**Callieri** et al., 2016, *Aquat Sci* 78, 215–225; **Alfreider** et al., 2017, *Environ Microbiol* 19, 2754–2768; **Alfreider** et al., 2018, *FEMS Microbiol Ecol* 94, fyy163; **Bock** et al., 2018, *Front Microbiol* 9, 1290), and variety of lakes from around the globe (**Hahn** et al., 2015, *Environ Microbiol* 17, 829–840). We showed that several phylogenetically narrow clusters, e.g. genera *Limnohabitans* and *Polynucleobacter* were widely distributed but included a variety of lineages and species, which were difficult to distinguish *in situ* even though they exhibit different metabolic capabilities and ecological preferences.

To study the ecology of individual lineages of the ubiquitous genus *Limnohabitans* *in situ*, we designed a **new CARD-FISH probe** and employed a **double hybridisation approach**. It allowed us distinguishing between RBT, LimA and LimE sub-populations of *Limnohabitans* and to unveil the previously unknown ecological preferences of LimA lineage, which appears to be mainly dependent on pulses of allochthonous organic matter (**Shabarova** et al., 2017, *Environ Microbiol* 19, 1296–1309). Even higher resolution between *Limnohabitans* lineages achieved with Reverse Line Blot Hybridization allowed for the identification of "generalist" lineages that were always present throughout the whole seasons as well as "specialists" that appeared in the Římov reservoir only for limited periods or irregularly (**Jezberová** et al., 2017, *Appl Environ Microbiol* 83, e01530–17).

Genus *Polynucleobacter* harbours several cryptic species that are almost indistinguishable by their 16S rDNA sequences. Therefore, we used two markers, the 16S–23S internal transcribed spacers and the glutamine synthetase gene to analyse the global phylogeography of this genus (**Hahn** et al., 2015, *Environ Microbiol* 17, 829–840). Our effort demonstrated that pelagic *Polynucleobacter* is composed of both ubiquitous strains with geographic distance-decay patterns as well as strains with restricted geographic distributions.

Research on the emergence of archaea in freshwaters using a new CARD-FISH probe revealed that the oxygenated hypolimnion of the deep lakes is largely inhabited by ammonia-oxidising *Thaumarchaeota* (**Coci** et al., 2015, *Archaea*, 590434; **Callieri** et al., 2016, *Aquat Sci* 78, 215–225). These archaea are often accompanied by a bacterium *Nitrospira*, a complete ammonia-oxidiser (comammox) (**Alfreider** et al., 2018, *FEMS Microbiol Ecol* 94, fyy163). Both prokaryotes not only differ in their complexity of ammonia-oxidising pathways but also in their CO<sub>2</sub>-fixation abilities (**Alfreider** et al., 2017, *Environ Microbiol* 19, 2754–2768). We also developed a strategy to overcome the quantitative bias of high-throughput sequencing of the bacterial 16S rDNA amplicons, a standard method used to reveal bacterial diversity. The newly proposed method based on inner standard calibrating NSG data allows for determining lineage contributions to microbial communities as well as to calculating their growth rates with less effort than needed for CARD-FISH analysis (**Piwoz** et al., 2018, *ISME J* 12, 2640–2654).

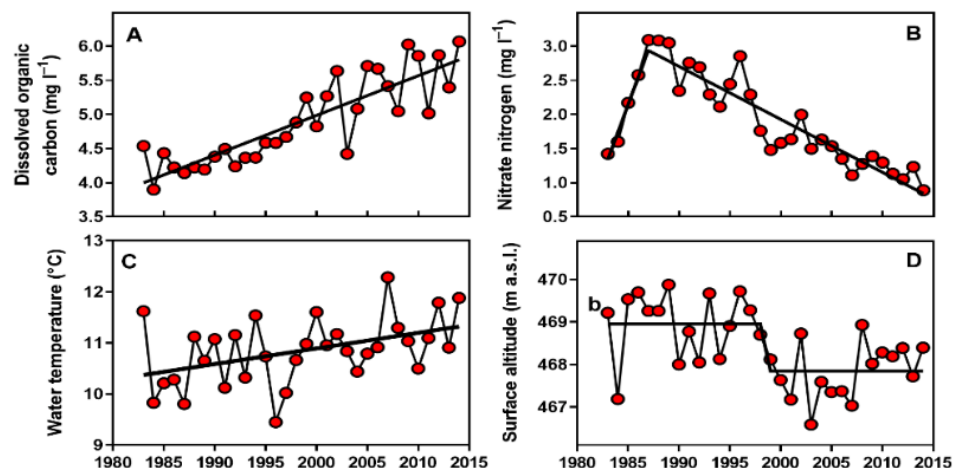
## 5. Phytoplankton ecology, reservoir limnology and time-series analyses

Tiny planktonic organisms play essential roles in nutrient cycling and energy fluxes through the aquatic food web. Using **various fluorescence techniques**, we measured, e.g. the relationship between light availability and phytoplankton

phosphatase production (Rychtecký et al., 2015, Microb Ecol. 69, 37–44) or diatom growth and viability (Znachor et al., 2015, Hydrobiologia 762, 253–265). We demonstrated, that *in situ* applications of fluorescent stains depicting physiological responses of the phytoplankton to the changing environment furnish important clues for deciphering processes beyond the spatiotemporal heterogeneity of aquatic systems.

Long-term data produced by routine site-based monitoring programs of freshwaters provide invaluable resources for investigating the effects of environmental change on biodiversity and ecosystem function, as demonstrated by our study of constraints on the biological recovery of the Bohemian Forest lakes from acid stress (Vrba et al., 2016, Freshw Biol 61, 376–395). Due to the unique availability of reliable long-term data, the Římov Reservoir was our primary research site. We analysed **time series** of weather, land use in the catchment, reservoir hydrochemistry and hydrology (Znachor et al., 2018, Sci Total Environ 624, 24–33). Most environmental variables exhibited temporal changes that included **contrasting trends, time-varying trends and trend reversals (Fig. 2A–C)**. After a series of heavy rains in the late 1990s, an administrative decision to increase the flood-retention volume of the reservoir resulted in a significant regime shift in reservoir hydraulic conditions trend in the 1999 (Fig. 2D).

**Figure 2:** Long-term trends in annual means of dissolved organic carbon (A), nitrate-nitrogen (B), water temperature (C) and surface altitude (D). Lines indicate the most parsimonious model.



We also studied the effects of environmental drivers on the composition, diversity and community stability of main morpho-functional phytoplankton groups (Znachor et al., 2019, Sci Total Environ 135626). Consistent with the previous analysis, we found significant differences in phytoplankton composition among the three periods defined by shifts and breakpoints in temporal trends in reservoir hydrochemistry and hydraulic conditions (1983–89, 1990–99, and 2000–14). Total nitrogen, inflow rate and surface level were the most important predictors of phytoplankton composition in the reservoir. Although cyanobacteria have been predicted to dominate the phytoplankton with rising temperature, this was not observed. Instead, diatoms became increasingly abundant. This is particularly interesting because, similarly to many freshwater systems worldwide, the enhanced thermal stability of the water column was also observed in the Římov reservoir. We hypothesise that the increased diatom dominance is driven by episodic high-inflow events, and, therefore, our future research will focus on deciphering the impact of extreme weather events on the reservoir ecology.

Combining environmental data with zooplankton time-series, we studied seasonal and intra-annual patterns of zooplankton temporal variation and community assembly processes including species interactions and the effects of driving factors such as food, temperature, nutrients and oxygen concentration (Gabaldón et al., 2019, *Freshwater Biol* 64, 1326–1341). Our results suggest that the zooplankton community is structured by both the biotic network at the seasonal scale and by the physical environment at the intra-annual scale.

## 6. Taxonomy, genomics, and ecotoxicology of cyanobacteria

Due to their ability of oxygenic photosynthesis and N<sub>2</sub> fixation, cyanobacteria are one of the most important groups of (not only) aquatic microbes. On the other hand, they form harmful blooms and produce a tremendous variety of bioactive compounds, including cyanotoxins. Surprisingly, the diversity of cyanobacteria is still poorly understood due to long-term taxonomic confusion that hinders interpretation of metagenomic and ecotoxicology studies. Members of our team have been among the researchers leading recent efforts towards the modern revision of the phylum-wide classification of cyanobacteria. Our studies combined a state-of-the-art **polyphasic approach** with **phylogenomic analyses** to identify the pitfalls in current cyanobacterial taxonomy and initiated its genome-based revision (**Mareš**, 2018, *Hydrobiologia* 811, 19–34; **Mareš** et al., 2019, *Front Microbiol* 10, 277). Several taxonomic studies have further focused on particular ecologically important taxa, such as the unicellular aquatic diazotrophs (**Mareš** et al., 2019, *J Phycol* 55, 578–610).

The progress in cyanobacterial genomics is partly obscured by the fact that most cyanobacterial strains are heavily contaminated by heterotrophic bacteria. Our team has successfully implemented rapid sequencing of cyanobacterial genomes from single cells/filaments, followed by mining for natural product biosynthetic gene clusters, *in silico* biosynthesis prediction, and evolutionary analysis (e.g. **Kust** et al., 2018, *ACS Chem Biol* 13, 1123–1129; **Mareš** et al., 2019, *Appl Environ Microbiol* 85, e02675–18). We have exploited our **unique culture collection** of several hundreds of cyanobacteria to select the strains for further in-depth studies by initial PCR and liquid chromatography/mass spectrometry (LC/MS) based screening (**Kust** et al., 2018, *Toxicon* 150, 66–73). The bioactive metabolites under study have included new analogues of well-known compounds such as microcystins (**Kust** et al., 2018, *Toxicon* 150, 66–73), and pederin-family compounds (**Kust** et al., 2018, *ACS Chem Biol* 13, 1123–1129). Besides, we have been in particular interested in a new group of ecotoxicologically relevant cyanobacterial lipopeptides, frequently found in soil and wetland cyanobacteria (**Mareš** et al., 2019, *Appl Environ Microbiol* 85, e02675–18), but recently also in freshwater phytoplankton. We have also participated in the development of detection techniques for the monitoring of these lipopeptides using LC/MS (**Urajová** et al., 2016, *J Chromatogr A* 1438, 76–83) and advanced molecular methods.

## 7. Methane dynamics in rivers

Methane (CH<sub>4</sub>) is an important component of the aquatic carbon cycle involved in many biogeochemical and physical processes. All of these processes are subject to large temporal and spatial heterogeneity. Understanding the variability of CH<sub>4</sub>-related processes (namely CH<sub>4</sub>-production, -oxidation, -emissions) is a key factor for making



more precise estimates of the global methane budget. The microbial CH<sub>4</sub>-oxidation forms an effective barrier, preventing even bigger amounts of this important greenhouse gas from being released into the atmosphere. Our intensive sampling campaigns have brought new insights into the main CH<sub>4</sub> pathways and general spatial trends along river systems, exemplified on the longitudinal transect of the Elbe river system from its headspring to its delta area (**Matoušů** et al., 2017, *Aquat Sci* 79, 443–458; **Matoušů** et al., 2019, *Aquat Sci* 81, 12; **Bednařík** et al., 2017, *Sci Total Environ* 584, 164–174). We found that in CH<sub>4</sub>-rich environments (e.g. harbours, human-affected river sections upstream from weirs), the CH<sub>4</sub> oxidation potential was very limited, or even undetectable. Thus, these habitats represent hot-spots of CH<sub>4</sub> emissions. On the contrary, natural upstream habitats with lower CH<sub>4</sub> concentrations were characterised by a very effective microbial oxidation, being able to remove more than 80% of CH<sub>4</sub> directly in the water column.

### 8. Ecology of complex microbial communities

The study of microbiomes associated with various eukaryotic hosts, including plants and invertebrates, and the role these interactions play in ecosystem functioning is currently one of the hot topics in ecology. This type of research requires an interdisciplinary approach combining knowledge of host physiology, microbial ecology, and systems biology. One of our model systems involved the unique trophic interaction between a rootless aquatic carnivorous plant and its associated microbiome consisting of bacteria, fungi, algae, and protozoa inhabiting a miniature ecosystem of traps. We showed that the traps, in terms of their ecophysiological function, can be compared to microbial cultivators or farms, which center around complex microbial consortia acting synergistically to convert complex organic matter, often of algal origin, into a source of utilisable nutrients for the plants (**Sirová** et al., 2018, *Microbiome* 6, 225; **Šimek** et al., 2017, *J Eukaryot Microbiol* 64, 336–348; **Pitsch** et al., 2017, *J Eukaryot Microbiol* 64, 322–335). A new promising model system, which we started to study intensively in 2019, is the association between larger freshwater planktonic crustaceans, especially *Daphnia* spp., and their microbiomes, with specific emphasis on energy transfer between trophic levels.

## Research activity and characterisation of the main scientific results

During 2015-2019, team members authored or co-authored **99 impact-factor papers** having cumulative IF=308. Based on the article influence score (AIS), **27 % of team's papers were published in journals positioned in the first quartile or better** of the journal's category. Among the top journals where the team's papers were published were **Trends in Ecology and Evolution (IF=15.236)**, **Frontiers in Ecology and the Environment (IF=10.935)**, **Global Change Biology (IF=8.88)**, **PloS Biology (IF=8.386)**, **Fish and Fisheries (IF=6.99)**, **Science of Total Environment (IF=5.589)** and **Ecology (IF=4.285)**.

### Obtaining a true picture of fish communities

We specialize in obtaining quantitative, semi-quantitative and qualitative information on the abundance, biomass, and composition of fish communities in large inland waters. For this purpose, we use a wide range of capturing and sampling devices and develop methods to survey quantitatively all size groups of all species in all important volumes of large waters. We continued to improve the methods of complex fish stock assessment by the gillnetting (**Šmejkal et al. 2015, PLOS One 10(3), e0122437**; **Blabolil et al. 2017, Ecol. Indic. 77, 368-376**), trawling (**Sajdlová et al. 2015, Fish. Res. 172, 105-113**; **Jůza et al. 2018, Acta Ichthyol. Piscat. 48(1), 105-108**), using combination of methods (**Blabolil et al. 2018, J. Fish Biol. 92(5), 1545-1559**), and especially hydroacoustics.

The main challenge in the use of scientific echosounders for surveying in meso- and eutrophic waters is the fact that significant part of fish lives either in shallow waters or shallow layers. Intensive work aimed on verification of depth and acoustic size of fish led to the discovery of multipath acoustic reflection over the surface (**Balk et al. 2017, Fish. Res. 188, 149-156**). Similar kind of interferences combined with increased reverberation was discovered in a shallow system like Austrian Lake Neusiedlersee and Bohemian ponds (**Koliada et al. 2019, Fish. Res. 218, 94-104**). Unexpected phenomenon of surface multipath reflections has very serious consequences for acoustic fish stock assessment. It proved correctly our long-termed effort of non-traditional ways of acoustic assessment of near-surface fish. We developed a mobile up-looking system that was found to be useful for recording surface-oriented fish and invertebrates (**Baran et al. 2017, Sci. Rep. 7, 4823**; **Baran et al. 2019, J. Limnol. 78(1), 60-70**). In the area of acoustic assessment of deeper layers, team members led the intercalibration of assessment methods by several state-of-the-art echosounders (**Drašík et al. 2017, Limnol. Oceanogr.: Methods 15(10), 836-846**). The contribution of the team into the development of fish stock assessment methods and general building of knowledge about fish in lakes and reservoirs was acknowledged in the proceedings of ECOFIL conference (**Kubečka et al. 2016, Fish. Res. 173, 1-3**) and the international group for standardization of sampling methods (**Bonar et al. 2017, Fisheries 42(3), 150-156**). Team expertise in application of scientific echosounders was applied also for the estimate of methane ebullition from the reservoirs (**Frouzová et al. 2015, Limnol. Oceanogr.: Methods 13(11), 609-616**; **Tušer et al. 2017, Water Resour. Res. 53(10), 8266-8276**) and further methane studies in the largest Czech river (**Matoušů et al. 2019, Aquat. Sci. 81(1), 12**; **Bednařík et al. 2019, Limnologica 79, 125716**).

Two studies enhanced the use of PIT-tagging and passive telemetry technology. Female fish expel some 20 % of their body mass as eggs during spawning. Consequently, the probability of PIT tag loss is ten times higher than with males (10 % vs. 1 %, Šmejkal et al. 2019, *Fish. Res.* **219**, 105325). This may lead to wrong estimates of population size, expecting equal retention probabilities. This study was the first to describe unequal tag retention probability in a cyprinid species. Use of tagging methodology is not limited only to fish, and we explored the potential to use this method for tracking amphibians, specifically great crested newt (*Triturus cristatus*). We found that this method is applicable in this species and does not negatively affect their behaviour (Weber et al. 2019, *PLOS One* **14**(7), e0219069). It may greatly help to investigate their ecology and movement between a terrestrial and aquatic environment.

### Diurnal vertical migrations of larval and juvenile fish

Combination of multiple quantitative tools led to studies with a relatively complex view of the distribution ecology in the early life history of fish. Diel vertical migrations (DVMs) belong among the most pronounced movements in the aquatic environment. They represent a typical behaviour even for early life stages of percid (Percidae) and coregonid (Coregonidae) fishes, like European perch (*Perca fluviatilis*), pikeperch (*Sander lucioperca*), ruffe (*Gymnocephalus cernua*) or maraena whitefish (*Coregonus maraena*) (Čech et al. 2017, *Hydrobiologia* **784**, 1-8; Sajdlová et al. 2017, *Hydrobiologia* **787**, 341-352; Jůza et al. 2019, *Limnologia* **76**, 19-27). While the general pattern of DVMs has been well described, the drivers of this movement remained poorly understood. Using a large black non-transparent foil (2500 m<sup>2</sup>) covering the water surface, we demonstrated for the first time that DVMs of bathypelagic early juveniles in a community of European perch were under direct control of the light intensity; that is, the migration is not genetically fixed behaviour (Sajdlová et al. 2018, *Freshw. Biol.*, **63**, 473-482). During the ongoing season the main habitat of occurrence of European perch juveniles, the hypoxic deep waters, could serve as a refuge against predatory fish including larger individuals of common carp (*Cyprinus carpio*) and common bream (*Abramis brama*) (Vejřík et al. 2016, *Freshw. Biol.*, **61**, 899-913; Vejřík et al. 2017, *PLOS One*, **11**(6), e0156430). The results of the RNA:DNA ratio of all three perch communities simultaneously occurring in canyon-shaped reservoirs (epipelagic perch fry – EPF, bathypelagic perch fry – BPF, littoral perch fry – LPF) showed that EPF bear the highest variability among individuals in contrast to BPF that has the lowest ratio as well as variability (Petrýl et al. 2015, *Int. Rev. Hydrobiol.* **100**, 13-20). It is likely that the evening migration to the epilimnion is absolutely essential for perch fry performing DVMs in these systems. All fish left experimentally in the deep habitat overnight were dead (Čech et al. 2017, *Hydrobiologia* **784**, 1-8). Analyses of six microsatellite loci and mtDNA Cytb showed that all spatially segregated communities of perch (EPF, BPF, LPF) belong to one population with the same phylogenetic origin (Kalous et al. 2017, *J. Appl. Ichthyol.* **33**, 306-313).

### Cohort strength variability in predatory fish

Fish recruitment in riverine reservoirs is not fully understood because the long-term data series required for standard stock–recruitment models are often lacking. In a study by Blabolil et al. (2016, *Fish. Res.* **173**, 45-52), we investigated two piscivorous species with different ecologies, asp (*Leuciscus aspius*) and pikeperch, over a 14-year period using a novel informative statistical approach based on

dimension reduction methods. Recruitment of asp fry was found to be affected by zooplankton abundance, predator density and temperature. Recruitment of pikeperch fry measured with seine and trawls was only affected by the number of predators, while recruitment of pikeperch fry estimated with gillnet data was also affected by temperature and water level fluctuation. Although gillnets are a commonly used sampling method, it seems to be inappropriate for developing a fry predicting model. Because many factors can affect cohort strength during the fish's early life period and year to year fluctuations in 0+ fish cohort strength are a common phenomenon, we further studied factors responsible for development of a 0+ pikeperch cohort (**Blabolil et al. 2019, Knowl. Manag. Aquat. Ecosyst. 420, 43**). Our study revealed that a strong 0+ pikeperch cohort at the time of hatching might not result in a strong cohort in general. Fish marking is essential to distinguish between stocked and naturally reproduced fish, but this procedure is challenging, especially in the early life stages. Therefore, two marking methods using oxytetracycline hydrochloride immersion for marking 3–5 day-old pikeperch larvae (**Blabolil et al. 2018, Biologia 73(5), 531-535**) and Alizarin Red S immersion for marking juvenile asp (**Halačka et al. 2019, Biologia 74(10), 1359-1362**) were investigated. Both studies confirmed the suitability of marking for use in studies focused on early growth, stocking success, and distribution etc.

### **Habitat use and spatial dynamics of fish**

Research in this direction was focused on disentangling fish spatial dynamics, with high emphasis on the understanding of fish diurnal migrations. This resulted in four papers dealing with fish diurnal horizontal and vertical movements in reservoirs (**Jůza et al. 2015, Hydrobiologia 762(1), 169-181**; **Říha et al. 2015, Hydrobiologia 747, 111-131**; **Jůza et al. 2018, Biologia 73, 647-658**; **Muška et al. 2018, Sci. Rep. 8, 5381**). These papers mapped out in great detail how adults of various species perform diurnal migrations and the proximate drivers of these movements. These results give a very unique and novel insight to short-term movements of fish and how fish movements can affect fish community structure. Findings also had important implications for fish sampling strategy. Research on a similar topic was performed in Lake Ontario by the team member Milan Říha during his research stay at Cornell University (USA; supported by Fulbright scholarship). The research resulted in a paper evaluating diel vertical migration of the pelagic fish community in the lake and the potential implications of these changes to lower trophic levels and the whole lake ecosystem (**Říha et al. 2017, J. Great Lakes Res. 43, 823-837**).

### **Aggregative behaviour of fish in open water habitat of a temperate reservoir**

While most of the authors focused their work on a mechanistic approach to the matter of schooling, the study of **Holubová et al. (2019, PeerJ 7, e6378)** explored the issue from a population point of view. Novel camera observation on the fish community carried out in the epipelagic habitat of a European temperate reservoir explored the relationship between density and aggregative features of predominantly cyprinid fish stock. Results demonstrated that schooling behaviour is triggered by the 'critical density' of fish in the habitat. School size as well as counts of schools and proportion of schooling individuals increased with the density of fish. Counts of clusters (observed units in time, including singletons, pairs and schools) and cluster size, on the other hand, showed a slowing tendency to increase. The slower increase implies the tendency of fish to create larger groups. Altogether, our findings suggest that fish density is a trigger factor in the formation of large fish schools. Fishing survey gear often causes damage to the fish and ecosystem and can be size or species selective.

Use of an underwater video camera (UVC) omits these flaws and can bring accurate information on species composition and density of daytime assemblages, moreover it can discover unknown details about behavioural patterns. **Holubová et al. (2019, Ecol. Indic. 105, 1-5)** tested the use of a remotely operated camera and proposed the use of the UVC as a valid sampling method even in the systems with higher trophies. No significant difference in species composition was found between traditionally used survey gear – purse seining and camera; nor was there a difference in the standardised fish abundance between hydroacoustics and camera when surveying an open water habitat of a temperate freshwater reservoir.

### **Reproductive success and predator-prey role reversals**

In the tributary of one of the largest Czech reservoirs, the Želivka Reservoir, several studies were conducted using a newly established predator-prey model system. Research was primarily focused on the behavioural analysis of the spawning run in the important predatory cyprinid species, the asp. Individual tagging and passive telemetry were used to determine individual spawning activity in the spawning ground. Males were found to arrive earlier on the spawning ground in seasonal and daily aspects (**Šmejkal et al. 2017, Sci. Rep. 7, 4737**). Furthermore, we identified the predator-prey reversal between small cyprinid bleak (*Alburnus alburnus*), and asp (**Šmejkal et al. 2017, Sci. Rep. 7, 6924**). Since bleak is an open water species with a preference for the upper water column, it does not graze adhesive asp eggs from the bottom but use the drifting stage, which is limited to tens of seconds only. Tagging and passive telemetry of bleak demonstrated that bleak arrives on the asp spawning ground before sunrise and leaves before sunset. Predominant nocturnal spawning activity of asp (approximately two thirds of the eggs are laid at night according to the model) was then linked to high predation on the eggs during the day and absence of egg eating bleak on the spawning ground during the night (**Šmejkal et al. 2018, Sci. Rep. 8, 15377**).

### **Foraging behaviour and food web interactions**

Predation is the primary top-down structuring force in aquatic ecosystems, and a change in predator-prey interactions involving fish may cause a change in the strength of trophic cascades and finally result in ecosystem shifts. We studied foraging behaviour of the European catfish (*Silurus glanis*), the largest European freshwater fish species, and revealed that this apex predator has enormous adaptability and learning ability to utilise various food sources (**Vejřík et al. 2017, Sci. Rep. 7, 15970**; **Vejřík et al. 2017, PLOS One 12, e0169000**). We conclude that the European catfish has a strong potential to influence overall food web structure in aquatic ecosystems (**Vejřík et al. 2019, J. Environ. Manag. 241, 374-382**). This finding is of great importance for fisheries management and nature conservation, as European catfish is now invasive in the freshwaters of western and southern Europe. In catfish-prey interactions we further explored the role of chemical cues perception in the contribution of prey species in the lake. In this study, we combined laboratory experiments and reviewed the community development, showing that species with no reaction to chemical cues or enhancement of their activity leads to their decline in the proportion of the fish community (**Šmejkal et al. 2018, Ecol. Evol. 8(9), 4544-4551**).

Using regurgitated pellets of the great cormorant (*Phalacrocorax carbo*) collected from roosting sites, we discovered that the size of fish consumed by cormorants increased significantly during the winter season (**Lyach et al. 2018, Bird Study 65, 249-256**). These results have applied importance for fisheries management, because they indicate that stocking of potentially vulnerable fish should be delayed to the time when



overwintering cormorants leave the area. Analysis of the nest sediment of the Common Kingfisher (*Alcedo atthis*) was used to reveal the role of non-fish prey in its diet (**Čech and Čech 2015, Bird Study 62(4), 457-465**). Unlike other fish-eating birds, in Common Kingfisher, the catch of non-fish prey appears to be accidental and is more likely a result of target misinterpretation than a Kingfisher regularly switching to prey other than fish.

Our analysis of meta-data from 26 lentic ecosystems revealed the dominance of non-native bottom-feeding fish species in altered ecosystems (reservoirs) in relation to natural lakes (**Bezerra et al. 2019, Ecosphere 10, e02757**), suggesting a generalised state of biotic homogenisation in lentic freshwaters under the influence of damming. Our other publications provide important information about inter- and intraspecific niche segregation (**Eloranta et al. 2017, Freshw. Biol. 62, 1401-1409; Vašek et al. 2018, Ecol. Freshw. Fish 27, 1054-1065**), resource use (**Blabolil et al. 2018, Biologia 73, 83-91; Lemoine et al. 2019, Mar. Ecol. Progr. Ser. 626, 161-175**) and predator–prey reversal (**Vejřík et al. 2016, PLOS One 11, e0156430**) in fish.

Results of **Vašek et al. (2017, Hydrobiologia 785, 327-335)** clearly show that fin clips and scales can be used as non-lethal alternatives to muscle tissue in stable isotope studies of threatened fish species. This finding is highly relevant to current ecological research, because there is an urgent need to eliminate unnecessary mortality of sampled animals.

### **Functional role of herbivorous fish and macrophytes in lakes**

Beside other activities, the team is intensively engaged in the role of fish in the food webs of aquatic ecosystems. In this field we use both, the bottom-up (food availability for fish under different conditions) and top-down (fish as predators on lower trophic levels) approaches and consequently individual (food selectivity) as well as the community-based approaches (bioenergetic modelling, food rations) are applied. Using a mesocosm experiment, we proved an important role of temperature in the herbivorous foraging strategy of fish (**Vejříková et al. 2016, Sci. Rep. 6, 39600**). Temperatures below 15 °C, even for a short time period, inactivate cellulase and cause diet limitations for herbivorous fish. Our results point to a marked impact of climate change on the fish herbivory. Herbivorous and omnivorous fish can move poleward and significantly affect macrophyte communities that evolved in cold waters. The significant impact of herbivory and competition on the macrophyte community has been demonstrated by a unique underwater field experiment (**Vejříková et al. 2018, Sci. Rep. 8, 12130**). The effect of removal of dominant competitors is immediate but short-term, whereas the impact of herbivory has a gradual but long-lasting effect. Our findings are applicable for the revitalisation of aquatic ecosystems that aims to increase macrophyte biodiversity. We also discovered that the presence of submerged macrophytes has a strong effect on individual niche variation among generalist fish species (**Vejříková et al. 2017, PLOS One 12, e177114**).

### **Factors shaping zooplankton communities**

Multiple factors operate simultaneously and interact in complex ways to shape community assembly. Understanding the organisation of communities including species interactions and the importance of driving factors (food, temperature, nutrients, oxygen concentration, etc.) is a central topic in ecology. To investigate which factors operate in the community structure two investigations were undertaken. In the studies of **Gabaldón et al. (2019, Freshwater Biol. 64(7) 1326-1341)** and **Znachor et al.**

(2018, *Sci. Total Environ.* **624**, 24-33), a long-term time series of zooplankton abundances and environmental conditions in the Rímov Reservoir were used and the main focus was to investigate which physical, chemical, and biotic factors structure zooplankton communities. Our results provide evidence that the network of interacting zooplankton taxa is composed of positive and negative relationships, which suggests facilitative, competitive, and predator–prey interactions. Although zooplankton species interactions have an influence year-round, the network of interacting species is more complex during the vegetative period and especially in summer, when species richness and abundance are high. In the second investigation (Gabaldón et al. 2017, *J. Limnol.* **76**(2), 292-304), a zooplankton community in a small mountain pond was studied during the spring growing season. We explored several physical conditions, such as high inflows, and the biotic dynamics of the main zooplankton groups (i.e. rotifers, cladocerans and copepods). Our results showed that high flood events commonly favour rotifers over crustaceans, probably due to rotifer species having great colonization ability and faster growth. Additionally, we found surprisingly that a repeated disturbance caused by high flood events does increase the species diversity of rotifers. Finally, our study also detected some cues that may indicate that diapausing egg bank is also playing an important role in the zooplankton community, favouring the dominance of rotifers. In order to explain patterns in fish productivity, crustaceans were investigated in a study in which the productivity of carp was recorded in two farming ponds, one vegetated and the other with scarce coverage of aquatic plants (Gabaldón et al. 2018, *Aquaculture* **485**, 111-118). In the pond with aquatic vegetation fish productivity was higher, *Daphnia* species started to grow significantly earlier, had higher relative abundances, and delayed the population decline. The results of this study indicate that macrophytes are likely to operate as a protection mechanism, not only for individual *Daphnia*, but also protect the resting egg bank. Accordingly, we consider that increasing macrophyte coverage should be considered in the management strategies for carp pond farming since littoral plant vegetation may act as a refuge for cladocerans as well as a productive ecotone improving the productivity of fishponds. Because *Daphnia* species play a key role in zooplankton communities of temperate ecosystems, mechanisms of the phenotypic plasticity of morphological parameters of the filtering screens were studied in *Daphnia galeata* (Macháček et al. 2016, *J. Plankton Res.* **38**(5), 1269-1280).

### Assessment of ecological quality using fish communities

The assessment of ecological quality in freshwater ecosystems is a key issue in many countries. We developed a national methodology of assessment of ecological potential in lakes and reservoirs which was successfully intercalibrated with other EU member states in the Central Baltic region (Ritterbusch et al. 2016, **EU publication 28022 EN, ISBN 978-92-79-59936-1, DOI:10.2791/396601**). National methodology itself was published by Blabolil et al. (2017, *Knowl. Manag. Aquat. Ecosyst.* **418**, 53) covering major environmental and pressure gradients in reservoirs in the Czech Republic. Fish data obtained by gillnet sampling were correlated with a proxy of eutrophication as a key indicator of anthropogenic pressure for selecting appropriate fish-based indicators, establishing scoring criteria and developing the index of ecological quality. Using the hindcasting approach and fish data from 144 French and Czech reservoirs between 2005 and 2013, a novel methodology based on fish communities to assess the ecological potential of central European reservoirs was developed (Blabolil et al. 2016, *Fish. Res.* **173**, 80-87). Our index predicts values that could be observed in the absence of pressures for each reservoir depending on their environmental

characteristics. The index accurately identifies reservoirs that are lightly, moderately and heavily affected by eutrophication. Ecological quality assessment of non-natural water bodies is, in contrast to natural systems, less developed and requires determining biological indicators that reliably reflect environmental conditions and anthropogenic pressures. Therefore, the study of **Vašek et al. (2016, Ecol. Indic. 63, 219-230)** was motivated to propose fish indicators appropriate for assessment of reservoir ecosystems in central Europe. Changes in water quality, total biomass and the taxonomic, trophic and size composition of fish communities were analysed along the longitudinal axes of four elongated, deep-valley reservoirs. The results suggested that fish communities are appropriate indicators of eutrophication and can be used for ecological quality assessment of non-natural lentic water bodies, such as reservoirs. Because lakes in Europe are subject to multiple anthropogenic pressures, effective assessment methods are needed. The challenge is to ensure consistency in ecological assessments over time, across ecosystem types and across jurisdictional boundaries. That is why we contributed to the development of a total anthropogenic pressure intensity (TAPI) index as a weighted combination of the most common pressures in European lakes (**Poikane et al. 2017, Sci. Total Environ. 586, 502-511**). This index was validated against 10 national fish-based water quality assessment systems using data from 556 lakes. We argue that fish-based multiple-pressure assessment tools should be seen as complementary to single-pressure tools offering the major advantage of integrating direct and indirect effects of multiple pressures over large scales of space and time. Fish were also used as proxies of ecological and environmental changes. The Horizon 2020 project, CLIMEFISH, initiated wide efforts to assess the influence of thermal changes on fish growth (**Estlander et al. 2017, Ecol. Evol. 7(2), 665-673**) and species interactions (**Souza et al. 2018, Sci. Total Environ. 640, 1332-1345; Souza et al. 2019, PeerJ. 2019, 2019(10), e7845**). Allan Souza, one of the contributors of CLIMEFISH, contributed also to the work demonstrating the importance of bivalve molluscs for freshwater biota (**Ilarri et al. 2018, Sci. Total Environ. 634, 1398-1405; Ilarri et al. 2019, Sci. Total Environ. 2019, 683, 185-192**) and the interactions between fish and molluscs (**Modesto et al. 2018, Fish Fish. 19(2), 244-259**). We explored the potential of three European riverine fish species as biomonitors of water quality through the analysis of diatoms in their guts. Our results clearly demonstrate that diatom indices calculated from fish gut contents are related to anthropogenic nutrient pollution and thus they are useful for inferring past water quality changes or determining reference conditions for rivers within the context of the EU Water Frame Directive (**Bešta et al. 2015, Hydrobiologia 742, 233-248**).

### Biological invasions

Biological invasions are an important component of global change in aquatic ecosystems and are considered one of the major threats to worldwide diversity. Invasive species are a hot topic in modern ecology, especially because native species are usually significantly influenced by their spreading. The classical case of ichthyological invasion with consequences to native fauna was observed in three Biesbosch reservoirs in the Netherlands. In these systems small benthic gobiid species spread rapidly since 2012. Based on our observations we described the first European pelagic occurrence and diet of invasive round goby (*Neogobius melanostomus*) juveniles and we have also found the largest individuals of round goby ever found in the pelagic area (**Jůza et al. 2016, Hydrobiologia 768, 197-209**). The methodology of passive sampling of invasive gobiids was tested and reported in paper by **Jůza et al. (2018, Fish. Res. 207, 175-181)**. Small fyke nets and gillnets were found to be suitable

methods for round goby monitoring in structured and also deep benthic habitats, however a large discrepancy was found in size structure of captured fish between both methods. Differences in sex dependent locomotor activity were found in round goby, when males were overestimated in the catches by passive sampling techniques (**Žák et al. 2018, J. Fish Biol. 93, 147-152**). On the other hand, in active sampling techniques, where the efficiency is not so strongly dependent on fish activity, the sex-ratio was relatively well balanced. Negative influence of round goby invasion on native fish species was demonstrated in the study by **Jůza et al. (2018, Biol. Invasions 20, 1523-1535)**. The change of benthic fish communities was compared between periods before and after the round goby invasion. Owing to a similar benthic lifestyle and high niche overlap, ruffe was the main species negatively influenced by round goby invasion. The competitive superiority of round goby over ruffe was found to be so strong that the once dominant species of the overall benthic fish community collapsed after only a few years of coexistence. The biology and ecology of gobiids was extensively studied especially in North America, where spreading, especially of round goby became a serious problem in the Great Lakes area. Our studies confirmed some patterns observed in North America and provided many novel findings in gobiid ecology and biology, and highlighted potential risks for native ichthyofauna.

Further research related to invasion science was focused on traits and characteristics that influence invasiveness, population dynamics of species invasions, and the development of novel invasion assessment tools and new concepts. In a study of crypticity of invasive species and their ecological functions a conceptual framework of crypticity in biological invasions was developed (**Jarić et al. 2019, Trends Ecol. Evol. 34(4), 291-302**). This study identified the underlying mechanisms, addressed their importance, and the potential of the framework to improve the recognition of the full risks and impacts of invasive species. In another study, based on the analysis of detailed freshwater fish data, it was revealed that novel hotspots of established alien species have arisen worldwide (**Vitule et al. 2019, Biodivers. Conserv. 28(11), 3037-3043**). The study demonstrated that researchers should also consider intra-country introductions when estimating and addressing the risks of alien species introductions. Some studies were also focused on specific alien fish species, including the assessment of the distribution and status of Mississippi paddlefish in Europe (**Jarić et al. 2019, J. Appl. Ichthyol. 35(1), 267-274**), and the population dynamics and drivers of boom-bust population dynamics of black bullhead (**Jaćimović et al. 2019, Fish. Manage. Ecol. 26(2), 153-164**).

### **Studies of extinction risk and extinction threats**

With the aim to determine species traits that influence sensitivity to climate change, a total of 443 European freshwater fish species were analysed regarding their susceptibility to future climate change (**Jarić et al. 2019, Glob. Change Biol. 25, 448-458**). Results helped identify the list of particularly vulnerable fish species and indicated that species most susceptible to climate change tend to occur in the Mediterranean region, have a smaller body size and small distribution area. Sturgeons and paddlefish represent one of the most highly threatened species groups worldwide, in urgent need of conservation and research efforts. A global survey of scientists specializing in sturgeon and paddlefish research, aimed to assess the state of knowledge of sturgeon and paddlefish life history, management and conservation, identified a high level of uncertainty and gaps in knowledge, as well as regional distribution of threats and particular conservation issues (**Jarić et al. 2018, J. Appl. Ichthyol. 34, 244-257**). Charismatic species such as sturgeons often attract higher conservation attention and

support, but they are also often exposed to higher levels of anthropogenic threats, such as unsustainable harvesting. In a study conducted within an international research team, a list of the most charismatic animals was identified based on surveys, questionnaires and online analysis, and their conservation status and public attitude analysed (**Courchamp et al. 2018, PLOS Biol. 16(4), e2003997**). The study found that these animals' popularity may be their downfall, with their high public profile leading to biased public perception and inappropriate complacency about their chances of survival. This study also identified appropriate policy measures to mitigate effects of this phenomenon, including conservation marketing and financial measures. With rare and highly threatened species, the only available data on their status are often their sighting records. In collaboration with an Australian research team, a new method was developed that is able to infer extinction of a species based on sighting records that vary in their reliability (**Brook et al. 2019, Ecology 100(9), e02787**). The method has the potential to be widely used in conservation science and practice. Furthermore, through a collaboration with research groups from China and UK, an analysis of sighting records and large-scale survey throughout Yangtze River indicated that one of the most iconic fish from this basin, Chinese paddlefish (*Psephurus gladius*), can be confirmed as extinct (**Zhang et al. 2019, Sci. Tot. Environ. doi:10.1016/j.scitotenv.2019.136242**). The study also revealed that the species probably became extinct during 2003-2005, and that it experienced functional extinction by 1993, which ultimately represented the key extinction driver. One of the key tools to assist decision-making in conservation investment and research focus is the IUCN Red List of Threatened Species. Analysis of linkage between IUCN listing and research effort in Data Deficient (DD) and Critically Endangered (CR) species indicated an effect of listing of DD species on subsequent research intensity, while such effect was observed in only a minority of CR species groups (**Jarić et al. 2017, PeerJ 5, e4025**). The research team also addressed a dark side of the current enthusiasm for compiling large datasets in support of evidence-based conservation, and demonstrated that data gathered in biodiversity databases can be poorly informative for the design and implementation of effective conservation strategies and actions (**Jarić et al. 2019, Biol. Conserv. 237, 248-252**). The study identified key mechanisms that contribute to this problem, and called for a cultural shift among conservation practitioners, consultants and others, to embrace the idea that documenting and acknowledging knowledge gaps and uncertainties is just as important as compiling data and taking known information into account.

### Conservation science

Conservation culturomics represents an emerging research area in the field of conservation science. It focuses on the study of human interactions with nature based on the quantitative analysis of voluminous digital data. The scope of research within this area mainly includes development of new tools and concepts and their potential applications. One of the important questions that conservation culturomics addresses is the level of attention directed at different species, environmental impacts, concepts, ecosystem services and different elements of the environment by society and science. Public attention is particularly relevant within the field of conservation, as societal preferences strongly impact support for conservation initiatives and their success. A study was carried out on the association between societal and research interests in four charismatic and threatened species groups, derived from a range of different online sources and social media platforms as well as scientific publications. It found a high level of concordance between scientific and societal taxonomic attention (**Jarić et**



**al. 2019, Sci. Tot. Environ. 648, 772-778**). Results of the study indicated that research is apparently not as disconnected from the interests of society as it is often reproached. In a study by **Correia et al. (2019, Front. Ecol. Environ. 17(5), 254-255)**, trends in public interest in conservation topics such as climate change and biodiversity based on Internet search frequency indicated that the positive trends in attention claimed in previous studies may not hold up under scrutiny due to unsupported assumptions. The study also called for caution when interpreting public interest based on culturomic data. The analysis of webpage retrieval by using scientific names and synonyms revealed that species data retrieval is often limited when synonyms are not considered, and that culturomic approaches should always account for species' scientific name synonyms (**Correia et al. 2018, Ecol. Indic. 90, 74-78**). Furthermore, a study by **Lennox et al. (2019, Conserv. Biol. doi:10.1111/cobi.13404)**, demonstrated good potential for the use of sentiment analysis in conservation science. Based on the analysis of 15,001 abstracts from papers published in conservation-focused journals in the last 20 years, trends in the sentiment scores were identified, and recommendations provided for further use of this approach in conservation science. Members of the team were also involved in research of habitat use and distribution of protected endangered species. A paper by **Baran et al. (2015, J. Fish Biol. 86, 805-811)** was focused on habitat requirement of protected alpine bullhead (*Cottus poecilopus*) and a paper by **Halačka et al. (2017, Folia Zool. 66, 163-166)** on discovery of a new population of Balkan spiny loach (*Sabanejewia balcanica*) in Jihlava River. These papers contribute to improved protection of these species and improved management of their populations.

## Research activity and characterisation of the main scientific results

Our research activity during the period of 2015-2019 was greatly shaped by projects and their scientific objectives. In the period 2015-2019, our team of 8 researchers published over 70 peer-reviewed articles.

Several of our publications focus on viruses that infect their hosts as a mixture of sequence variants (Koloniuk et al., 2018, *Viruses*, 10(4), 204; Fránová et al, *Viruses*, 2019, 11(11), 982). It is under investigation whether that was a result of simultaneous infection by the mixture or rather than by individual variants were introduced into the host gradually.

Using high-throughput sequencing, we described co-infection of sweet and sour cherry trees with diverse genomic variants of two closely related viruses, namely prunus virus F (PrVF) and cherry virus F (CVF) (Koloniuk et al., 2018, *Viruses*, 10(4), 204). Both viruses are most homologous to members of the *Fabavirus* genus (*Secoviridae* family). The comparison of CVF and PrVF RNA2 genomic sequences suggested that the two viruses may significantly differ in their expression strategy. Indeed, similar to comoviruses, the smaller genomic segment of PrVF, RNA2, may be translated in two collinear proteins while CVF likely expresses only the shorter of these two proteins. Linked with the observation that identity levels between the coat proteins of these two viruses were significantly below the family species demarcation cut-off, these findings supported the idea that CVF and PrVF represent two separate *Fabavirus* species. Further studies should reveal their natural vectors and their impact on plant health.

Strawberry crinkle disease is one of the major diseases that threaten strawberry production. Although the biological properties of the agent, strawberry crinkle virus, have been thoroughly investigated, its complete genome sequence has never been published. We have published the complete sequences of two divergent isolates of strawberry crinkle virus coinfecting a single strawberry plant (Koloniuk et al., 2018, *Arch Virol*, 163(9), 2539–2542). The strawberry is a difficult type of sample in terms of the lab work and often low virus concentrations. Besides, we had to deal with rather low titres of rhabdoviruses, their nucleotide similarity, and a ten-fold lower concentration of one of the isolates. That work has led us to the discovery of another rhabdovirus species infecting strawberry – strawberry virus 1 (StrV-1). Genomic sequences of three distinct StrV-1 genotypes co-infecting a single *F. ananassa* host were obtained using combined Illumina and Ion Proton high-throughput sequencing. StrV-1 was transmitted to herbaceous plants via *Aphis fabae* and *A. ruborum*, further mechanically transmitted to *Nicotiana occidentalis* 37B and sub-inoculated to *N. benthamiana*, *N. benthamiana* DCL2/4i, *N. occidentalis* 37B and *Physalis floridana* plants (Fránová et al, *Viruses*, 2019, 11(11), 982). One of significant achievements was the successful virus transmission by aphid vectors in cooperation with the Entomology Institute of BC CAS. Since then we are keeping cultures of several aphid species naturally hosted on strawberry plants. Viral vectors are extensively used in two current research projects.

During an explanation of the cause of stunting and death of clover plants, two novel rhabdoviruses were identified and characterized, including their biological and molecular properties (Fránová et al., 2019, *Plant Pathology*, 68(7), 1276-1286). Developed generic cytorhabdovirus PCR test might assist plant pathologists during testing of infected samples with not-yet-described cytorhabdoviruses.

Black and red currants (*Ribes* spp.) are widely grown berry crops. Notwithstanding the progress in currant virology, there are gaps in knowledge, one of which was addressed through the characterization of a new closterovirus complex, affecting both black and red currants. Besse et al. (2010, *Julius-Kühn-Archiv*, 214–219) observed similar particles in currants however, only a small genomic region around 300 bp was known for the virus. We studied in depth, a closterovirus species, tentatively named 'blackcurrant leafroll associated virus 1' (BcLRaV-1), identified in black and red currants including its particle morphology, genome organization and the evolutionary forces acting on the virus. The work was published in collaboration with five other laboratories (Koloniuk et al., 2018, *Viruses*, 10(7), 369). Further research activities are continued with collaborators include studies of the BcLRaV-1 biological and transmission properties.

Research on fungal and lichen viruses brought us several interesting results. In 2015, we presented the first proof as to the natural presence of CaMV in algae and the first demonstration of algae being artificially infected with this virus (Petrzik et al., 2015, *PLoS ONE* 10(3): e0120768). *Trebouxia aggregata* (Archibald) Gärtner, a lichen symbiotic alga, has been identified as a host of the well-known herbaceous plant virus *Cauliflower mosaic virus* (CaMV, family *Caulimoviridae*). The alga had been isolated from *Xanthoria parietina* more than 70 years ago and has been maintained in a collection since that time. The virus from *T. aggregata* was mechanically transmissible into a herbaceous host and induced disease symptoms there. Its genome differed by 173 nt from the closest European CaMV-D/H cauliflower isolate. No site under positive selection was found on the CaMV genome from *T. aggregata*. We, therefore, assume that the virus's presence in this alga was not sufficiently long to fix any specific changes in its genome. Apart from this symbiotic alga, CaMV capsid protein sequences were amplified from many other non-symbiotic algae species maintained in a collection (e.g., *Oonephris obesa*, *Elliptochloris* sp., *Microthamnion kuetingianum*, *Chlorella vulgaris*, *Pseudococcomyxa* sp.). CaMV-free *Chlorella vulgaris* was treated with CaMV to establish the virus infection. The virus was still detected there following five passages. The virus infection is morphologically symptomless on *Chlorella* algae and the photosynthesis activity is slightly decreased in comparison to CaMV-free alga culture.

Exploration of lichen virome led us to the discovery of two novel chrysovirus in an endolichenic fungus (Petrzik et al., 2019, *Viruses*, 11(12), 1120). We have proved firstly that not the matrix fungus but an endolichenic fungus was the main host and replication site of this virus. These are the first descriptions of mycoviruses from a lichen environment. This result was brought from cooperative work with the histology group (fluorescent microscopy) of the Entomology Institute of BC CAS.

Collaborative research on mycoviruses with the Laboratory of genetics, Wageningen University, resulted in the discovery of several different viruses infecting the obligate alkalophilic fungus *Sodiomyces alkalinus* (Hrabáková et al., 2017, *PLoS One*, 12(11), e0187799–20). The fungus, a soda-lake species, with an ability to grow optimally at very high ambient pH. All discovered viruses showed efficient vertical transmission through asexual and sexual spores. In addition, they did not evoke apparent phenotypic host alteration. This is the first description of a viral infection in an alkalophilic fungus.

A novel dsRNA virus was identified in the mycorrhizal fungus *Thelephora terrestris* (Ehrh.) (Petrzik et al., 2016, *Virology*, 489, 12–19). This virus, named *Thelephora*

terrestris virus 1 (TtV1), had a distinct genome organization and was suggested to classify as a member of a newly created genus “Phlegivirus”. Twenty species of oribatid mites were identified in soil material in the vicinity of *T. terrestris*. TtV1 was detected in large amounts in *Steganacarus* (*Tropacarus*) *carinatus* (C.L. Koch, 1841) and in much smaller amounts in *Nothrus silvestris* (Nicolet). This is the first description of mycovirus presence in oribatid mites.

Several articles published in collaboration were of great taxonomic importance in a field of mycology. Two large polypore genera *Phellinus* and *Inonotus* were distinguished for more than 100 years. In 2002, molecular taxonomy showed that some of the typical *Phellinus* cluster with *Inonotus*, despite their phellinus’s morphology. The pieces of evidence of two new independent lineages were provided that deserve new generic names (Zhou et al., 2016, Fungal Diversity, 77(1), 335-347). In another article (Han et al., 2016, Fungal Diversity, 80(1), 343-373), molecular phylogeny showed that morphologically different polypores formed one distinct “antrodia” clade. The inner structure of the clade is enigmatic; quite dissimilar genera often show so similar sequences that it relativizes accepted genus concepts. We were the first who tried to solve the problem on a broad, world-wide base. Accepted was the “splitters concept”: to confirm existing genera we validated them with multigene approach.

Our research on phytoplasmas was submitted as four publications. Obtained results provide valuable tools to understand the epidemiology of these economically important pathogens.

A quarantine organism, *Candidatus* Phytoplasma mali, is the causal agent of apple proliferation, one of the most important apple diseases in Europe. The genetic diversity of this pathogen in Central and Southern Europe has already been reported; however, almost no data exist from Eastern Europe. In our study (Fránová et al., 2019, Folia Microbiologica, 64, 373-82), *Ca. P. mali* strains identified in 14 apple trees from the Bulgarian germplasm collection were characterized by restriction fragment length polymorphism (RFLP) and sequence analysis of four genomic loci. Unexpectedly, when using nitroreductase and rhodanese- like gene sequences, the Bulgarian strains clustered separately from the other European strains. Apart from the identification of different *Ca. P. mali* strains, the paper also recommends the unification of the rpX-subgroup nomenclature to avoid future confusion.

In collaboration with Portuguese and Italian colleagues, phytoplasma affecting cultivated blackberry (*Rubus* L. subgenus *Rubus* Watson) in the Iberian Peninsula was identified for the first time. Portuguese phytoplasma strain (named black Port phytoplasma) was characterized at the molecular level as a distinct new taxon. The 16S rRNA gene sequence revealed that it is related to ‘*Candidatus* Phytoplasma rubi’ (16SrV-E ribosomal subgroup) and RFLP analysis revealed a unique profile following *Mse*I endonuclease digestion of R16F2n/R2 amplicons that distinguished it from strains belonging to previously established 16SrV phytoplasma subgroups. Phylogeny of the 16S rRNA sequences, multigene sequence analyses of 16S-23S, tuf, rplV-rpsC, rplF-rplR, rplO-SecY-map and uvrB-degV loci as well as variability of unique oligonucleotide sequences defined for ‘*Ca. P. rubi*’, confirmed the uniqueness of this Portuguese phytoplasma strain. Given that the Primocane Fruiting Blackberry genotype was produced at the University of Arkansas, USA, and the plant material was then multiplied in the UK and Portugal, the finding of a new phytoplasma strain deserves further studies on phytoplasma dissemination and its economic impact on blackberry production (Fránová et al., 2016, IJSEM, 66, 1438-1446). A survey for

phytoplasma diseases was conducted in a sweet and sour cherry germplasm collection and in cherry orchards within the Czech Republic during 2014–2015 (Fránová et al., 2018, Journal of Phytopathology, 166(1), 59–66). Phytoplasmas were detected in 21 symptomatic trees. Multiple infections of cherry trees by diverse phytoplasmas of 16Srl and 16SrX groups and 16Srl-A, 16Srl-B, 16Srl-L, 16SrX-A subgroups were detected by RFLP. Nevertheless, phylogenetic analysis placed subgroups 16Srl-B and 16Srl-L inseparable together onto one branch of phylogenetic tree. This is the first report of subgroups 16Srl-A and 16Srl-L in *Prunus* spp., and subgroup 16SrX-A in sour cherry trees. Additionally, novel RFLP profiles for 16Srl-A and 16Srl-B-related phytoplasmas were found in cherry samples. Phytoplasmas with these novel profiles belong, however, to their respective 16Srl-A or 16Srl-B phylogenetic clades.

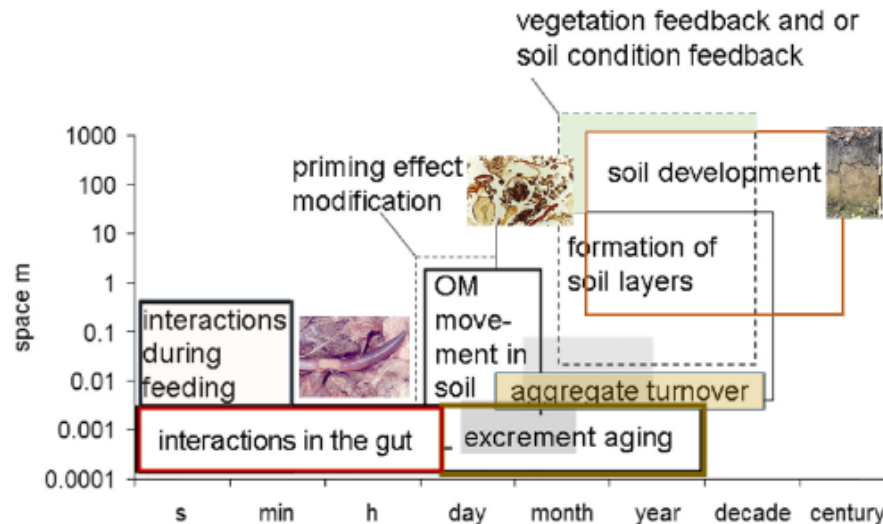
*Primula acaulis* (L.) Hill. plants showing stunting, leaf-yellowing, and virescence were first discovered in the Czech Republic. RFLP analyses and sequencing enabled classification of the detected phytoplasmas into the aster yellows group, ribosomal subgroup 16Srl-B, tufl-B, rpl-B, groE- LIB-III and SecY-IB subgroups (Fránová et al., 2016, J Phytopathol, 164(3), 166–176). Phylogeny of the 16S rRNA gene sequences as well as sequence analysis of several chromosomal regions, such as the 16S-23S ribosomal operon, ribosomal proteins, spc ribosomal protein operon, genes for elongation factor EF-Tu, molecular chaperonin large subunit GroEL, immunodominant membrane protein, ribosome recycling factor, uridylyl transferase, ATP- and Zn<sup>2+</sup>-dependent proteases not only confirmed its affiliation with the '*Candidatus* Phytoplasma asteris' species but also enabled its detailed molecular characterization. The less researched regions of phytoplasma genome (amp, adk, hflB, pyrH-frr genes) could be valuable as additional markers for phytoplasma through differentiation especially within the 16Srl-B ribosomal subgroup.

During 2015-2019, we have published nine reports in which we extended known diversity of novel plant viruses in different plants species (Koloniuk et al., 2018, Arch Virol, 163(12), 3447–3449; Koloniuk et al., 2018, Arch Virol, 163(8), 2213–2218; Koloniuk et al., 2015, Arch Virol, 160(6), 1585–1588; Koloniuk et al., 2018, Arch Virol, 163(5), 1387–1389; Lenz et al., 2018, Arch Virol, 163(12), 3439–3442; Petrzik et al., 2015, Arch Virol, 161(2), 491–493; Petrzik et al., 2016, Arch Virol, 161(2), 491–493; Petrzik et al., 2016, Arch Virol, 161(5), 1405–1409; Petrzik et al., 2016, Arch Virol, 161(4), 1083–1086). Those publications aim to fill in gaps in virus taxonomy and provide a better understanding of the direction of virus evolution in nature.



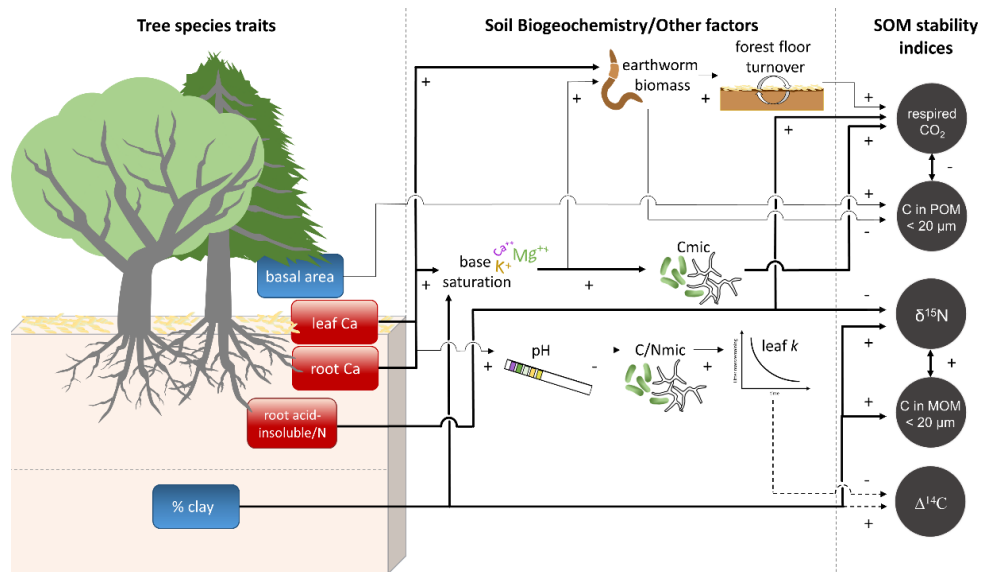
## Research activity and characterisation of the main scientific results

As already mentioned, the central question our team examines is the role of soil biodiversity in biogeochemical cycles. In particular, we focus on the C cycle. This is not only because soil C, specifically soil organic matter (SOM), is one of the most principal pools of C on the Earth, containing three times more C than the atmosphere, which as such plays a key role in the global C cycle and has tremendous potential in global change mitigation, but also because the C cycle in soil drives all the other biogeochemical processes and affects virtually all soil properties. Soil biota plays a central role in litter decomposition and SOM transformation. In natural ecosystems, decomposer food webs process almost all organic matter produced during photosynthesis. Decomposition is affected by many abiotic and biotic drivers (Angst et al. 2019. *Biology and Fertility of Soils* 55: 525-538). One of the key questions of recent ecosystem research is how the ecosystem processes correspond with community functioning. Studies that compare the microbial community composition and process rate are still not numerous enough to allow wider generalization and the message may not be very clear yet. For example, our study of *Salix* twigs decomposition showed that twigs of various diameter harbour very different communities, but have very similar decomposition rates (Angst et al. 2018. *FEMS Microbiology Ecology* 94: fiy126). Besides microbial processes, interactions between microbes and other soil organisms play important roles in SOM transformation. Plants play an important role, by providing organic matter of different quality which in turn shapes microbial community dynamics and activity as has been shown by Jílková et al. (Jílková et al. 2018. *Soil Biology and Biochemistry* 117: 1-4; Jílková et al. 2019. *Soil Biology and Biochemistry* 138: 107595) and Mastný et al. (Mastný et al. 2018. *Soil Biology and Biochemistry* 121: 221-230) in temperate coniferous forest soils, and Józefowska et al. (Józefowska et al. 2017. *Geoderma* 292: 9-16) in various forest plantations. The effect of trees on the overall C storage in soil is largely driven by the leaf traits of the trees. On the other hand, the stability of SOM is largely dependent on the root traits of the trees (Angst et al. 2019. *Global Change Biology* 25: 1529-1546). Interestingly, the latter study shows that the rate of C storage may not correspond with C stability. The study of Józefowska et al. (Józefowska et al. 2017. *Geoderma* 292: 9-16) also shows that the effect of plants can be to a large extent indirect and modified by interactions with soil fauna.



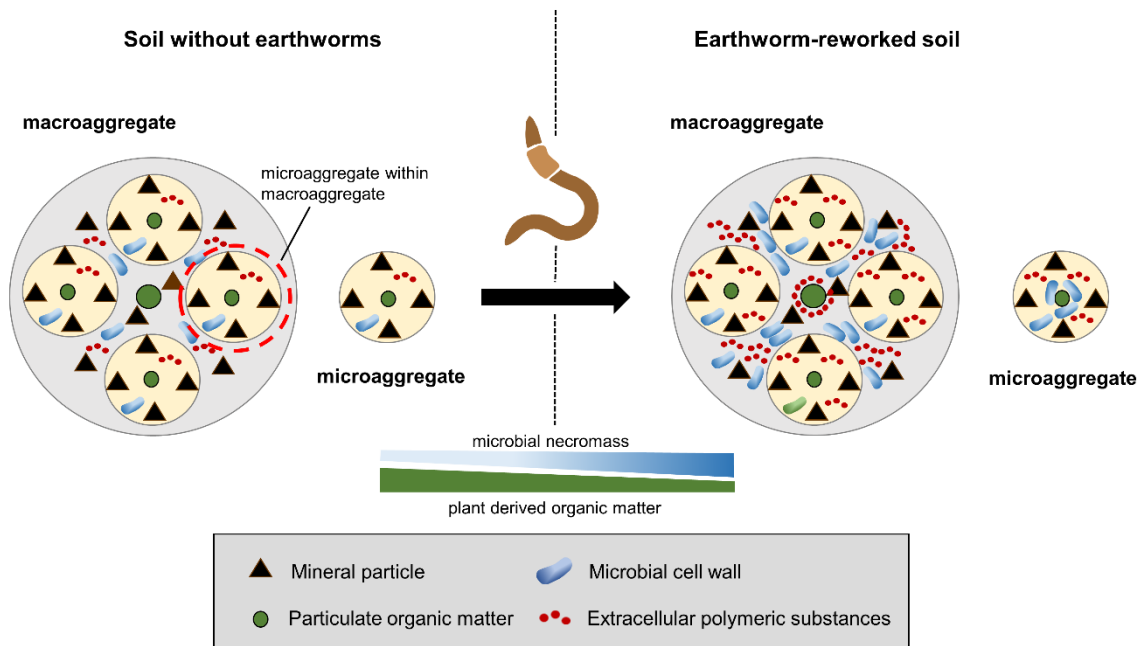
**Fig. 1.** Spatiotemporal interactions of key processes mediated by interactions between soil fauna, microflora and soil environment (based on Frouz 2018. *Geoderma* 332: 161-172).

Our group traditionally pays great attention to invertebrate-microbial interactions and their role in SOM transformation. Frouz (Frouz 2018. *Geoderma* 332: 161-172) brings a conceptual synthesis on the role of soil macro- and mesofauna on litter decomposition and SOM stabilization (Fig. 1). Soil fauna can consume a substantial amount of the annual litterfall. This fauna can cause both an increase in the mineralization of SOM and the release of C as CO<sub>2</sub> into the atmosphere (typically in the early stages of decomposition) and at the same time can support the stabilization of C in the soil which reduces decomposition and promotes C storage in the soil (Frouz et al. 2015. *European Journal of Soil Biology* 8: 18-24). Although mechanisms by which fauna enhances microbial decomposition have already been well understood, the stabilization of SOM in soil and slow down of mineralization by soil fauna is less understood. Frouz et al. (Frouz et al. 2015. *Soil Biology and Biochemistry* 91: 23-31) used artificial litter and fauna manipulation to identify the major principles responsible for major mechanisms contributing to the macrofauna-mediated slowdown of litter decomposition, related to the production of microbial necromass and various polycondensation reactions. In addition to these mechanisms, some soil fauna (such as earthworms) mix organic matter with soil matrix which represents another important mechanism of soil C stabilization, namely the binding of organic matter on mineral particles (Angst et al. 2017. *Geoderma* 289: 29-35; Jílková et al. 2019. *Soil Biology and Biochemistry* 138: 107595; Angst et al., 2019 *Global Change Biology* 25(4), 1529-1546) (Fig. 2). This is particularly important in earthworms where the stabilization of SOM is connected to physical protection rather than to the chemical changes of organic matter. Angst et al. (Angst et al. 2019. *Communications Biology* 2: 441) summarized the role of earthworms as being biochemical reactors that convert labile plant compounds into stabilized soil microbial necromass (Fig. 3). An important step in this process is embedding the organic matter in the clay matrix inside soil aggregates which allows the formation of microbial biomass, and consequently necromass to attach to clay minerals. Beside microbial necromass, aggregation controls the stability of other compounds such as lignin and lipids in clay-sized particulate and mineral-associated SOM (Angst et al. 2017. *Biogeochemistry* 132: 307-324).



**Fig. 2.** Interactions of major drivers responsible for stability of SOM (based on Angst et al. 2019. *Global Change Biology* 25: 1529-1546).

These studies show the central role of soil aggregates in SOM stabilization which represents a significant contribution to an ongoing shift in the paradigm of SOM stabilization in the current scientific literature. At the same time, practical outcomes of these studies have led to the conclusion that any disturbance of soil structure leads to destabilization and the loss of SOM. The results are crucial for the inclusion of soil fauna in the recent C models, which is one of the hot topics of soil and ecosystem ecology. We are also working on the upscaling of these mechanisms to the field level. Leaf traits are very good predictors of the whole site rate of C storage in young soils, but their effect to a large extent interferes with substrate quality (Józefowska et al. 2017. *Geoderma* 292: 9-16). In mature soils, we found that soil organic C stocks in topsoil and subsoil were controlled by the parent material, C input in the rhizosphere, and microbial-derived compounds. More specifically, microbial-derived compounds, root necromass, parent material, and particulate organic matter derived from roots explained more than 90% of the variation in soil organic C stocks, independent of soil depth (Angst et al. 2018. *Soil Biology and Biochemistry* 122: 19-30). We elaborated on this difference between initial and mature soils even further. It has been shown that young soils developing *de novo* in disturbed areas such as post-mining heaps have a higher C sequestration rate in order of magnitude. We have even elucidated the mechanisms responsible for this phenomenon (Frouz 2017. *Land Degradation and Development* 28: 664-672). These results have important implications for C-related soil management strategies, in that any strategy seeking to increase soil C stocks would need to manipulate the above-mentioned factors.



**Fig. 3.** Conceptualization of the earthworm effect on SOM stabilization (Angst et al. 2019. *Communications Biology* 2: 441).

Another important aspect of the C cycle, beside C storage in the soil, is its release into the atmosphere (Fig. 4). This can be done through respiration in the form of CO<sub>2</sub> and other compounds, among them methane (CH<sub>4</sub>) being the most important. Production of these gases has a direct impact on the greenhouse effect and global warming. Our results show that even the release of CO<sub>2</sub> from soil may be a complex process affected by environmental factors (Jílková et al. 2015. *Soil Biology and Biochemistry* 86: 50-57; Jílková et al. 2016. *FEMS Microbiology Ecology* 92: fiw141) as well as by many interactions with soil matrix that may potentially feedback to soil respiration and CO<sub>2</sub> production (Frouz and Bujalský 2018. *Scientific Reports* 8: 10099). However, an even more complicated situation is in CH<sub>4</sub> which after production can be oxidized by CH<sub>4</sub>-oxidizing bacteria. Aerobic CH<sub>4</sub> oxidation affected by many environmental factors (Jílková et al. 2016. *FEMS Microbiology Ecology* 92: fiw141) has been believed to be a crucial process of CH<sub>4</sub> degradation, thus decreasing CH<sub>4</sub> fluxes from soils. In our research we have, however, confirmed that anaerobic oxidation of CH<sub>4</sub> might be of equal importance to CH<sub>4</sub> consumption in soils and sediments, which can substantially

improve C-cycling models (Gauthier et al. 2015. *Soil Biology and Biochemistry* 80: 167-174).



**Fig. 4.** Soil respiration measurement in the field by Veronika Jílková (Otto Wichterle award winner).

Understanding drivers of soil biota diversity is another important topic of our research. As mentioned in the previous section, soil biota plays a crucial role in many ecosystem processes, which is why we have paid great attention to the factors that determine soil biota community composition in various environments from nutrient hot spots, across agricultural fields and pristine national reserves in the Czech Republic, to ice-free parts of Antarctica (Jílková et al. 2015. *Geoderma* 259-260: 56-61; Jílková et al. 2019. *Geoderma* 334: 55-62; Tsiafouli et al. 2015. *Global Change Biology* 21: 973-985; Chroňáková et al. 2019. *FEMS Microbiology Ecology* 95: fiz130; Smykla et al. 2018. *Soil Biology and Biochemistry* 116: 265-276). We also pay great attention to the exploration of the general theoretical principles of community assembly such as the role of deterministic and stochastic processes in community assembly (Lin et al. 2017. *Water Research* 123: 134-143) and the role of traits in a species position in the community. Because of the central role of interactions between vegetation and soil biota in biogeochemical cycles, we have also focussed on the role of vegetation in the establishment of soil biota community (Chroňáková et al. 2019. *FEMS Microbiology Ecology* 95: fiz130). A side effect of our community studies has been progress in the exploration of soil biodiversity. During our community research, we have described several new species and clarified taxonomic issues (e.g., Bohunická et al. 2015. *Phytotaxa* 197: 84-103). Although this is not the main field of our research, this activity is highly needed for all the other fields of soil biology because soil biodiversity in contrast to the aboveground biota is substantially underexplored.

Particular attention has been paid to the role of environmental drivers that are related to ongoing climate change and human activity-induced changes in land use. An



example of such factors could be fire, as the occurrence of fire has increased worldwide as a consequence of climate stochasticity associated with global change. Fire is a frequent and pervasive disturbance that affects forest ecosystems, altering the main soil properties as well as biological communities. Understanding the effects of fire on the soil microbiome is important to be able to predict the consequences in the ecosystem functions they deliver. We have investigated post-fire temporal variations in microbial-mediated soil ecosystem functions and have shown that fire modulates ecosystems functioning through the phylogenetic structure of soil bacterial communities (Pérez Valera et al. 2019. *Soil Biology and Biochemistry* 129: 80-89). This provides predictions that might be important in the current context of increasing fire frequency and intensity worldwide.

An important issue of recent biology is invasive species, which is also a hot issue for us (Patoka et al. 2016. *Biological Invasions* 18: 457-461). Many invasive plants can substantially affect soil and soil biota communities. For example, in one of our studies (Štrobl et al. 2019. *Diversity and Distributions* 25: 1800-1813) we showed by using a multi-taxonomic approach and various sampling methods, that native woodlots and those of invasive *Robinia pseudoacacia* host different assemblages of soil biota. The parallel presence of both types of woodlots supports high arthropod diversity in otherwise simplified agricultural landscapes by creating a more complex mosaic of habitats.

An important human activity extensively affecting soil is agriculture which has been a subject of our extensive research for a long time. The effect of agriculture is highly contextual. For example, aboriginal farming by native people in the rainforests of Papua New Guinea is very sustainable and has little effect on the local ecosystem (Kukla et al. 2019. *Land Degradation and Development* 30: 166-177). On the other hand, our study on a European gradient showed that agriculture intensification reduces soil biodiversity which may hamper the provisioning of ecosystem services (Tsiafouli et al. 2015. *Global Change Biology* 21: 973-985).

An important part of our research is related to the effect of anthropogenic pressure on soil biota and the role of soil biota in ecosystem recovery. These aspects have a strong overlap with the exploration of human impact on the community of soil biota which has already been described above in reference to agriculture (Tsiafouli et al. 2015. *Global Change Biology* 21: 973-985; Kukla et al. 2019. *Land Degradation and Development* 30: 166-177) and other anthropogenic impacts such as soil industrial pollution (Jílková et al. 2017. *Journal of Soils and Sediments* 17: 2127-2135). These studies can bring sensitive indicators of the impacts of human activity on the ecosystem properties and functions. Beside community changes, we may also see other important indicators of human pressure on the soil such as antibiotic resistance of soil bacteria which is a subject of our intensive research. Tetracycline antibiotics represent the main load of antimicrobial agents contaminating soil via organic fertilizers in the intensive livestock system. We have shown that tetracycline resistance genes persist in soil amended with cattle faeces independently from chlortetracycline selection pressure (Kyselková et al. 2015. *Soil Biology and Biochemistry* 81: 259-265). On the other hand, native soil microorganisms hinder soil enrichment with antibiotic resistance genes following manure applications (Pérez Valera et al. 2019. *Scientific Reports* 9: 6760).

We have also paid great attention to the exploration of the role of soil biota in ecosystem recovery, and in the development of various reclamation and decontamination measures. Our studies vary from the exploration of the soil biota role

in soil formation under various tree species plantations as described above (Józefowska et al. 2017. *Geoderma* 292: 9-16) to modification of reclamation technologies such as site grading which shows that rough wave-like heaped overburden promotes the establishment of woody vegetation, while levelling promotes grasses during unassisted post-mining site development (Frouz et al. 2018. *Journal of Environmental Management* 205: 50-58). All these findings may lead to an improvement of reclamation technologies used in the reclamation of post-mining land and similar areas.

Finally, we are also working on the development of cutting-edge decontamination and analytical technologies with the use of nanotechnologies and composite materials. Special attention has been paid to the development of magnetically responsive materials such as adsorbents for the removal of important organic and inorganic pollutants and radionuclides (e.g., Angelova et al. 2016. *Journal of Cleaner Production* 137: 189-194; Mullerova et al. 2019. *Materials Chemistry and Physics* 225: 174-180) or silver nanoparticles (Janáček et al. 2018. *Applied Clay Science* 162: 10-14). Alternatively, magnetic materials can be used as carriers for the immobilization of biologically active compounds and cells (Baldíková et al. 2017. *Materials Science and Engineering C-Materials for Biological Applications* 1: 214-221; Baldíková et al. 2017. *Journal of Magnetism and Magnetic Materials* 427: 29-33) or isolation of biologically active compounds (Placková et al. 2017. *Plant Journal* 89: 1065-1075). A new analytical procedure “Magnetic textile solid phase extraction” has been developed for target analyte preconcentration from diluted solutions (Šafařík et al. 2018. *Journal of Industrial Textiles* 48: 761-771; Šafařík et al. 2019. *Food Chemistry* 274: 215-219).

We have kept several culture collections of microorganisms to collect, store, protect and provide a diversity of microorganisms and to conduct applied-oriented research. Research is oriented to natural products discovery and the identification of novel bioactive compounds from actinomycetes using genomic and bioinformatic tools (in-house) combined with comparative metabolomics (outsourced). We previously described a new compound Colabomycin E, showing weak antibiotic, but strong anti-inflammatory activities (Petříčková et al. 2014. *ChemBioChem* 15: 1334-1345). Based on this knowledge about the biosynthesis of similar polyketides of the manumycin family, we have proposed and applied the screening procedure targeting gene coding for unique enzymes (Petříčková et al. 2015. *Frontiers in Microbiology* 6: 814). The compound was patented in the USA (Petříček et al. 2016. U.S. Patent No. 9,447,062. Washington, DC).

## Research activity and characterization of the main scientific results

### ***Sequence composition and evolutionary dynamics of repetitive DNA in plant genomes***

Taking advantage of the decreasing costs of Illumina sequencing and our previous experience with using short-read data for global repeat characterization in individual species, we embarked on more ambitious projects to elucidate patterns of repetitive DNA evolution in various plant taxa. Since one of the most intriguing questions in plant genomics was whether and how repetitive elements drive genome size evolution, we focused on groups of closely related species with well-resolved phylogenies that differ substantially in their nuclear DNA content. Our strategy employed the RepeatExplorer pipeline for repeat quantification directly from unassembled low-pass genomic Illumina reads. As such, it did not require genome assemblies or species-specific reference databases. Therefore, it could be applied to any non-model species selected for some interesting biological features. Our pilot study included 23 species from the legume tribe Fabeae (including the genera of *Pisum*, *Lathyrus*, *Vicia*, and *Lens*) possessing medium-sized genomes that vary 7.6-fold in size. The study revealed that although the Fabeae genomes are rich in diverse groups of repeats, most of the genome size variation between these species was driven by a differential accumulation of a single lineage of LTR-retrotransposon (Macas et al., 2015). Besides its specific findings, this study introduced several methodological concepts for comparative analysis of repeat composition between relatively large numbers of species that were later adopted by other research groups for their investigations of plant and animal genomes.

In collaborative studies with I. Leitch (Kew gardens, UK) and I. Schubert (IPK Gatersleben, Germany) we then focused on investigating species that represent extremes of the 2400-fold genome size variation observed in plants: the giant genomes of *Fritillaria* lillies and miniature genomes of carnivorous *Genlisea* species. In contrast to the prominent role of the single retrotransposon lineage in the medium-sized genomes of Fabeae, the extremely large genomes of *Fritillaria* species were found to be made of highly heterogeneous, relatively low-abundance repeat-derived DNA. This suggests a scenario where amplified repeats continually accumulate due to infrequent DNA removal (Kelly et al., 2015). In *Genlisea*, some species possess extremely reduced genomes (e.g., *G. nigrocaulis*), while others have almost 20-fold larger genomes due to the accumulation of repetitive DNA. Nevertheless, even the miniature genome of *G. nigrocaulis* still contains ~16% of repetitive DNA, including a potentially active lineage of mobile elements that is absent in its related species. These findings demonstrated that repetitive DNA persists even in the smallest plant genomes characterized by rapid turnover and frequent elimination of genomic DNA (Vu et al., 2015). The rapid genome evolution in this genus was also evident from centromere and telomere sequence alterations (Tran et al., 2015). Taken together, these results suggest that differently-sized plant genomes differ in their patterns of repetitive DNA turnover, with the largest ones entering one-way path to “genome obesity” by losing their ability for repeat removal. A follow-up study is underway to test this hypothesis on a much broader set of species, including all known groups of plants with extremely large genomes.

An interesting by-product of the comparative studies of repeats in different taxa emerged from the frequent observations of repeat profiles that reflect phylogenetic

relationships of the species. In a collaborative study, Dodsworth et al. (2015) demonstrated that the output of the RepeatExplorer pipeline that was run in the comparative mode could be used to infer phylogenetic trees. This approach is unique in utilizing information from the whole repetitive portion of the genome that is usually ignored in phylogenetic studies and may be informative even in taxa where there is little genetic differentiation in standard phylogenetic markers (Dodsworth et al., 2015).

### ***Development of bioinformatics tools for analyzing repetitive DNA in eukaryotic genomes***

We continued in improving the RepeatExplorer pipeline by optimizing its code for faster execution, ability to process larger datasets, and better visualization of the results. We also developed a module for automated annotation of the identified repeats that substantially reduces user effort previously needed for evaluating the pipeline output. The module relies on REXdb, a new database of conserved protein domains encoded by mobile elements that was assembled in frame of our study of phylogenetic relationships of plant LTR-retrotransposons (Neumann et al., 2019). This study examined thousands of elements retrieved from 80 species representing major groups of green plants (Viridiplantae) and led to the establishment of improved, lineage-centered classification system reflecting phylogenetic relationships as well as structural features of the elements. The high citation rate of the paper (>30 citations within the first year after its publication) suggests that this classification system is being quickly adopted by the plant genomics community.

We introduced a new pipeline, TAREAN, specifically designed for unsupervised identification of satellite repeats in unassembled short reads (Novak et al., 2017). TAREAN shares the repeat identification algorithm (graph-based clustering) with RepeatExplorer, but employs a newly developed algorithm for examining cluster graph shapes to identify circular graphs typical for tandemly organized repeats such as satellite DNA. This approach is unique in using relatively small numbers of unassembled Illumina reads for identification of satellites with monomers that could be much longer than the read lengths.

A substantial part of our bioinformatics activities was devoted to making our tools available to the scientific community by deploying them on an easy-to-use Galaxy platform and running a public front-end server connected to an HPC computer cluster (<https://repeatexplorer-elixir.cerit-sc.cz/>). These computing resources are managed by the ELIXIR-CZ consortium and are currently used by over a thousand registered users from research institutions worldwide, providing about 30,000 CPU-days/year of computational power. To further support the users of our tools, we organize annual workshops at our institute, including practical training and help with solving specific problems with the user-provided data. Thanks to these efforts, our tools are being extensively used by many non-bioinformaticians to, for example, employ cytogenetic techniques that utilize identified repeats as FISH landmarks for discrimination and mapping of chromosomes. RepeatExplorer and TAREAN-identified satellite repeats are also increasingly used for diagnostics purposes as highly-sensitive and specific molecular markers for various parasitic helminths (Grant et al., 2019).

### ***Satellite DNA investigated using novel computational tools and ultra-long sequence reads***

The efficiency of our tools for the identification and characterization of satellite repeats was demonstrated in *Vicia faba*, a long-established model for cytogenetic studies in plants. Despite the previous extensive investigations of this species, there were only three satellite repeats identified previously. However, our analysis combining the RepeatExplorer and TAREAN pipelines resulted in the characterization of over twenty novel satellites. This study also revealed unexpected diversity of *V. faba* centromeric satellites, contrasting replication profiles of the identified repeats and a high proportion of satellites with extremely long monomers (Avila Robledillo et al., 2018). A similar approach proved to be efficient in the crucifer *Ballantinia antipoda*, revealing a satellite repeat with an atypical dispersed distribution on the chromosomes (Finke et al. 2019), and in *Aegilops speltoides* and *Plantago lagopus* where it facilitated the development of FISH markers for investigation of B-chromosomes (Wu et al., 2019; Kumke et al., 2016). Considering these results together with reports from other research groups utilizing the same tools in both plant and animal genomes, it appears that their findings will challenge some of the established characteristics of satellite repeats, including their strict preference for short monomers and the presence of very few satellite repeat families in the genome. It is likely that these characteristics might be biased by the experimental methods that were previously used for satellite DNA identification (e.g., the cloning of ladder-like bands from restriction digests of genomic DNA) and also by the low sensitivity of these methods.

While the efficient identification of satellite repeats is important for capturing their sequence diversity in various species, this information alone is not sufficient for our understanding of molecular mechanisms driving the evolution of satellite DNA. The other pieces of the puzzle is how these repeats are organized into long arrays spanning hundreds of kilobases, what is the distribution of higher-order repeats (HORs) and sequence variants along these arrays, and how is satellite DNA interspersed with other types of repeats. This kind of information became accessible only recently with the introduction of long-read sequencing platforms. Therefore, we started to utilize Oxford Nanopore sequencing that has the best potential for generating ultra-long (>100 kb) reads to investigate these features in a group of plant species selected for specific composition or features of their satellite DNA. The first study was performed in the repeat-rich legume species *Lathyrus sativus* that possess a set of highly amplified satellite families differing in their distribution on chromosomes. We developed a set of computational scripts for evaluating various properties of satellite repeat arrays detected in ultra-long reads and revealed that most of the satellite repeats in this species originated by an expansion of short arrays of tandemly repeated sequences present in mobile elements. We also showed that this satellite amplification preferentially occurred in pericentromeric regions of *L. sativus* chromosomes that bear distinct epigenetic modifications (Vondrak et al., 2019).

### ***Sequence composition and structure of plant centromeres***

Plant centromeres are genomic regions known to be predominantly comprised of repetitive DNA; however, the evolutionary implications of the repeat accumulation, its molecular mechanisms, and contribution to centromere specification are largely unknown. Early studies performed in model species like *Arabidopsis* and rice resulted in a relatively simple model of the plant centromere. This model states that



centromeres are mostly made of a single family of satellite repeat whose sequence co-evolves with a centromere-specific variant of the protein histone H3, also known as CenH3 or CENP-A. However, our previous work in pea (*Pisum sativum*) revealed much more complex centromeres characterized by multiple separated CenH3 domains located within extended primary constrictions of the metaphase chromosomes, which contrasted with a single CenH3 chromatin domain present in the simple primary constrictions of typical monocentric plant chromosomes. Moreover, pea CenH3 domains were found associated with a diverse set of fifteen centromeric satellite repeats sharing no sequence similarities. In follow-up studies, we investigated these expanded (or “meta-polycentric”) centromeres in three related genera (*Pisum*, *Lathyrus* and *Vicia*). We found that they are confined to *Pisum* and *Lathyrus*, and that their occurrence coincides with the presence of two copies of CenH3 genes in these genera (Neumann et al., 2015). Although the relevance and eventual mechanism of their centromere expansion are yet to be elucidated, “meta-polycentric” chromosomes are of great interest as potential evolutionary intermediates between monocentric and holocentric chromosomes. This hypothesis is supported by our finding of epigenetic modifications typical for pericentromeric chromatin in the extended primary constrictions; these modifications are similarly distributed along the whole holocentric chromosomes (Neumann et al., 2016).

Besides the studies on the centromeres described above, our data on repeat composition and distribution of various molecular markers in pea chromosomes contributed to the construction of the first chromosome-scale assembly of the *Pisum sativum* genome (Kreplak et al., 2019). Since the centromeric regions are still poorly represented in this first version of the assembly, we continue in our collaboration with the *Pisum* Genome Consortium with the aim of refining the assembly and using it to more advanced studies of the pea centromeres.

In collaboration with our partner laboratories, we also investigated repeat composition of centromeric chromatin in holocentric plant species *Rhynchospora pubera*. We revealed the first case of plant holocentric chromosomes that accumulated a specific satellite repeat in their CenH3-containing chromatin, suggesting that its properties could be very similar to the centromeric chromatin of the monocentric species (Marques et al., 2015). In the subsequent study, the same family of centromeric satellites was found conserved in other *Rhynchospora* species; however, in these species we also identified additional non-centromeric satellites with different patterns of chromosomal distribution (Ribeiro et al., 2017).

Finally, we have initiated a long-term project aiming at elucidating the mechanisms of holocentric chromosome evolution, using the plant genus *Cuscuta* as a model. This genus is unique in including species differing in their centromere type (monocentric and holocentric), and our phylogenetic analysis revealed that the holocentric chromosome organization originated there relatively recently from monocentric ancestors.

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## Research activity and characterisation of the main scientific results

### Trace metal (loid) toxicity and deficiency

Many trace metals are essential micronutrients, and all of them can become toxic when they become bioavailable in excess. Bioavailable concentrations of trace metals in the environment and agriculture are vastly different (with natural and anthropogenic causes) in various habitats, ranging from deficient to toxic levels. Therefore, research has focused on response to trace metals by plants and photosynthetic model organisms (algae and bacteria) in terms of uptake, transport, sequestration, speciation, deficiency, toxicity and detoxification. Most early and even numerous recent studies have used environmentally not relevant conditions. This applies in particular to extremely high, environmentally and agriculturally not relevant concentrations of toxic metals and metalloids. Further, individual processes often were not mechanistically interconnected, so that causes and consequences of metal(loid) effects remained unclear. In this contribution, recent insights are shown, mostly in the (sub-)nanomolar range of metal concentrations, with a simulation of natural light- and temperature cycles and trying to interconnect individual effects.

The submerged rootless water plant *Ceratophyllum demersum* turned out to be a useful shoot model, allowing to assess effects on photosynthetic tissues without interaction with root toxicity. In this model it could be shown that metal(loid) (As, Cd, Cr, Cu, Ni) concentrations that were previously considered as not having any effect actually have a strong impact on the plants, and with a different sequence of events than observed at very high concentrations. In addition, soybean (*Glycine max*) was used as a model for terrestrial plants and crops. We used a combination of various biophysical and biochemical methods for measurements *in vivo* (e.g. photosynthesis biophysics, formation of reactive oxygen species, metal transport), *in situ* (e.g. quantitative (sub)cellular distribution and speciation of metals, mRNA levels) as well as on isolated proteins (for identification and characterization of metalloproteins) and metabolites (metabolomics). For example, using metalloproteomics via HPLC-ICPMS of protein extracts from stressed plants, changes in target sites of metal binding to proteins from deficient to toxic concentrations could be analysed. X-ray absorption (XANES) and fluorescence ( $\mu$ XRF) spectroscopy provided information about metal(loid) speciation and distribution. The combination of techniques clearly showed metal(loid)-induced changes in the metabolism, already at very low concentrations, and allowed for new insights into the mechanisms of sublethal toxicity of As, Cd and Cu. In the years 2015-2019, the most important results of this area of our research have been published in the following publications:

Andresen E, Kappel S, Stärk HJ, Riegger U, Borovec J, Mattusch J, Heinz A, Schmelzer CEH, Matoušková Š, Dickinson B, Küpper H (2016) **Cadmium toxicity investigated at the physiological and biophysical levels under environmentally relevant conditions using the aquatic model plant *Ceratophyllum demersum* L.** New Phytologist 210, 1244-1258.

Cadmium is an important environmental pollutant and poisonous to most organisms. We aimed to unravel mechanisms of Cd toxicity in the model water plant *Ceratophyllum demersum* exposed to low (nM) concentrations of Cd as they appear in nature. Experiments were conducted under environmentally relevant conditions, including nature-like light and temperature cycles, and low biomass to water ratio. We measured Chl fluorescence kinetics, reactive oxygen species, oxygen exchange, pigments, metal

binding to proteins, and determined accumulation of starch and metals. The inhibition threshold concentration for most parameters was 20 nM. Below that, hardly any stress symptoms were observed. The first site of inhibition were photosynthetic light reactions (maximal quantum yield of photosystem II (PS II) reaction centre measured as  $F_v/F_m$ , light-acclimated PSII activity  $\Phi_{PSII}$ , total Chlorophyll). Trimers of the PS II light harvesting complexes (LHCI) decreased more than LHC monomers and detection of Cd in the monomers suggests replacement of Mg by Cd in the Chl molecules. As a consequence of dysfunctional photosynthesis and energy dissipation, reactive oxygen species (superoxide, hydrogen peroxide) appeared. Cadmium had negative effects on the macrophytes at much lower concentrations than reported previously, emphasizing the importance of studies applying environmentally relevant conditions. A chain of inhibition events could be established.

Mishra S, Alfeld M, Sobotka R, Andresen E, Falkenberg G, Küpper H (2016) **Analysis of sublethal arsenic toxicity to *Ceratophyllum demersum*: Subcellular distribution of arsenic and inhibition of chlorophyll biosynthesis.** Journal of Experimental Botany 67, 4639-4646.

Arsenic (As) pollution is a serious concern worldwide. Recent studies under environmentally relevant conditions revealed that pigments were the first observable target of toxicity, preceding all photosynthetic parameters and oxidative stress in the aquatic plant *Ceratophyllum demersum*. Lethal toxicity was initiated by the change of As species and distribution pattern in various tissues. Here the localization of As was investigated at the sub-cellular level through X-ray fluorescence using a submicron beam and a Maia detector. It was found that with such data useful tissue structural information can be obtained from the ratio of tomograms of photon flux after the sample and the Compton scattering. The  $\mu$ XRF tomograms showed that As predominantly accumulated in the nucleus of the epidermal cells in young mature leaves exposed to sublethal 1  $\mu$ M As. This may point to toxicity effects of arsenic in the nucleus such as interference with nucleic acid synthesis by replacement of P by As. At higher cellular concentrations the vacuole was the main storage site of As, particularly in mature leaves. The analysis of precursors of chlorophyll and degradation metabolites revealed that the observed decrease of chlorophyll was associated with hindered biosynthesis and not due to degradation. Coproporphyrinogen III disappeared already at 0.5  $\mu$ M As. Subsequent precursors, e.g. protoporphyrin IX, Mg-protoporphyrin, Mg-protoporphyrin methyl ester and Divinyl protochlorophyllide, were significantly decreased at this concentration as well, indicating that the pathway was blocked further upstream of tetrapyrrole synthesis.

Jaime-Pérez N, Kaftan D, Bina D, Bokhari SNH, Shreedhar S, Küpper H (2019) **Mechanisms of sublethal copper toxicity damage to the photosynthetic apparatus of *Rhodospirillum rubrum*.** Biochimica et Biophysica Acta (BBA) - Bioenergetics 1860, 640-650, DOI: <https://doi.org/10.1016/j.bbabi.2019.06.004>

Magnesium ( $Mg^{2+}$ ) is the ubiquitous metal ion present in chlorophyll and bacteriochlorophyll (BChl), involved in photosystems in photosynthetic organisms. In the present study we investigated targets of toxic copper binding to the photosynthetic apparatus of the anoxygenic purple bacterium *Rhodospirillum rubrum*. This was done by a combination of in vivo measurements of flash photolysis and fast fluorescence kinetics combined with the analysis of metal binding to pigments and pigment-protein complexes isolated from Cu-stressed cells by HPLC-ICPMS (ICP-sfMS). This work concludes that *R. rubrum* is highly sensitive to  $Cu^{2+}$ , with a strong inhibition of the

photosynthetic reaction centres (RCs) already at 2  $\mu\text{M}$   $\text{Cu}^{2+}$ . The inhibition of growth and of RC activity was related to the formation of Cu-containing BChl degradation products that occurred much more in the RC than in LH1. These results suggest that the shift of metal centres in BChl from  $\text{Mg}^{2+}$  to  $\text{Cu}^{2+}$  can occur in vivo in the RCs of *R. rubrum* under environmentally realistic  $\text{Cu}^{2+}$  concentrations, leading to a strong inhibition of the function of these RCs.

Andresen E, Lyubenova L, Hubáček T, Bokhari SNH, Matoušková Š, Mijovilovich A, Rohovec J, Küpper H (print: 2020, published online 24 November 2019) **Chronic exposure of soybean plants to nanomolar cadmium reveals specific additional high-affinity targets of Cd toxicity.** Journal of Experimental Botany 71, 1628-1644, DOI: <https://doi.org/10.1093/jxb/erz530>)

Solving the global environmental and agricultural problem of chronic low-level Cd exposure requires better mechanistic understanding. Here, soybean (*Glycine max*) plants were exposed to Cd concentrations ranging from 0.5 nM (background concentration, control) to 3  $\mu\text{M}$ . Plants were cultivated hydroponically under non-nodulating conditions for 10 weeks. Toxicity symptoms, net photosynthetic oxygen production, photosynthesis biophysics (Chl fluorescence: Kautsky and OJIP), Cd binding to proteins (metalloproteomics by HPLC-ICPMS) and Cd ligands in LHCII (XANES), accumulation of elements, Chl and metabolites were monitored. There was a distinct threshold concentration of toxicity onset (140 nM) as visible from the strongly decreased growth, the switch-like pattern for nutrient uptake and metal accumulation and photosynthetic fluorescence parameters like  $\Phi_{\text{RE10}}$  (OJIP) and saturation of the PS. XANES analyses of isolated LHCII revealed that Cd was bound to N or O (and not S) atoms. Nutrient deficiencies due to inhibited uptake could be caused by transporter blockage by Cd ions. The changes in specific fluorescence kinetic parameters indicate electrons not being transferred from PSII to PSI. Inhibition of photosynthesis combined with inhibition of root function could explain why the amino acid and carbohydrate metabolism decreased in favour of molecules involved in Cd-stress tolerance (e.g. anti-oxidative system, detoxifying ligands).

### Metal transport and sequestration

*Noccaea caerulescens* and *Arabidopsis halleri* were used as a hyperaccumulator model species to study the regulation of metal transport genes, and biochemical properties of one selected metal transporter (NcHMA4) were studied in the protein purified from *N. caerulescens*. *A. thaliana* was used as a model species in a collaboration with groups in Germany to study the mechanisms of metal loading and sequestration in seeds. This work led to the following publications.

Mishra S, Mishra A, Küpper H (2017) **Protein Biochemistry and Expression Regulation of Cadmium/Zinc Pumping ATPases in the Hyperaccumulator Plants Arabidopsis halleri and Noccaea caerulescens.** Frontiers in Plant Science, <https://doi.org/10.3389/fpls.2017.00835>.

P<sub>1B</sub>-ATPases are decisive for metal accumulation phenotypes, but mechanisms of their regulation are only partially understood. Here we studied the Cd/Zn transporting ATPases NcHMA3 and NcHMA4 from *Noccaea caerulescens* as well as AhHMA3 and AhHMA4 from *Arabidopsis halleri*. Protein biochemistry was analysed on HMA4 purified from roots of *N. caerulescens* in active state. Metal titration of NcHMA4 protein with an electrochromic dye as charge indicator suggested that HMA4 reaches maximal ATPase activity when all internal high-affinity  $\text{Cd}^{2+}$  binding sites are occupied. Although

HMA4 was reported to be mainly responsible for xylem loading for heavy metals for root to shoot transport, the current study revealed high expression of NcHMA4 in shoots as well. Further, there were additional 20 kD and 40 kD fragments at replete  $\text{Zn}^{2+}$  and toxic  $\text{Cd}^{2+}$ , but not at deficient  $\text{Zn}^{2+}$  concentrations. Altogether, the protein level expression analysis suggested a more multifunctional role of NcHMA4 than previously assumed. Organ-level transcription analysis through quantitative PCR of mRNA in *N. caerulea* and *A. halleri* confirmed the strong shoot expression of both NcHMA4 and AhHMA4. Further, in shoots NcHMA4 was more abundant in 10  $\mu\text{M}$   $\text{Zn}^{2+}$  and AhHMA4 in  $\text{Zn}^{2+}$  deficiency. In roots, NcHMA4 was up-regulated in response to deficient  $\text{Zn}^{2+}$  when compared to replete  $\text{Zn}^{2+}$  and toxic  $\text{Cd}^{2+}$  treatment. In both species, HMA3 was much more expressed in shoots than in roots, and HMA3 transcript levels remained rather constant regardless of  $\text{Zn}^{2+}$  supply, but were up-regulated by 10  $\mu\text{M}$   $\text{Cd}^{2+}$ . Analysis of cellular expression by quantitative mRNA in situ hybridisation (QISH) showed that in *A. halleri*, both HMA3 and HMA4 mRNA levels were highest in the mesophyll, while in *N. caerulea* they were highest in the bundle sheath of the vein. This is likely related to the different final storage sites for hyperaccumulated metals in both species: epidermis in *N. caerulea*, mesophyll in *A. halleri*.

Eroglu S, Giehl RFH, Meier B, Takahashi M, Terada Y, Ignatiev K, Andresen E, Küpper H, Peiter E, von Wirén N (2017) **Metal Tolerance Protein 8 mediates manganese homeostasis and iron re-allocation during seed development and germination.** Plant Physiology 174, pp. 1633–1647.

Metal accumulation in seeds is a prerequisite for germination and establishment of plants but also for micronutrient delivery to humans. To investigate metal transport processes and their interactions in seeds, we focused on *METAL TOLERANCE PROTEIN8* (*MTP8*), a tonoplast transporter of the manganese (Mn) subclade of cation diffusion facilitators, which in *Arabidopsis* (*Arabidopsis thaliana*) is expressed in embryos of seeds. The x-ray fluorescence imaging showed that expression of MTP8 was responsible for Mn localization in subepidermal cells on the abaxial side of the cotyledons and in cortical cells of the hypocotyl. Accordingly, under low Mn availability, MTP8 increased seed stores of Mn, required for efficient seed germination. In mutant embryos lacking expression of *VACUOLAR IRON TRANSPORTER1* (*VIT1*), MTP8 built up iron (Fe) hotspots in *MTP8*-expressing cells types, suggesting that MTP8 transports Fe in addition to Mn. In *mtp8 vit1* double mutant seeds, Mn and Fe were distributed in all cell types of the embryo. An Fe transport function of MTP8 was confirmed by its ability to complement Fe hypersensitivity of a yeast mutant defective in vacuolar Fe transport. Imbibing *mtp8-1* mutant seeds in the presence of Mn or subjecting seeds to wet-dry cycles showed that MTP8 conferred Mn tolerance. During germination, MTP8 promoted reallocation of Fe from the vasculature. These results indicate that cell type-specific accumulation of Mn and Fe in seeds depends on MTP8 and that this transporter plays an important role in the generation of seed metal stores as well as for metal homeostasis and germination efficiency under challenging environmental conditions.

Andresen E, Peiter E, Küpper H (2018) **Trace metal metabolism in plants.** Journal of Experimental Botany 69, 909-954

Many trace metals are essential micronutrients, but also potent toxins. Due to natural and anthropogenic causes, vastly different trace metal concentrations occur in various habitats, ranging from deficient to toxic levels. Therefore, one focus of plant research is on the response to trace metals in terms of uptake, transport, sequestration,

speciation, physiological use, deficiency, toxicity, and detoxification. In this review, we cover most of these aspects for the essential micronutrients copper, iron, manganese, molybdenum, nickel, and zinc to provide a broader overview than found in other recent reviews, to crosslink aspects of knowledge in this very active research field that are often seen in a separated way. For example, individual processes of metal usage, deficiency or toxicity often were not mechanistically interconnected. Therefore, this review also aims to stimulate the communication of researchers following different approaches, such as gene expression analysis, biochemistry, or biophysics of metalloproteins. Further, in this review recent insights will be highlighted, emphasizing data that were obtained under physiologically and environmentally relevant conditions.

### **Regulation of photosynthetic light harvesting and pigment-protein complexes**

Regulation of light harvesting efficiency and of energy flows through photosynthetic membranes is one of the key issues in autotrophic organisms. The process of photosynthesis starts in the light-harvesting antenna proteins and many regulatory mechanisms operate around these proteins as well. In most photosynthetic organisms the light-harvesting system consists of tens of different proteins with specialized roles within the photosynthetic apparatus. In eukaryotic autotrophs the multi-gene LHC family of proteins provides structures forming the outer antenna systems for both photosystems. Despite the tremendous progress in understanding the photosynthetic pigment-protein complexes, in plants the vast landscape of photosynthetic diversity remains little explored. Eukaryotic algae with plastids of secondary endosymbiotic origin are one of the largest sources of pigment diversity in photosynthetic processes. We built on our experience in working with non-model organisms to understand better how these organisms change their light-harvesting architecture in response to their environmental pressures. Carotenoids are of extreme importance in photosynthesis to both collect light energy and protect from light damage. The studied organisms provide a wide selection of different carotenoid-LHC combinations. This provides insight into the flexibility of the basic protein scaffold, which enables the use of diverse carotenoids in essentially the same roles. Here the contrasting systems from diatoms, using fucoxanthin for light harvesting, and from eustigmatophytes, using violaxanthin for both light harvesting and as a basis of protective xanthophyll cycle, proved very useful. The work requires an interdisciplinary approach, which integrates biochemical methods for protein and protein-complex purifications with biophysical methods for analysis of the pigment complements and their function. The following publications provide a sample of our work in this direction. Our most important work concerned the presence and properties of red-shifted antenna systems based on Chl a which aid in harvesting light in shaded environments.

Herbstová M, Bína D, Koník P, Gardian Z, Vácha F, Litvín R (2015) **Molecular basis of chromatic adaptation in pennate diatom *Phaeodactylum tricornutum***. *Biochimica et Biophysica Acta-Bioenergetics*, 1847(6-7), 534-543. DOI: 10.1016/j.bbabi.2015.02.016

The remarkable adaptability of diatoms living in a highly variable environment assures their prominence among marine primary producers. The present study integrates biochemical, biophysical and genomic data to bring new insights into the molecular mechanism of chromatic adaptation of pennate diatoms in model species *Phaeodactylum tricornutum*, a marine eukaryote alga possessing the capability to shift its absorption up to ~700 nm as a consequence of incident light enhanced in the red component. Presence of these low energy spectral forms of Chl a is manifested by

room temperature fluorescence emission maximum at 710 nm (F710). Here we report a successful isolation of the supramolecular protein complex emitting F710 and identify a member of the Fucoxanthin Chlorophyll a/c binding Protein family, Lhcf15, as its key building block. This red-shifted antenna complex of *P. tricornutum* appears to be functionally connected to photosystem II. Phylogenetic analyses do not support relation of Lhcf15 of *P. tricornutum* to other known red-shifted antenna proteins thus indicating a case of convergent evolutionary adaptation towards survival in shaded environments.

Litvín R, Bína D, Herbstová M, Gardian Z (2016) **Architecture of the light-harvesting apparatus of the eustigmatophyte alga *Nannochloropsis oceanica***. Photosynthesis research 130, 137-150. DOI: 10.1007/s11120-016-0234-1

Proteomic, spectroscopic, and phylogenetic analysis of light-harvesting protein (Lhc) function in oleaginous *Nannochloropsis oceanica* (Eustigmatophyta, Stramenopila) is presented. *N. oceanica* utilizes Lhcs of multiple classes: Lhcr-type proteins (related to red algae LHCl), Lhcv (VCP) proteins (violaxanthin-containing Lhcs related to Lhcf/FCP proteins of diatoms), Lhcx proteins (related to Lhcx/LhcSR of diatoms and green algae), and Lhc proteins related to Red-CLH of *Chromera velia*. Altogether, 17 Lhc-type proteins of the 21 known from genomic data were found in our proteomic analyses. Besides Lhcr-type antennas, a RedCAP protein and a member of the Lhcx protein subfamily were found in association with Photosystem I. The free antenna fraction is formed by trimers of a mixture of Lhcs of varied origins (Lhcv, Lhcr, Lhcx, and relatives of Red-CLH). Despite possessing several proteins of the Red-CLH-type Lhc clade, *N. oceanica* is not capable of chromatic adaptation under the same conditions as the diatom *Phaeodactylum tricornutum* or *C. velia*. In addition, a naming scheme of *Nannochloropsis* Lhcs is proposed to facilitate further work.

### Photophysics of light-harvesting pigment-protein complexes

Building on the topics covered in the previous section, advanced biophysical methods are needed to understand the details of energy flow inside of the light harvesting pigment-protein complexes. The major issue here is the interaction of pigment molecules within the proteins, specifically chlorophylls and carotenoids. Many algae use carotenoids with conjugated carbonyl group in their LHC proteins. A typical example is the FCP antenna from diatoms which contains fucoxanthin. For carbonyl carotenoids, the excited-state structure is complicated as the conjugated carbonyl group introduces an intramolecular charge transfer (ICT) state into the excited-state manifold. This feature makes the excited state dynamics of carbonyl carotenoids dependent on solvent polarity, which in turn allows to finely tune their properties by protein binding site if they are embedded into proteins. Light-harvesting proteins with carbonyl carotenoids are known to have high energy transfer efficiencies for energy flow from carotenoid to chlorophyll. In our work we have shown that this is also true for proteins in which the carbonyl carotenoid is replaced with a noncarbonyl carotenoid violaxanthin but the protein scaffold itself is phylogenetically related to FCP.

Analysis of the nature of the ICT state is complicated by its apparent coupling with the  $S_1$  state. It has been recently shown by pump-dump-probe spectroscopy that  $S_1$  and ICT are in fact two distinct states in equilibrium. We have applied the method to the systems coming from our biochemical lab, starting with the FCP from diatoms, showing that the  $S_1$  state is actually the energy donor in FCP.



In case of closely spaced or even overlapping absorption bands and very fast energy transfer processes the resolution of the pump-probe approach is limited. Complex two-dimensional electronic spectroscopy method was used to analyze such system, bacterial FMO protein, in collaboration with a partner in Lund, Sweden. Apart from high temporal and spectral resolution the method can also identify coupled pigments within the larger pigment complements of typical LHC proteins. We are currently carrying out further experiments using this method also on pigment-protein complexes mentioned in the section above (VCP).

Kesan G, Litvín R, Bína D, Durchan M, Šlouf V, Polívka T (2016) **Efficient light-harvesting using non-carbonyl carotenoids: Energy transfer dynamics in the VCP complex from *Nannochloropsis oceanica***. *Biochimica et Biophysica Acta-Bioenergetics*, 1857(4), 370-379. DOI: 10.1016/j.bbabi.2015.12.011

Violaxanthin–chlorophyll a protein (VCP) from *Nannochloropsis oceanica* is a Chl a-only member of the LHC family of light-harvesting proteins. VCP binds carotenoids violaxanthin (Vio), vaucheriaxanthin (Vau), and vaucheriaxanthin-ester (Vau-ester). Here we report on energy transfer pathways in the VCP complex. The overall carotenoid-to-Chl a energy transfer has efficiency over 90%. Based on their energy transfer properties, the carotenoids in VCP can be divided into two groups; blue carotenoids with the lowest energy absorption band around 480 nm and red carotenoids with absorption extended up to 530 nm. Both carotenoid groups transfer energy efficiently from their S<sub>2</sub> states, reaching efficiencies of ~70% (blue) and ~60% (red). The S<sub>1</sub> pathway, however, is efficient only for the red carotenoid pool for which two S<sub>1</sub> routes characterized by 0.33 and 2.4 ps time constants were identified. For the blue carotenoids the S<sub>1</sub>-mediated pathway is represented only by a minor route likely involving a hot S<sub>1</sub> state. The relaxed S<sub>1</sub> state of blue carotenoids decays to the ground state within 21 ps. Presence of a fraction of non-transferring red carotenoids with the S<sub>1</sub> lifetime of 13 ps indicates some specific carotenoid-protein interaction that must shorten the intrinsic S<sub>1</sub> lifetime of Vio and/or Vau whose S<sub>1</sub> lifetimes in methanol are 26 and 29 ps, respectively. The VCP complex from *N. oceanica* is the first example of a light-harvesting complex binding only non-carbonyl carotenoids with carotenoid-to-chlorophyll energy transfer efficiency over 90%.

Bína D, Durchan M, Kuznetsova V, Vácha F, Litvín R, Polívka T (2019) **Energy transfer dynamics in a red-shifted violaxanthin-chlorophyll a light-harvesting complex**. *Biochimica et Biophysica Acta-Bioenergetics*, 1860(2), 111-120. DOI: 10.1016/j.bbabi.2018.11.006

Photosynthetic eukaryotes whose cells harbor plastids originating from secondary endosymbiosis of a red alga include species of major ecological and economic importance. Since utilization of solar energy relies on the efficient light-harvesting, one of the critical factors for the success of the red lineage in a range of environments is to be found in the adaptability of the light-harvesting machinery, formed by the proteins of the light-harvesting complex (LHC) family. A number of species are known to employ mainly a unique class of LHC containing red-shifted chlorophyll a (Chl a) forms absorbing above 690 nm. This appears to be an adaptation to shaded habitats. Here we present a detailed investigation of excitation energy flow in the red-shifted light-harvesting antenna of eustigmatophyte *Trachydiscus minutus* using time-resolved fluorescence and ultrafast transient absorption measurements. The main carotenoid in the complex is violaxanthin, hence this LHC is labeled the red-violaxanthin-Chl a protein, rVCP. Both the carotenoid-to-Chl a energy transfer and excitation dynamics

within the Chl a manifold were studied and compared to the related antenna complex, VCP, that lacks the red-Chl a. Two spectrally defined carotenoid pools were identified in the red antenna, contributing to energy transfer to Chl a, mostly via  $S_2$  and hot  $S_1$  states. Also, Chl a triplet quenching by carotenoids is documented. Two separate pools of red-shifted Chl a were resolved, one is likely formed by excitonically coupled Chl a molecules. The structural implications of these observations are discussed.

West RG, Bina D, Fuciman M, Kuznetsova V, Litvín R, Polívka T (2018) **Ultrafast multi-pulse transient absorption spectroscopy of fucoxanthin chlorophyll a protein from *Phaeodactylum tricornutum***. *Biochimica et Biophysica Acta-Bioenergetics*, 1859(5), 357-365. DOI: 10.1016/j.bbabi.2018.02.011

We have applied femtosecond transient absorption spectroscopy in pump-probe and pump-dump-probe regimes to study energy transfer between fucoxanthin and Chl a in fucoxanthin-Chl a complex from the pennate diatom *Phaeodactylum tricornutum*. Experiments were carried out at room temperature and 77 K to reveal temperature dependence of energy transfer. At both temperatures, the ultrafast ( $< 100$  fs) energy transfer channel from the fucoxanthin  $S_2$  state is active and is complemented by the second pathway via the combined  $S_1$ /ICT state. The  $S_1$ /ICT-Chl a pathway has two channels, the fast one characterized by sub-picosecond energy transfer, and slow having time constants of 4.5 ps at room temperature and 6.6 ps at 77 K. The overall energy transfer via the  $S_1$ /ICT is faster at 77 K, because the fast component gains amplitude upon lowering the temperature. The pump-dump-probe regime, with the dump pulse centered in the spectral region of ICT stimulated emission at 950 nm and applied at 2 ps after excitation, proved that the  $S_1$  and ICT states of fucoxanthin in FCP are individual, yet coupled entities. Analysis of the pump-dump-probe data suggested that the main energy donor in the slow  $S_1$ /ICT-Chl a route is the  $S_1$  part of the  $S_1$ /ICT potential surface.

Thyrhaug E, Tempelaar R, Alcocer M, Židek K, Bina D, Knoester J, Jansen T, Zigmantas D (2018) **Identification and characterization of diverse coherences in the Fenna-Matthews-Olson complex**. *Nature Chemistry*, 10(7), 780-786. DOI: 10.1038/s41557-018-0060-5

The idea that excitonic (electronic) coherences are of fundamental importance to natural photosynthesis gained popularity when slowly dephasing quantum beats (QBs) were observed in the two-dimensional electronic spectra of the Fenna–Matthews–Olson (FMO) complex at 77 K. These were assigned to superpositions of excitonic states, a controversial interpretation, as the strong chromophore–environment interactions in the complex suggest fast dephasing. Although it has been pointed out that vibrational motion produces similar spectral signatures, a concrete assignment of these oscillatory signals to distinct physical processes is still lacking. Here we revisit the coherence dynamics of the FMO complex using polarization-controlled two-dimensional electronic spectroscopy, supported by theoretical modelling. We show that the long-lived QBs are exclusively vibrational in origin, whereas the dephasing of the electronic coherences is completed within 240 fs even at 77 K. We further find that specific vibrational coherences are produced via vibronically coupled excited states. The presence of such states suggests that vibronic coupling is relevant for photosynthetic energy transfer.

## Development of methods and instruments for the research of the team

In our research, often the established standard methods turn out not to be ideal for the tasks. For this reason, already since many years we have developed our own new techniques, new instruments and upgrades of instruments. For instrument developments, this was done in cooperation with instrument manufacturing companies. In the following publications were the most important in this area of our work 2015-2019.

Küpper H, Bokhari SNH, Jaime-Pérez N, Lyubenova L, Ashraf N, Andresen E (2019) **Ultra-trace metal speciation analysis by coupling of sector-field ICP-MS to high-resolution size exclusion and reversed-phase liquid chromatography**. *Analytical Chemistry* 91, 1710961-10969, DOI: 10.1021/acs.analchem.9b00222)

Techniques for metal speciation analysis with sub-nanomolar (ppt) detection limits in complex matrices, with simultaneous quantification of matrix elements, have become a necessity for investigating targets of trace metal binding to macromolecules and pigments at environmentally relevant concentrations. In this work we optimised the analysis of such metal binding in a custom-built HPLC-ICPMS system. Key elements of the optimisation were the choice of components for the metal-free HPLC-DAD system and sector-field ICP-MS detection (ICF-sfMS) with desolvating injection, and optimisation of sample handling. Protein analysis was done using ammonium bicarbonate buffer and size exclusion chromatography (SEC-ICP-sfMS), with possible addition of anion exchange chromatography. Detection of metal exchange in pigments (chlorophylls and bacteriochlorophylls) was based on reversed-phase chromatography with a methanol-acetone gradient and coupling to the ICP-sfMS via a dedicated organic matrix interface (RPC-ICP-sfMS). The resulting HPLC-DAD-ICP-sfMS system has detection limits in the picomolar range in protein buffer, limited by the maximal achievable purity of buffers/solvents and not by system sensitivity. Tests for method optimisation showed that sonication, meant to increase protein solubilisation, leads to artefacts of metal loss from metalloproteins. Examples for Cd binding to soybean proteins and chlorophyll, Cr binding to *Arabidopsis thaliana* proteins, La binding to *Desmodemus quadricauda* proteins and Cu binding to *Rhodospirillum rubrum* proteins and pigments are shown. These application examples demonstrate that the system is sensitive enough to detect binding of metals to proteins and pigments at background concentration levels of typical nutrient solutions made from analytical grade chemicals, equivalent to ultra-trace metal concentrations in non-polluted environments.

Küpper H, Benedikty Z, Morina F, Andresen E, Mishra A, Trtílek M (2019) **Analysis of OJIP chlorophyll fluorescence kinetics and QA re-oxidation kinetics by direct fast imaging**. *Plant Physiology* 179, 369-381, DOI: <https://doi.org/10.1104/pp.18.00953>

Chlorophyll fluorescence kinetic analysis has become an important tool in basic and applied research on plant physiology and agronomy. While early systems recorded the integrated kinetics of a selected spot or plant, later systems enabled imaging of at least the slower parts of the kinetics (20 ms time resolution). For faster events, such as the rise from the basic dark-adapted fluorescence yield to the maximum (OJIP transient), or the fluorescence yield decrease during re-oxidation of plastoquinone A (Q<sub>A</sub>) after a saturating flash, integrative systems are used because of limiting speed of the available imaging systems. In our new macroscopic and microscopic systems, the OJIP or Q<sub>A</sub> re-oxidation fluorescence transients are directly imaged using an ultrafast camera. The

advantage of such systems compared to non-imaging measurements is the analysis of heterogeneity of measured parameters, for example between the photosynthetic tissue near the veins and the tissue further away from the veins. Further, in contrast to the pump-and-probe measurement, direct imaging allows for measuring the transition of the plant from the dark-acclimated to a light-acclimated state via a quenching analysis protocol in which every supersaturating flash is coupled to a measurement of the fast fluorescence rise. We show that pump-and-probe measurement of OJIP is prone to artifacts, which are eliminated with the direct measurement. The examples of applications shown here, zinc deficiency and cadmium toxicity, demonstrate that this novel imaging platform can be used for detection and analysis of a range of alterations of the electron flow around photosystem II.

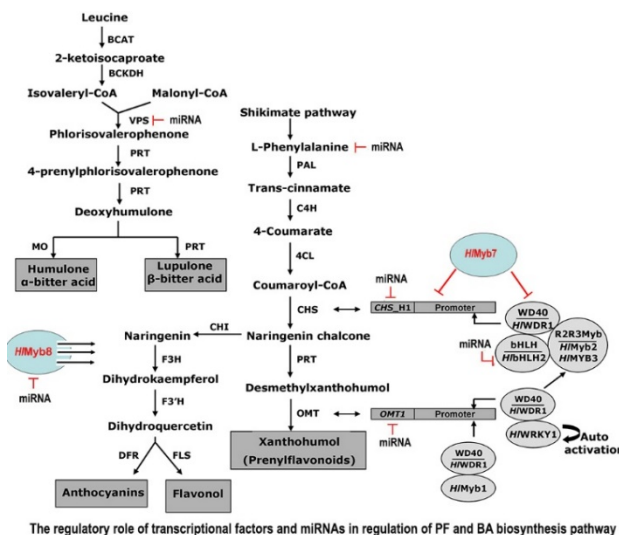
## Research activity and characterisation of the main scientific results

### 1A: Analysis of regulatory network of transcription factors of *Humulus lupulus* involved in lupulin metabolome pathways.

The team members of Department of Molecular Genetics from past decades and especially in the evaluated period have been extensively working on secondary metabolite [bitter acids (BA) and prenylflavonoids (PF)] biosynthesis pathway in hop. As a model system, Arabidopsis has facilitated us to elucidate many details of the PF and BA biosynthesis pathway, in particular structural and regulatory genes, network nodes [transcription factors (TFs) and their target sequences] and edges (TF–TF dimers/trimer and TF–DNA target interactions), and the interconnectedness of the other pathways. These advances in our understanding and construction of regulatory network have been made possible by the multidisciplinary approach using state-of-the-art genetic analyses, biochemical assays, investigation of promoter activity and DNA–protein interactions and bioinformatics technique.

Our research started as enduring great hardships in pioneer work to isolate and characterize members of an oligofamily of chs\_H1 genes (designated as "true" chalcone synthases) that codetermine the biosynthesis of prenylated chalcones in hop (e.g. Matoušek et al., 2006, J Agric Food Chem. 54, 7606–7615). In following years, huge amount of research effort has been placed by our group into the characterization of regulatory network, which regulates PF and BA biosynthesis pathway in hop. To this end, we showed that the lupulin gland-specific PF and BA biosynthesis pathways are regulated in high complexity manner with the involvement of key chs\_H1 gene and the simultaneous interaction of a specific combination of several types of TFs, mainly from Myb (M), bHLH (B), WDR (W) and bZip families (Matoušek et al., 2010, J Agric Food Chem. 58, 902-912; Matoušek et al., 2012, BMC Plant Biol. 20, 27). The parallel activation of CHS\_H1 promoter has been shown to be driven by either highly organized ternary MBW complexes (Hls-Myb3/HlbHLH2/HIWDR1 or HIMYB2/HlbHLH2/HIWDR1) or binary complexes (HlbHLH2 /HIWDR1) through protein-protein interactions (Matoušek et al., 2010, J Agric Food Chem. 58, 902-912; Matoušek et al., 2012, BMC Plant Biol. 20, 27).

In the evaluated period the well characterized flavonoid biosynthesis pathway of *Arabidopsis* (*Arabidopsis thaliana*) together with hop mRNA profiles further facilitated us to identify orthologous TFs in hop such as HIWRKY1, HIMy7 (homologue of



AtMyb4) and HlMyb8 (homologue of AtMyb12). Based on functional analyses, we showed with priority that HlWRKY1 (homologue of AtWRKY75) transcription factor forms a binary complex with HlWDR1 and as a master transcription factor activates structural genes of terminal steps (together with ternary MBW complex) of the PF and BA biosynthesis pathways (Matoušek et al., 2016, *Plant Mol Biol.* 92, 263–277). In this work the function of lupulin in biotic stress reaction based on molecular genetic analyses was predicted. The expression of HlWRKY1 transcription factor can be activated by a protein kinase and modulated by autoactivation and by RNA silencing machinery (Matoušek et al. 2016, *Plant Mol Biol.* 92, 263–277). The TF HlMyb8 is a close homologue of AtMyb12 of *Arabidopsis* and was found to be very strong activator of the promoters of chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS) and chalcone flavanone isomerase (CHI). The complementation analysis in *Arabidopsis* and transcriptome profiling of HlMyb8 overexpression line of hop revealed that it has prominent role in flavonol production by diverting the flux of CHS gene product with negative regulatory effect to PF and BA biosynthesis pathway (Kocábek et al., 2018, *Plant Sci.* 269, 32–46). Similarly, the functional analysis and transcriptome profiling of HlMyb7 (AtMyb4 homologue) overexpression line revealed that it acts as transcriptional repressor either by targeting MBW complexes or by inhibiting HlWRKY1/HlWDR1 complex through the presence of inhibitory EAR domain (Matoušek et al., 2012, *BMC Plant Biol.* 20, 27).

The use of advanced computational tools, which we intensively employed in the evaluation period has been accelerated the accumulation of reports on miRNAs-based regulation of biosynthesis and accumulation of secondary metabolites in plants. Therefore, further work on functional characterization of miRNAs-target networks using computational approach was undertaken for understanding post-transcriptional regulatory mechanism of the lupulin metabolome biosynthesis pathway and response to Citrus bark cracking viroid (CBCVd) pathogenesis in the collaboration of Agronomy Department of the Biotechnical Faculty, University of Ljubljana, Slovenia). Strikingly, four secondary metabolite unigenes encoding for enzyme valerophenone synthase (VPS), phenylalanine ammonia lyase (PAL), chalcone synthase (chs\_H1), O-methyltransferase-1 (OMT1) and two transcriptional factors (HlMyb8 and HlHHLH2) encoding unigene involved in BA and PF biosynthesis pathways were identified as the target of six different conserved hop miRNAs in our study (Mishra et al., 2015, *BMC Genomics.* 17, 919).

From the past, several independent and cooperative hop breeding programmes around the world have been directed towards the development of new improved cultivars with advantageous traits such as higher yield, enriched lupulin, high metabolome content to satisfy the demand of the brewing industry, which have been largely constrained by sexual incompatibility of developed lines, limited genetic resources, long cumbersome process and appearance of unintended characteristics. As an alternative to enhance PF and BA content in hop, first pioneering efforts were made by our group to develop overexpression hop transgenic lines. Gain-of-function





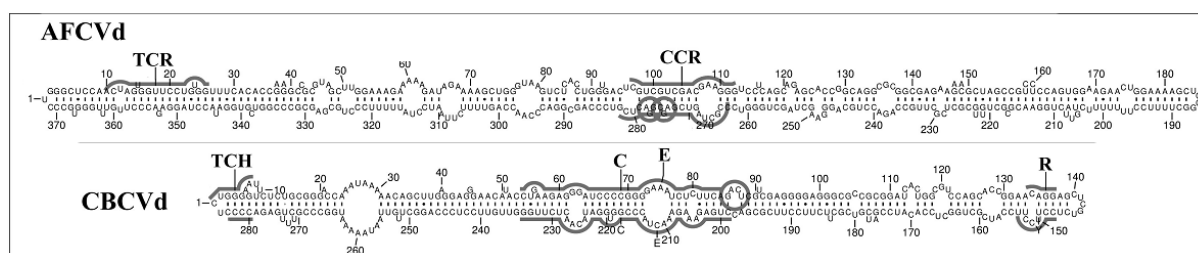
lines were generated by overexpressing the coding sequence of HIWRKY1 and HIWDR1 using dual gene expression cassette vectors (Matoušek et al. 2016, Plant Mol Biol. 92, 263–277; Mishra et al. 2018, BMC Genomics. 19, 739). On the basis of transcriptome and metabolome analysis, we have demonstrated that the overexpression of HIWRKY1 and HIWDR1 (WW\_OE) in leaves of hop modulates diverse metabolic sectors in favour of the enhanced accumulation of PF and BA as well as those associated with stress response and primary metabolism in hop (Mishra et al., 2018, BMC Genomics. 19, 739). In this study, we conclude that WW\_OE leads to a large-scale modulation in transcriptome of hop that might be responsible for major metabolic reprogramming to favour the high-level accumulation of PF, BA and other phenolics. In conclusion, the parallel advances in our ability to access information on draft genome sequences of hop, transcriptomics data, previous and new functional studies of regulatory component coupled with their transformation into hop, it seems likely that, an integrative approach such as application of CRISPR/Cas9 genome editing technique is required for rational manipulation of regulatory networks to enhance lupulin metabolome content in hop. Nevertheless, currently our research programme focuses on the standardization of CRISPR/Cas9 genome editing technique in hop genome to deliver new tool for agronomy that have potential to increase secondary metabolite content in other plant species. To this end, we have used phytoene desaturase (PDS) gene as a model gene for establishing genome editing in hop. PDS is one of the rate-limiting enzymes in the carotenoid biosynthesis pathway and knockout of PDS gene leads to albino phenotype in plant. CRISPR construct for HIPDS gene was developed as per previously published report (Xing et al., 2014, BMC Plant Biol. 14: 327). The pKSE401\_HIPDS\_CRISPR construct was transformed in *Agrobacterium tumefaciens* strain LBA4404, which was used to generate hop transgenic plants. The undertaken study will be the first report of CRISPR/Cas9 based genome editing in the hop. The initial results, especially obtaining of mutant phenotype and confirming editing events, show that genome editing through CRISPR/Cas9 can be applied as an efficient tool for hop genome modification.

### **1B: Genetic and molecular analysis of lupulin gland development in hop using wide transcriptomic comparisons.**

Hop is cultivated for female cones containing in their glandular trichomes secondary metabolites, such as bitter acids or prenylflavonoids valuable for brewing industry and for pharmaceutical and medicinal applications. Amount and composition of lupulin glands form together with their biosynthetic capacity the quantitative genetic trait co-determining the overall yield of lupulin gland. With the acceptance of new project in 2018 (GAČR19-19629S), we included research connected to lupulin glands initiation and morphogenesis. As an integral part of the first step of this project, we have performed the genome-wide transcriptome profiling of leaf, flower, bracts and lupulin gland tissue samples of two contrasting cultivars differing notably in lupulin gland (LG) density (Osvald 72: low LG density; Vital: high LG density) (Mishra et al., 2020, Int J Mol Sci. 21; 2330). From these results we cloned candidate genes hop homologues like Glabra2, Mixta1, Glabrous, Cyclin2B, Wo, WRKY44, WRKY75, Mic1, Myb36, Myb61, homologues encoding HD domains and other “candidates” which can regulate the glandular trichome development in hop (Matoušek et al., 2020, Nath et al., 2020; manuscripts under preparation).

## 2A: Investigation of the mechanisms of plant-viroid interaction and viroid-induced pathogenesis with implementation of NGS-based data.

Over the three decades our group has contributed significantly in plant-viroid interactions field, which includes characterization of the PSTVd variant AS1, vsRNA expression (Matoušek et al., 2007, Biol. Chem. 388, 1-13), analysis of potato and hop viroid thermomutants (e.g. Matoušek et al., 2001, Virology 287, 349–358), viroid stability and transmissibility through weed species (Matoušek et al., 2007, J Virol. 81, 11891–11899). Within the evaluated period we performed degradome analyses of mRNAs linked to viroid pathogenesis network caused by type member of Pospiviroidae potato spindle tuber viroid (PSTVd) (Matoušek et al., 2015, J. Plant Physiol. 183, 85–94). In the degradome analysis, we analyzed plant morphogenesis-regulating transcription factor SANT/HTH-Myb (*S/Myb*) as potential viroid target that was silenced due to viroid pathogenesis. For the first time we clearly showed that induction of degradome of *S/Myb* is directly connected to viroid pathogenesis network, but it is not directly targeted by viroid-specific RNA. In addition, we discovered the necrotic function and additional morphogenesis functions of *S/Myb* (Matoušek et al., 2015, J. Plant Physiol. 183, 85–94). In order to gain in depth knowledge of role of miRNAs in hop and their response to Citrus bark cracking viroid (CBCVd) pathogenesis, our group in the collaboration of Agronomy Department of the Biotechnical Faculty University of Ljubljana, Ljubljana, Slovenia performed the high-throughput sequencing of two small RNA (sRNA) libraries prepared from healthy and CBCVd-infected hop plants. We used small RNA analysis pipeline and identified 49 novels and 67 conserved miRNAs in hop. Among them we found 36 conserved miRNAs and 37 novel miRNAs, which were differentially expressed in the response to CBCVd-infection. Furthermore, target genes prediction of newly identified hop miRNAs, using the psRNATarget program with default parameters against the transcript sequences of the hop genome as a reference set facilitated us to identify 311 potential targets. These identified targets were classified into different gene families with various biological functions, including the control of hop development, signal transduction, phase change, metabolism, secondary metabolite production (including bitter acids and prenylflavonoid biosynthesis pathways), protein translocation, and responses to biotic/abiotic stresses (Mishra et al., 2016, BMC Genomics. 17:919).



Secondary structures of analyzed parasitic RNAs.

More recently, during evaluated period, we employed comprehensive transcriptome analyses to dissect host-viroid interactions and to identify gene expression changes associated with disease development in hop in response to Citrus bark cracking viroid (CBCVd) infection. This work contributed to in-depth understanding of mechanism of CBCVd-pathogenesis in hop. Our results showed that the expression of genes associated with plant immune responses (protein kinase and mitogen-activated protein kinase), hypersensitive responses, phytohormone, photosynthesis, pigment metabolism, protein metabolism, sugar metabolism, and modification, and others were

altered, which underscores the underlying mechanism of systemic symptom development upon CBCVd-infection in hop (Mishra et al., 2019, *Viruses*.10:570). Furthermore, the differentially expressed gene dataset combined with RT-qPCR, illustrated that major transcription factors families were differentially modulated in a coordinated way to fine-tune the defense response and disruption of transcription factors regulatory networks could be an alternative mechanism of CBCVd-induced pathogenesis in hop (Nath et al., 2019, *Viruses*. 11, 419).

The information obtained from our viroid-responsive RNA sequencing data (Mishra et al., 2019, *Viruses*. 10: 570; Štajner et al. 2019, *Int J Mol Sci*. 20, 3154) provided us fascinating angle of role of mediator complex in viroid pathogenesis and robust framework for formulating experiments of discerning the expression profile of MED subunits in model plants (*N. benthamiana*, *N. tabacum*, tomato and hop) with different viroid species infection to dissect the response of MED subunits in viroid-host interaction. The gene expression analysis of MED subunits showed the profound modulation of gene expression of four MED subunits (MED4, MED14, MED19 and MED27) in single CBCVd and AFCVd transformed *N. tabacum* and in single CBCVd, AFCVd and PSTVd infected *N. benthamiana*, whereas the other MED subunits showed the plant species or viroid-specific negligible, weak or moderate expression changes. In tomato, MED12 and MED13 showed the significant changes in gene expression contrary to other MED subunits, which did not show any distinction in expression pattern. The elevated and tailored expression of the MED subunits observed in our study coupled with several previous reports about massive modulation of genes involved in immune responses, primary and secondary metabolism, hormone signaling pathways provided the circumstantial support for the prominent role of MED complex in orchestrated transcriptional reprogramming of host genes in response to viroid infection. Our group has for the first time reported the response of MED subunits to a viroid-infection using different viroid-host combinations (Nath et al., 2020, *Int J Mol Sci*. 21:2498).

## **2B: Investigation of mechanisms of viroid propagation and elimination by the use of integrated omics data.**

With priority, we analysed multiple interaction of different viroid species which are endangering cultivation of worldwide hops. These hop isolates of viroid parasitic RNAs included citrus back cracking viroid (CBCVd), hop stunt viroid (HSVd), apple fruit crinkle viroid (AFCVd) and hop latent viroid (HLVd). Our biolistic co-inoculation experiments showed the multiple and compatible infections but the unstable co-expression as quantified after hop dormancy, probably also due to some silencing and epigenetic mechanisms. The high plant mortality, morphological disorders and depression of metabolome genes during multiple infections showed synergisms and gene dysregulations (Matoušek et al., 2017, *J Plant Physiol*. 213, 166-177). Viroid populations often contain a complex mixture of sequence variants, and environmental stress (including transfer to different hosts) has been shown to result in a significant increase in sequence heterogeneity and formation of quasispecies. We used deep-sequencing to analyze vsRNAs from plants infected with different PSTVd variants to examine the composition of mutant spectra of PSTVd quasispecies in infected tomato plants. Several novel and known PSTVd variants were recovered, found to be competent in replication addition to common strand-specific mutations, suggested that viroid quasispecies serve as a source of adaptation to new hosts, and moreover

changes in selective pressures induce the rapid emergence of new variants (Brass JR, Matoušek et al., 2017, RNA Biol. 4, 317-325).

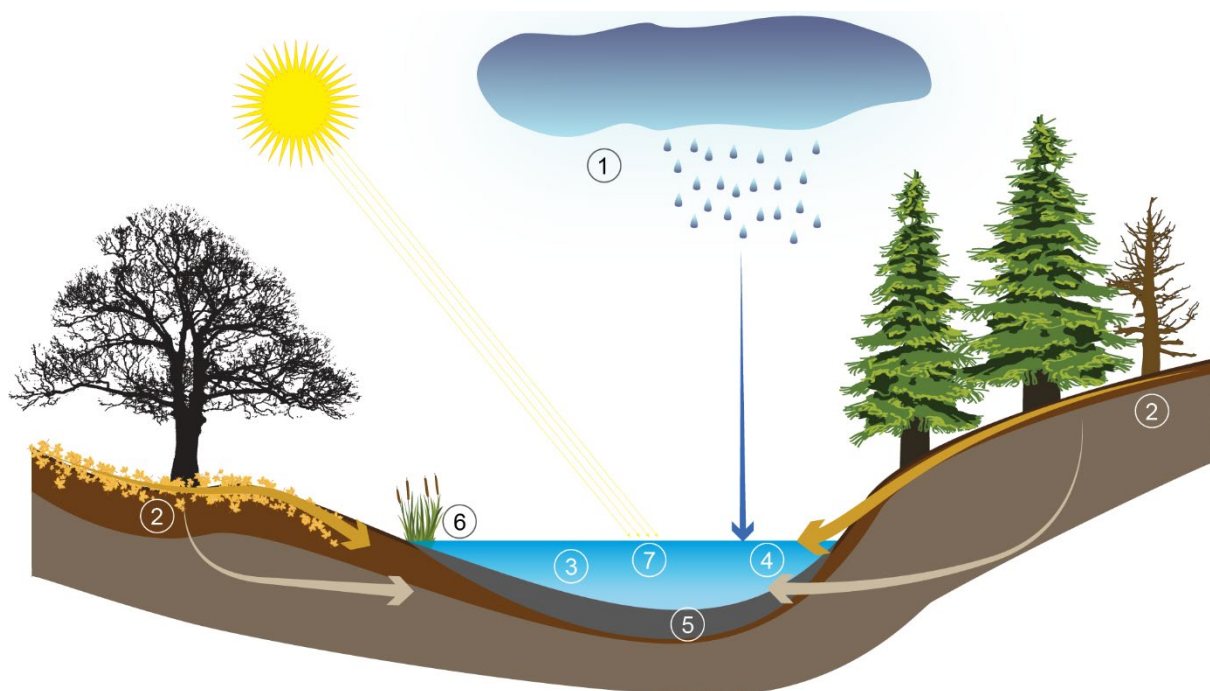
Viroids represent a significant danger due to their fast spreading, difficult eradication from plant tissues and fast rate of adaptation. Already in previous evaluation protocol we mentioned the importance to analyse the phenomenon of viroid suppression or elimination from generative tissues, where we observed earlier the elimination of hop latent viroid (HpLVd) during maturation of hop pollen, (Matoušek J., et al. 2008, Biological chemistry 389: 905-918)- suggesting an intricate and intriguing molecular interaction(s) of viroid parasitic RNA during pollen development which result in the elimination HpLVd during male gametophyte development. There are some practical consequences for hop breeding process, where viroid-free hybrid plants can be selected from seeds. Some hop viroids like HpLVd, AFCVd and CBCVd are not transmissible through hop pollen to seeds and to offspring, while others like HSVd belong to transmissible parasitic RNAs. Within new international project (GAČR 18-10515J) partly covering this evaluation period, we analyzed the molecular background of elimination of two hop viroids AFCVd and CBCVd from male gametophyte cells. As both viroids, as we found experimentally, are able to replicate in pollen model, *N. tabacum*, the mechanism of eradication is in parallel analyzed by NGS, complex RNA and protein expression profiling using infected and transformed tobacco anther tissues at different developmental stages. Therefore, the in-depth understanding of viroids transmission through the generative phases comes from our current works overlapping the evaluation period. We found significantly low levels of viroid RNA in developing pollen, which further diminished in mature pollen of AFCVd and CBCVd *N. benthamiana* infected individually with Apple fruit crinkle viroid (AFCVd) and Citrus bark cracking viroid (CBCVd) (Steinbachová, L., Matoušek, J. et al. 2018, International Conference on Viroids and Viroid-Like RNAs) and *N. tabacum* infected with AFCVd (Matoušek et al. 2020, Int J Mol Sci. 21, 3029) compared to whole anther and leaf tissues, suggesting the activation of molecular events which promote viroid elimination from infected male gametophyte. Our results showed that the viroid replication pathway involving DNA-dependent polymerase II complex is depressed in comparison to leaves and simultaneously specific and unspecific degradation proceeds in pollen and in the growing pollen tubes. The results were achieved (1) by observation of viroid integrity using hybridization and thermodynamic techniques and by quantification with strand-specific RT-qPCR method, which we developed during evaluation period to quantify viroid propagation (2) by profiling of viroid derived small RNAs, mRNA degradome and proteomic analyses of infected pollen; (3) by identification of transcripts and proteins, which significantly alter as a result of viroid infection by integrating omics data. Our experimental evidences indicated that intimate molecular interaction(s) of the viroid parasitic RNA and its recognition and elimination on the level of male gametophyte has a critical checkpoint in the early stage of pollen tube growth, where traces of viroid are eliminated by the process involving ARGONAUTE proteins and TUDOR nuclease leading to the complete degradation of circular and linear viroid forms (Matoušek et al., 2020 Int J Mol Sci. 21, 3029).

## Research activity and characterisation of the main scientific results

The research of the HEM Team combines several interconnected topics, including (1) atmospheric pollution, (2) catchment processes (soil-water and bedrock-water interactions), internal (physical, chemical, photochemical, and biological) processes in waters, (3) sedimentation and sediment-water interactions, (4) hydrological and climatic effects on water composition (isotopes, nutrients), (5) macrophytes, and (6) modelling (Figure 1).

The team members were principal investigators or principal co-investigators of 14 research project grants of the International Atomic Energy Agency (IAEA) (1×), Czech Science Foundation (GACR) (8×), Ministry of Education, Youth and Sports of the Czech Republic (MSMT) (1×), Ministry of Agriculture of the Czech Republic (MZE-NAZV) (1×), and Technology Agency of the Czech Republic (TACR) (3×) during 2015–2019. Financial support from these grants was 2.1 mil. € (i.e., 420 k€ annually).

The scientific and publication activity of HEM members resulted in 86 papers in international impacted journals and 12 papers in other peer reviewed journals from 2015–2019.



**Figure 1** – Research topics of Hydrochemistry and Ecosystem Modelling Team combines individual research activities: 1 – atmospheric pollution, 2 – catchment processes (soil and vegetation properties), 3 – aquatic chemistry, 4 – isotope chemistry, 5 – sedimentation and sediments, 6 – aquatic macrophytes, and 7 – photochemistry, improving our knowledge on biogeochemical cycles of elements and providing data sets for ecosystem modelling and forecasting responses to anticipated environmental changes.

### Atmospheric pollution

#### Effect of long-range transport of atmospheric pollutants and climate change on ecosystems of alpine lakes

1) We studied process of biological recovery of alpine lakes in the Tatra Mountains. Within original findings, the most important conclusion is that the delayed return of planktonic crustaceans to some chemically recovered lakes could be a consequence

of the shortened water residence time of these lakes due to increasing frequency of heavy rains during the last three decades. The climate-related changes in hydrology together with the ecology of planktonic crustaceans, thus contribute to the delays in the biological recovery of the Tatra Mountain lakes (Stuchlík et al., 2017, *Water Air Soil Poll.* 228, 184).

2) We studied responses in water chemistry and macroinvertebrates in a strongly acidified mountain stream in the Brdy Mountains in 1999 and again in 2010. The signs of the biological recovery from acidification included the first occurrences of less acid-tolerant macroinvertebrate taxa (Beneš et al., 2017, *Biologia* 72, 1049-1058).

3) We studied the impacts of land use policy on the recovery of mountain catchments from acidification in the Jizera Mountains. We found that the existing land use policy, institutes of 'protected headwater area' and 'zones of hygienic protection' (Water Act 138/1973) were ineffective in this situation. In a catchment scale, the proposed scenario of structured forestry zones (respecting riparian buffers, stability of steep slopes, significant fog drip areas, peat spots) can decrease the annual load of sulphur and nitrogen by approx. 30%. (Křeček et al., 2017, *IFOREST* 10, 680-686, Křeček et al. 2019, *Land Use Policy* 80, 439-448, Křeček et al., 2019, *Freshw. Sci.* 38, 257-269).

4) We studied atmospheric deposition of organochlorine compounds, polycyclic aromatic hydrocarbons and heavy metals at European high-elevation sites. We found that: (i) the Tatra Mountain fish belonged among the most contaminated fish in European mountain lakes (Dočkalová et al., 2015, *Biologia* 70, 516-529), and (ii) deposition of organochlorine compounds was mostly associated with intensive agricultural activities during the last decades (Arellano et al., 2015, *Atmos. Chem. Phys.* 15, 6069-6085). Our study also contributed to the most comprehensive description of atmospheric deposition of polycyclic aromatic hydrocarbons to high-elevation European lakes (Arellano et al., 2018, *Atmos. Chem. Phys.* 18, 16081-16097).

#### Effects of recovery from acidification and climate change on soil and water chemistry

1) We explained differences in relative changes in N, P, and DOC leaching from alpine catchments recovering from acidification as the primary reason of their catchment characteristics, especially the soil type and amount (scree vs. alpine soils): The rate and extent of  $\text{NO}_3^-$  decrease, while DOC and P increase in lakes during recovery was positively correlated with scree area in their catchments (Kopáček et al., 2015, *Environ. Sci. Technol.* 49, 2895-2903; Kopáček et al., 2015, *Biogeochemistry* 125, 315-335).

2) The study on  $\text{NO}_3^-$  and DOC leaching from forested catchment revealed their high sensitivity to hydrology and inter-annual climate characteristics (Kopáček et al., 2016, *Ecol. Ind.* 63, 196-208).

3) We explained why calcium and sulphate leaching decoupled ( $\text{SO}_4^{2-}$  continued in decrease while  $\text{Ca}^{2+}$  started to increase) in some alpine lakes during their recovery from acidification. The reason for this change was elevated mechanical weathering of granodiorite rocks due to climate change (decreasing snow cover in winter, while increasing frequency of heavy rains and freeze-thaw cycles during the last 3 decades) and dissolution of liberated accessory calcite (Kopáček et al., 2017, *Environ. Sci. Technol.* 51(1), 159-166). We showed that this process also increased P leaching from apatite in freshly crashed rocks, resulting in increasing chlorophyll-a concentrations in



alpine lakes with high scree proportion in their catchments (Kopáček et al., 2019, *Aquat. Sci.* 81:70).

4) Biological recovery of mountain forest lakes in the Czech Republic was mostly related to decreased acidic deposition (Vrba et al., 2016, *Freshwater Biol.* 61, 376-395). In contrast, the present biological recovery of numerous alpine lakes from acidification is, besides decreasing acidic deposition, also supported by climatically elevated weathering and alkaline dust deposition from arid areas in Northern Hemisphere (Moser et al., 2019, *Global Planet Change* 178, 77-95).

#### Long term trends in atmospheric deposition and water chemistry – monitoring and modelling

1) We developed a conceptual model enabling reconstruction of complete precipitation chemistry (including acid neutralizing capacity and pH) back to 19<sup>th</sup> century, based on emission trends in S and N compounds and industrial dust in the Czech Republic and central Europe (Kopáček et al., 2016, *Water Res.* 103, 30-37). Deposition models of S and N compounds enabled their reconstruction for individual years in any place in the Czech Republic, based on its longitude, latitude, and elevation (Oulehle et al., 2016, *Atmos. Environ.* 140, 456-468).

2) These deposition models enabled reconstruction of seasonal changes in lake water chemistry of mountain lakes (Oulehle et al., 2017, *Sci. Total. Environ.* 536, 1019-1028) and together with long-term monitored data to evaluate effects of agriculture, industry and acidic deposition on element fluxes in the Vltava river throughout the whole 20<sup>th</sup> century (Kopáček et al., 2017, *Water Res.* 125: 374-383).

3) The S and N deposition models were also used to reconstruct acidic deposition and nutritional effects of N-inputs to ~650 research forest plots throughout the Czech Republic. In collaboration with forest and soil scientists, these data were used to evaluate effects of acids and N inputs on tree characteristics, growth, and health during the 20<sup>th</sup> century (Cienciala et al., 2016, *Sci. Total. Environ.* 573, 541-554; Altman et al., 2017, *Sci. Total. Environ.* 609, 506-516; Cienciala et al., 2018, *Sci. Total. Environ.* 619-620, 1637-1647) and on the present soil chemistry (Šantrůčková et al., 2019, *Sci. Total. Environ.* 687, 96-103).

4) Long-term monitoring data and analysis of sediment core from Plešné Lake enabled us to participate in a comparative study of long-term Hg and Pb sediment archives (Norton et al., 2016, *Environ. Chem.* 13, 517-527) and long-term dynamics of watershed leaching and lake sediment sequestration of rare earth elements (Norton et al., 2016, *J. Paleolim.* 55(3), 209-222).

#### **Catchment processes**

##### Effects of forest disturbance on element cycling in terrestrial and aquatic ecosystems

1) We studied cycling and terrestrial losses of nutrients (N, P, C), base cations (BCs), and Al from undisturbed and bark-beetle killed forests. Natural forest dieback in an unmanaged mountain catchment increased availability of N forms in soils and BCs concentrations on soil sorption complex (Kaňa et al., 2019, *Ecol. Ind.* 97, 319-328) and accelerated leaching of NO<sub>3</sub><sup>-</sup>, BCs, Al, and dissolved organic C (DOC) to receiving waters (Kopáček et al., 2017, *Sci. Total. Environ.* 584-585, 971-981; Oulehle et al., 2019, *Ecosystems* 22, 725-741).

2) The elevated intensity of in-lake processes (assimilation and denitrification of  $\text{NO}_3^-$  and photochemical and microbial reduction of allochthonous organic acid anions) increased lake water alkalinity and accelerated its biological recovery from acidification (Kopáček et al., 2019, *Limnol. Oceanogr.* 64, 1614-1626).

3) Tree dieback affected microclimate characteristics (soil and air temperature, soil moisture and air humidity) in the unmanaged mountain forest catchment similarly to climate change in a nearby undisturbed forest during the last two decades (Kopáček et al., 2019, *Sci. Total. Environ.* 720, 137518).

4) Using the data on long-term monitoring of soil and water composition prior to, during, and after tree dieback, we developed a new conceptual model explaining changes in DOC leaching from disturbed forests. This model is based on combined cycles of N, DOC, and protons ( $\text{H}^+$ ) in soils (Kopáček et al., 2018, *Environ. Sci. Technol.*, 52, 6291-6299; Kaňa et al., 2015, *PLoS ONE* 10(7): e0134165).

5) The isotope hydrology was applied in the Bohemian Forest catchments, focusing on long-term variations and cycling of nutrients, geochemistry and hydrogeology of lakes in catchments with disturbed and undisturbed forests (Vystavna et al., 2020, *J. Hydrol.* 585, 124834; Vystavna et al., 2018, *Hydrol. Process.* 32 (24), 3650-3661).

### **Aquatic chemistry**

One of our important topics was the cycling of phosphorus (P) in aquatic ecosystems (as a key nutrient for their eutrophication) and the export of P from river basins to waters. Major results include: (i) P binding mechanisms on suspended particles is dominated by iron (Fe) and aluminium (Al) hydroxyoxides (Jan et al., 2015, *J. Soils Sediments* 14, 1620-1629; Borovec et al., 2018, *Sci. Total. Environ.* 624, 1316-1324); (ii) The particulate and dissolved P forms exported from soils to waters greatly vary in their eutrophication potential (Krasa et al., 2015, *Adv. Geoecol.*, 44, 21-34; Hejzlar et al., 2016, *Waste Forum* 4, 225-233; Kopáček et al., 2017, *Wat. Res.* 125, 374-383); (iii) The cycle and utilization of P in reservoirs, as well as the related biological processes, are significantly influenced by reservoir hydrodynamics (Vystavna et al., 2017, *PLoS ONE* 12, e0186917; Gabaldon et al., 2017, *J. Limnol.* 76, 292-304; Znachor et al., 2018, *Sci. Total Environ.* 624, 23-33; Gabaldon et al., 2019, *Freshw. Biol.* 64, 1326-1341).

### **Sediment of lakes and reservoirs, paleolimnology**

#### Paleolimnological and paleoecological identification of Younger Dryas causes in mountain lakes sediment.

We identified traces of the cosmic-impact event and the Laacher See Eruption in the lake sediment from Stará jímka paleolake (the Bohemian Forest). Our results are consistent with the Younger Dryas impact hypothesis and bring an evidence of one or more cosmic airburst events occurring at this time. Discovery of Laacher See tephra was the original finding in the area of the Czech Republic (Kletetschka et al., 2018, *J. Geol.* 126, 561-575).

#### **The research of hydraulics and sediments**

This study was strongly influenced by the demand from water managers and companies dealing with the issues of the influence of sediments of inflow parts of reservoirs on water quality. In the long canyon-shaped reservoirs, particle deposits in

the inflow and transitional parts of the water body play an important, but poorly understood role. To evaluate the importance, volume and distribution of sediments in reservoirs, an algorithm was developed to evaluate their thickness from the sonar record (2 different software). Furthermore, new types of sampling devices (3 utility models) were developed, enabling the implementation of laboratory manipulation experiments with intact chambers with a diameter of 20 cm. A major benefit came with the development of a new methodology for determining the concentrations of sorption-active Fe and Al in sediments (Jan et al, 2015, J. Soils Sediments, 15, 1620-1629), enabling a correct and accurate estimation of their behaviour in aquatic ecosystems. The method was subsequently extended to a tool for predictions of P sorption / desorption on particles (Borovec and Jan, 2018, Sci. Total. Environ. 624, 1316-1324). Comprehensive knowledge was finally summarized in the national methodology for localization of sediments and determination of their properties for the purpose of remediation, which has been certified and implemented by the Ministry of Agriculture.

## **Macrophytes**

### Reproduction recovery and mycorrhiza of *Isoëtes* species in strongly acidified lakes

We studied two aquatic quillworts, which survived the strong water acidification during 1960s–1990s in Plešné and Černé Lake (the Bohemian Forest, Central Europe), but failed to reproduce. We studied the relationships between the recent population recovery and an improvement of lake water quality. The lake water acidification and temperature have a lagged effect on population dynamics via offspring recruitment (Čtvrtlíková et al., 2016, Ecol. Indic. 70, 420-430). To improve our knowledge of plant nutrition, sediment biology and sediment chemistry in pristine quillwort lakes, we studied the incidence and diversity of root-associated fungi in the isoetid functional group of macrophytes in Norway. We isolated new arbuscular mycorrhizal fungal species *Rhizoglyphus melanum* (Sudová et al., 2015, Mycol. Prog. 14, 1-8) and provided spore photographs and sequences of *A. rugosa* (Pereira et al., 2016, Phytotaxa 260, 14-24), both associated with freshwater isoetids in Norway.

### Macrophytes as the main focus of management strategies aimed at biodiversity restoration and conservation

Our experimental study in an artificial lake, created by flooding of an opencast mine, showed that competition (removal of dominant species), or herbivory (preventing fish and crayfish) have significant positive impacts on species richness, composition and cover of macrophyte community (Vejříková et al., 2018, Sci. Rep. 8, 12130). These findings may be applicable during revitalisations of aquatic ecosystems, aiming to the increasing macrophyte biodiversity.

Our study on fishpond macrophyte assemblages under different fish farming management showed significant difference in species richness and abundance of macrophytes between the nursery and main fishponds (Francová et al., 2019, Aquat. Bot. 159, 103131). These results are of interest for conservation of aquatic habitats. Additionally, we have developed floating green islands as a perspective alternative for improving an ecological potential of freshwaters and as a support of littoral habitats in regulated reservoirs (Function sample – Floating island with submerged vegetation for enhancement of littoral in reservoirs).

### **Photochemistry**

The new photochemically induced process was described in freshwaters. Photochemical degradation of dissolved organic matter results in the release of previously organically bound metals (mostly Al and Fe). These metals rapidly hydrolyse, forming insoluble particles with large surface area and positively charged adsorbing sites. Dissolved phosphate adsorbs on them and becomes insoluble. This process increases P-limitation of primary production in headwater environments that receive waters rich in soil dissolved organic matter (Porcal et al., 2017, *Chemosphere* 167, 374-381; Porcal and Kopáček, 2018, *Chemosphere* 193, 1018-1026).

### **Ecosystem analysis and modelling**

The forecast of the development of processes in the aquatic ecosystem of shallow lakes during climate change was within the framework of international cooperation (EC 7<sup>th</sup> FP project REFRESH) on the experiment with mesocosms in a climate gradient across Europe. It has been found that increasing temperature and water level fluctuations have many diverse effects: (i) decrease in diversity and resilience of the zooplankton community (Baho et al., 2015, *Sustainability* 7, 1142-1160; Tavsanoğlu et al., 2017, *Aquat. Ecol.* 51, 257-273); (ii) increase in periphyton biomass in summer (Mahdy et al., 2015, *Aquat. Sci.* 78, 499-510); (iii) retention of nutrients (P and N) increasing in shallow systems with macrophytes (Coppens et al., 2016, *Hydrobiologia* 778, 13-32); and (iv) the ratio between gross primary production and respiration of an aquatic ecosystem depending on the trophic level (Scharfenberger et al. 2019, *Limnol. Oceanogr.* 64, 616-631).

## Research activity and characterisation of the main scientific results

### The LUKEŠ lab

#### Diplonemids – new major players of oceanic plankton

Diplonemids are biflagellated protists that are a sister clade to mostly parasitic kinetoplastids. Until 2015, they were considered a (very) rare component of marine ecosystems. This has changed thanks to the *Tara* expedition, in which one of us (J.L.) participated. Novel barcoding approaches and deep sequencing of an unprecedented set of samples revealed that diplonemids are very abundant (**de Vargas et al., 2015, *Science* 348, 1261605**; **Caputi et al., 2019, *Global Biogeochem. Cycles* 33, 391-419**). We have first introduced this obscure group to the public (**Lukeš et al., 2015, *Curr. Biol.* 25, R702-704**) and subsequently analysed in detail its extreme diversity and abundance (**Flegontova et al., 2016, *Curr. Biol.* 26, 3060-3065**). Next, we have participated in the analysis of single cell-genome sequencing of these protists (**Gawryluk et al., 2016, 26, 3053-3059**; **Okamoto et al., 2019, *J. Euk. Microbiol.* 66, 519-524**), after which we turned our attention to dissect their mitochondrial genomes characterized by extreme editing and *trans*-splicing (**Valach et al., 2016, *RNA Biol.* 13, 1204-1211**; **Faktorová et al., 2018, *RNA Metabolism* (chapter) pp. 145-176**; **Kaur et al., 2020, *Nucl. Acids Res.* 48, 2694-2708**). These molecular studies went hand in hand with morphological and life cycle studies (**Tashyreva et al., 2018 *Protist* 169, 158-179**; **Tashyreva et al., 2018, *mBio* 9, e02447-17m**; **Prokopchuk et al., 2019, *Protist* 170, 259-282**), which allowed us to bring new species into culture and analyse their behavior (**Fig. 1**).

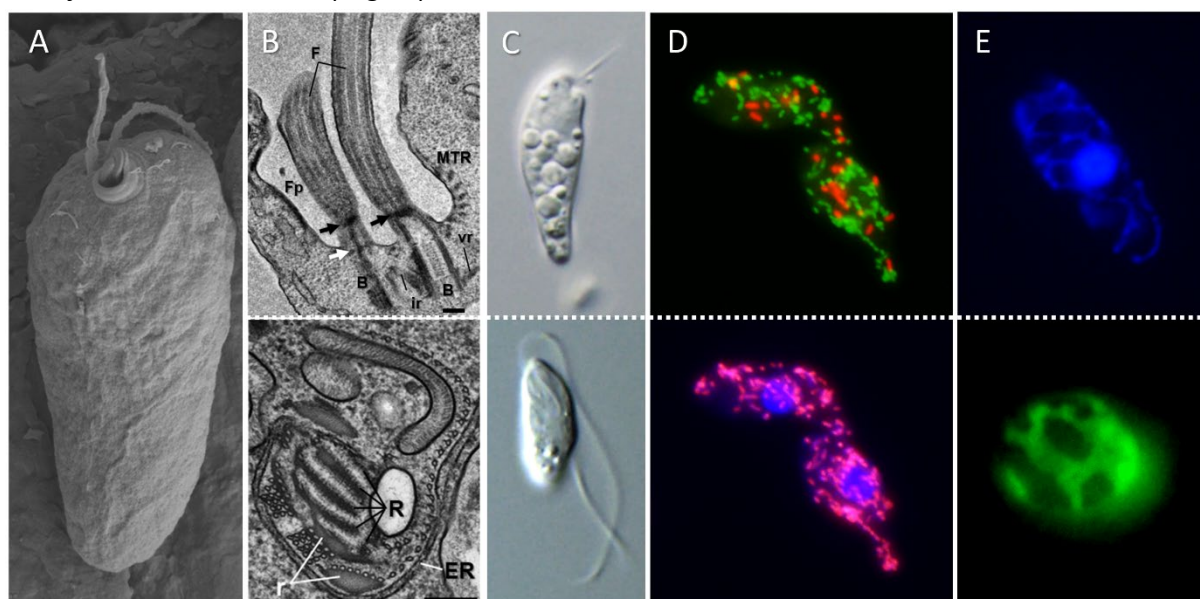
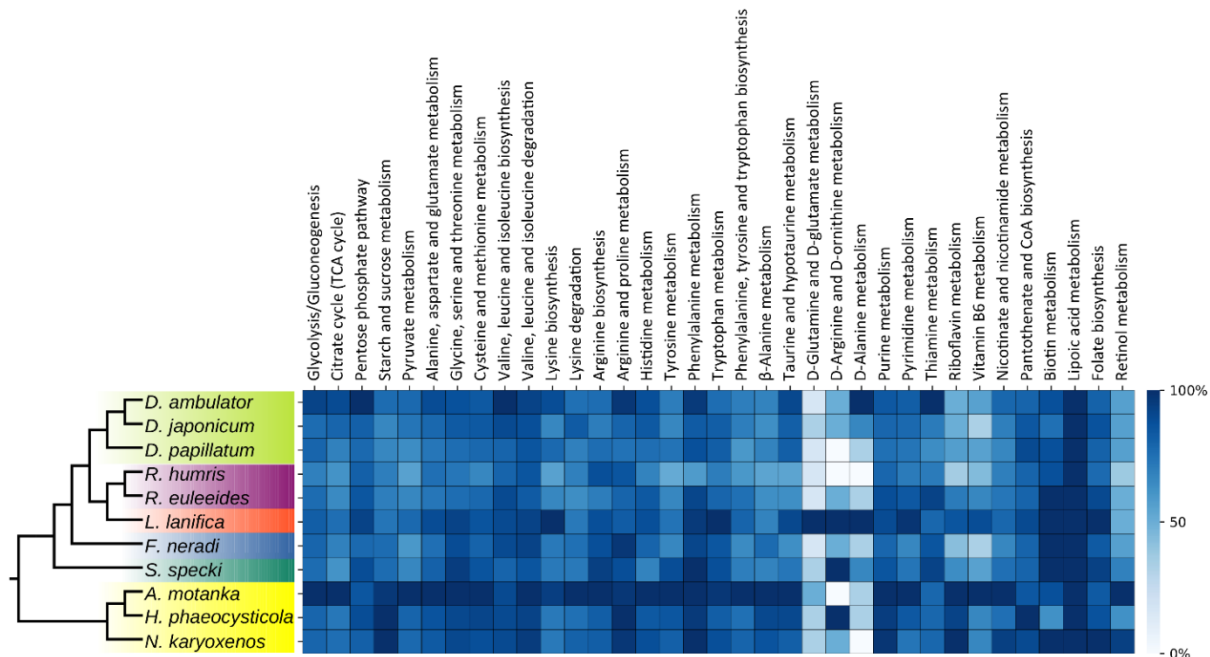


Fig. 1. Description of new diplonemid species based on scanning (A) and transmission (B) electron microscopy, light microscopy (C), fluorescence in situ hybridization of endosymbionts (D) and fluorescence staining of organelles (E).

This way, we noticed the presence of highly reduced endosymbionts with a number of unique features (**George et al., 2020, *Curr. Biol.* 30, 925-933**). We have made first successful steps toward genetic manipulation of diplonemids (**Kaur et al., 2018, *Env. Microbiol.* 20, 1030-1040**). Initial analyses of the nuclear genome coupled with metabolomics, transcriptomic and biochemical studies (**Škodová-Sveráková et al., 2020, *Protist* 171, 125717**) will allow initial insight into their metabolism (**Fig. 2**). Our

long-term aim is to reveal, via this holistic approach, the so far totally unknown ecological function of diplomonids in the oceanic ecosystem.



**Fig. 2.** Completeness (from 0 to 100%) of pathways of carbohydrate, amino acid and vitamin metabolism in various diplomonids estimated using KEGG database.

### Euglenids – poorly known photosynthetic relatives of parasitic trypanosomes

Although photosynthetic euglenids are studied for more than a century, their nuclear genome was sequenced only now by a consortium in which we participated (**Ebenezer et al., 2019, *BMC Biol.* 17, 11**). We have also shown for the first time that their mitochondrial genome is streamlined and transcriptome lacks the editing encountered in sister clades of diplomonids or kinetoplastids (**Dobáková et al., 2015, *Genome Biol. Evol.* 7, 3358-3367; Faktorová et al., 2016, *F1000 Res.* 5, 392**). We have covered euglenozoans in frame of a highly cited revised classification of eukaryotes (**Adl et al., 2019, *J. Euk. Microbiol.* 66, 4-119**). Next, we turned our attention to the protein composition of the plastid, showing a proteome acquired from multiple other organisms, lending credence to the proposed “Shopping Bag” evolutionary hypothesis (**Novák Vanclová et al., 2020, *New Phytol.* 225, 1578-1592**), as well as the mitochondrion, which turned out to contain the highest number of proteins documented in this omnipresent organelle, even exceeding mitochondrial counts recorded in multicellular organisms, and demonstrating features presumed to be exclusive to its parasitic relatives (**Hammond et al., 2020, *Mol. Biol. Evol.* in press**). Furthermore, we have analysed the metabolic capabilities, both *in silico* (**Butenko et al., 2020, *BMC Biol.* 18, 23**) and experimentally (**Škodová-Sveráková et al., 2020, *Pathogens* 9, 317**) and contributed to the development of genetic tools for these protists (**Faktorová et al., 2020, *Nat. Methods* 17, 481-494**).

### RNA editing in the mitochondrion of trypanosomes

The dissection of RNA editing of the uridine insertion/deletion type in the mitochondrion of *Trypanosoma brucei* is a long-term research direction of our lab. Lately, we have focused on its phylogenetic distribution (**Carnes et al., 2015, *PLoS Negl. Trop. Dis.* 9, e3404; Verner et al., 2015, *Int. Rev. Cell Mol. Biol.* 315, 73-151**), composition of editing complexes (**Huang et al., 2015, *RNA* 21, 2088-2102; Mehta et al., 2020, *RNA*,**



in press), frequency of mis-editing (David et al., 2015, *mBio* 6, e01498-15; Gerasimov et al., 2018, *Nucl. Acids Res.* 46, 765-781) and the interaction between proteins and RNA (Dixit et al., 2017, *mBio* 8, e02288-16; Dixit and Lukeš, 2018, *RNA* 24, 1594-1606; Dixit et al., 2020, *Meth. Mol. Biol.* 2116, 303-323) participated in reviews on this intricate mechanism (Read et al., 2015, *WIREs RNA* 7, 33-51; Aphasizheva et al., 2020, *Trends Parasitol.* 36, 337-355).

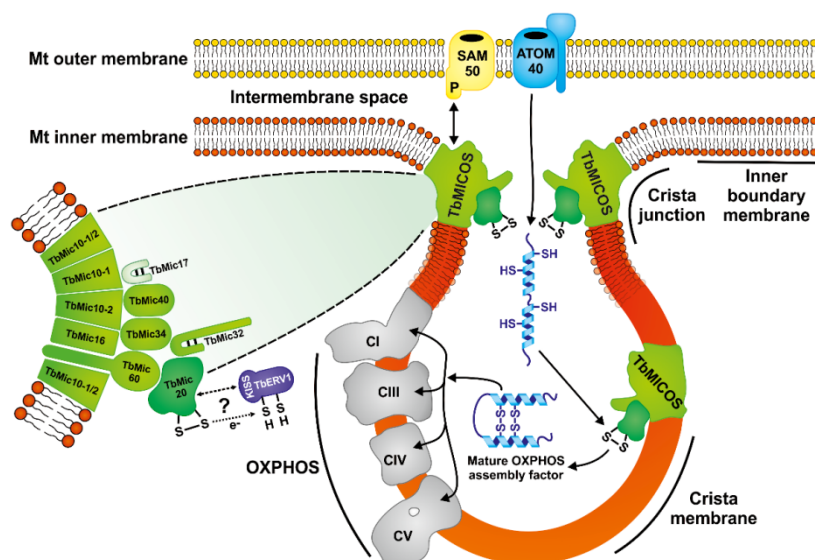
#### Diversity and evolution of trypanosomatid flagellates

Another long-term goal of our lab is to isolate new strains of trypanosomatids (Seward et al., 2016, *Protist* 168, 71-79; Králová et al., 2019, *Int. J. Parasitol.* 49, 1075-1086), describe their morphology, taxonomy and diversity (d'Avila-Levy et al., 2015, *Mem. Inst. Osw. Cruz* 110, 956-965; Yurchenko et al., 2016, *J. Euk. Microbiol.* 63, 198-209; Flegontova et al., 2018, *Env. Microbiol.* 20, 878-889; Votýpka et al., 2019, *J. Euk. Microbiol.* 66, 600-607), their relationship with their insect hosts (Hamilton et al., 2015, *mBio* 6, e01356-15) and endosymbiotic bacteria (Kostygov et al., 2016, *mBio* 7, e01985-15; Kostygov et al., *Front. Microbiol.* 8, 1940, 2017; Silva et al., *Parasitology* 145, 1287-1293, 2018; Harmer et al., *Parasitology* 145, 1311-1323, 2018; Dheilly et al., *PLoS Path.* 15, e1008028, 2019) and their newly discovered viruses (Akopyants et al., 2016 *Genome Announc.* 4, e00711-16; Akopyants et al., 2016 *Genome Announc.* 4, e00715-16; Grybchuk et al., 2018, *PNAS* 115, E506-515; Grybchuk et al., 2018, *mBio* 9, e01932-19). Moreover, we have sequenced and are currently sequencing the genomes of several interesting representatives or members of new lineages (Kraeva et al., 2015, *PLoS Pathog.* 11, 1005127; Flegontov et al., 2016, *Sci. Rep.* 6, 23704; Tanifuji et al., 2017, *Sci. Rep.* 7, 11688; Skalický et al., 2017, *PNAS* 114, 11757-11762), dissect peculiarities of their metabolism (Škodová-Sveráková et al., 2015, *Mol. Microbiol.* 96, 55-67; Oppendoes et al., 2016, *J. Euk. Microbiol.* 63, 657-678; Ishemgulova et al., 2017, *PLoS One* 12, e0174165; Butenko et al. 2018, *Curr. Genomics* 19, 150156; Bianchi et al., 2019, *Mol. Biochem. Parasitol.* 232, 111199), monitor their distribution and prevalence in the mammalian hosts (Jirků et al., 2015, *Int. J. Parasitol. Wld. & Dis.* 4, 277-282; Votýpka et al., 2015, *Int. J. Parasitol.* 45, 741-748), among other aspects of their biology. Finally, we are trying to summarize all the data and be the authority for human-non-pathogenic trypanosomatids (Votýpka et al., 2015, *Trends Parasitol.* 31, 460-469; Lukeš et al., 2018, *Trends Parasitol.* 34, 466-480; Maslov et al., 2019, *Parasitology* 146, 1-27).

#### Unique features of the trypanosomatid and apicomplexan mitochondria

Another continuous line of inquiry of our lab is the dissection of aerobic protist mitochondria, which we believe differ significantly from the typical “eukaryotic” mitochondrion. Due to the available model systems, we study this organelle in kinetoplastid and apicomplexan parasites. In *Chromera*, the photosynthetic predecessor of malaria-causing *Plasmodium*, we have described divergent respiratory chain (Flegontov et al., 2015, *Mol. Biol. Evol.* 32, 1115-1131) and mitochondrial genome (Oborník & Lukeš, 2015, *Annu. Rev. Microbiol.* 69, 129-144; Woo et al., 2015, *eLife* 4, e06974), and its host-parasite interface (Bartošová-Sojtková et al., 2015, *PLoS Pathog.* 11, e1005080). More recently, we have focused on a range of unusual features discovered in the mitochondrion of *T. brucei*, such as its novel tRNA modifications (Sample et al., 2015, *Nucl. Acids Res.* 43, 4262-4273), kinetoplast DNA maintenance (Týč et al., 2015, *mBio* 6, e02425; Lin et al., 2015, *Parasit. Vectors* 8, 665), metabolism (Gnipová et al., 2015, *Euk. Cell* 14, 297-310), iron-sulfur cluster

synthesis (Benz et al., 2016, *Int. J. Parasitol.* 46, 641678; Tonini et al., 2019, *PLoS Pathog.* 14, e1007326) and iron metabolism (Horáková et al., 2015, *FEBS J.* 282, 4157-4175; Basu et al., 2016, *Biochim. Biophys Acta* 1860, 363-370), mitochondrial translation (Hashimi et al., 2016, *PLoS Pathog.* 12, e1005492; Týč et al., 2017, *Mitochondrion* 34, 67-74), heme synthesis pathway (Cenci et al., 2016, *BMC Evol. Biol.* 16, 109; Horáková et al., 2017, *J. Biol. Chem.* 292, 6998-7010), peculiar distribution and activities of catalase (Kraeva et al., 2017, *Inf. Genet. & Evol.* 50, 121-127; Horáková et al., 2020 *FEBS J.* 287, 964-977), kinetoplast DNA segregation (Pena Diaz et al., 2017, *PLoS Pathog.* 13, e1006310), mitochondrial radical oxygen species formation (Speijer et al., 2020, *mBio* 11, e00955-20), changes of mitochondrial proteome during the life cycle (Zíková et al., 2017, *PLoS Pathog.* 13, e1006679), protein import (Pena Diaz et al., 2018, *PLoS One* 13, e0196474) etc. Particularly exciting is intense research on the function and structure and function of the cristae-shaping MICOS complex and its non-canonical role in mitochondrial biogenesis (Fig. 3) (Kaurov et al., 2018, *Curr. Biol.* 28, 3393-3407; Eichenberger et al., 2019, *Mol. Microbiol.* 12, 1731-1743). We are also interested in multifarious factors underlying the diversity of cristae shapes found throughout eukaryotes (Pánek et al., 2020, *Curr. Biol.* 30, R575-588). We are also trying to find out the mechanisms behind major departure from the canonical genetic code, in one branch of trypanosomatids (unpubl. data).



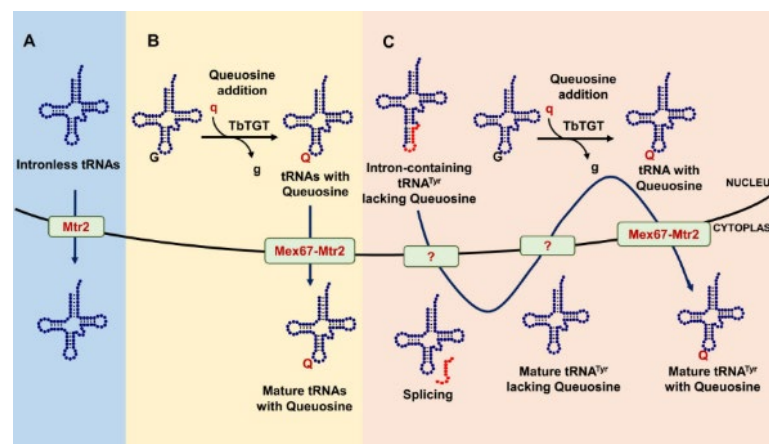
**Fig. 3.** Working model on MICOS function in oxidative folding of respiratory chain assembly factors in the intermembrane space. In this way, MICOS facilitates population of the crista membrane with protein complexes involved in oxidative phosphorylation (OXPHOS).

## The PARIS lab

### tRNA processing and subcellular trafficking in *Trypanosoma brucei*

Pre-tRNAs are transcribed and processed in the nucleus and exported to the cytoplasm, where they get charged with corresponding amino acid for protein synthesis. Using *T. brucei* as our model system, we showed that nuclear export is not just unidirectional. Using combination of cell fractionation and molecular approaches, we described the re-export of the only intron containing tyrosyl-tRNA from the nucleus of *T. brucei*. We see the retrograde pathway as a result of the differential intracellular localization of the cytosolic splicing machinery and the queuosine (Q) modification enzyme tRNA guanine transglycosylase (TGT) in the nucleus (**Lopes et al., 2016, RNA 22, 1190-1199, Kessler et al., 2018, RNA Biol. 15, 528-536**).

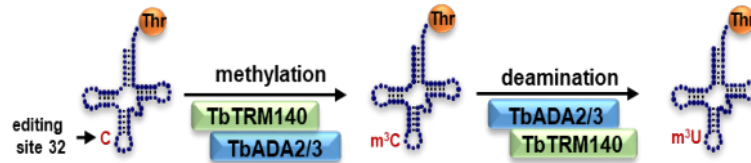
To fully understand this pathway, we needed to identify the export factors responsible for tRNA transport between the nucleus and the cytoplasm. Among the first tested were Exportin-t and Exportin-5, which transport tRNAs in other eukaryotes. Surprisingly, these proteins had no effect on tRNA translocation in *T. brucei*. Therefore, we continued diligently our search and discovered that the canonical mRNA exporters TbMex67 and TbMtr2 have additional role in nuclear export of tRNAs. Subcellular localization of different tRNAs shows that TbMtr2 serves as a general tRNA nuclear exporter, whereas TbMex67 is more specialized, exporting queuosine modified tRNAs. This recent study demonstrates the dynamics of tRNA trafficking in *T. brucei* and its potential impact not only on the availability of tRNAs for protein synthesis but also on their modification status (Fig. 4) (**Hegedüsová et al., 2019, Nucl. Acids Res. 47, 1207-1213**).



**Fig. 4.** Possible pathways for primary export and re-export of tRNAs, where TbMex67 and TbMtr2 serve distinct roles. (A) TbMtr2 has a more general role in tRNA nuclear export exporting intronless, mature, modified or unmodified tRNAs. (B) TbMex67 exporting queuosine-containing tRNAs. (C) Intron-containing tRNA<sup>Tyr</sup> is exported by an unknown exporter (question mark).

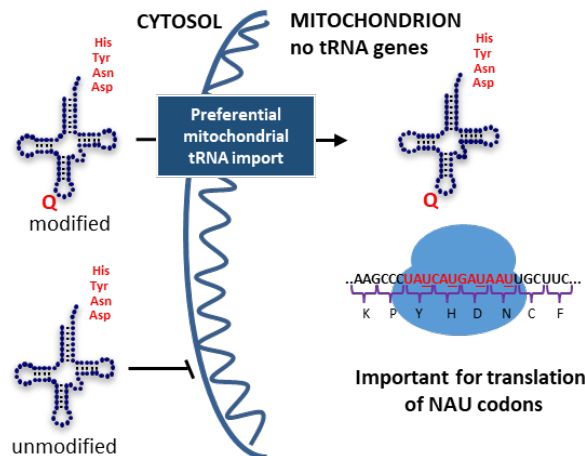
The importance subcellular compartmentalization during tRNA maturation was the subject of another study concerning the functionally interdependent events at the single position of threonyl-tRNA in *T. brucei*. In this joint effort, we show that cytosine 32 in the anticodon loop is methylated to 3-methylcytosine (m3C) as a pre-requisite for C-to-U deamination. Formation of m3C *in vitro* requires the presence of both the m3C methyltransferase TRM140 and the deaminase ADAT2/3. Once formed, m3C is deaminated to 3-methyluridine (m3U) by the same set of enzymes. ADAT2/3 is a highly mutagenic enzyme, but we also show that when co-expressed with the methyltransferase, its mutagenicity is kept in check. This helps to explain how *T. brucei* escapes 'wholesale deamination' of its genome while harbouring both enzymes in the

nucleus. In addition, this observation has implications for the control of the activation-induced cytidine deaminase, another mutagenic deaminase, which is responsible for diversification of the human antibodies and provides a rationale for its regulation (Fig.5) (Rubio et al., 2017, *Nature* 542, 494-497).



**Fig. 5** Editing and methylation at a single site by functionally interdependent activities.

Significant progress was achieved in the functional characterization of TbTGT, the enzyme responsible for Q-tRNA modification in *T. brucei*: 1) We show that TbTGT is a heterodimeric complex (TbTGT1 and TbTG2) responsible for Q-tRNA formation in the nucleus and has a role in the retrograde export. 2) Since the mitochondrial genome of trypanosomes does not encode any tRNAs, they must import the whole set from the cytoplasm. Interestingly, when compared to 50% of Q-tRNAs in the cytosol, *T. brucei* mitochondria contain almost fully modified tRNA. This is explained by preferential transport of modified tRNAs as documented by *in vitro* import assay. Moreover, Q-tRNAs are important in the translation of mitochondrial NAU codons, which makes sense in the context of the extensive U-insertion RNA editing in the trypanosome mitochondrion. Taken together, our results indicate that nutrient-driven queuosine tRNA modification in *T. brucei* regulates cytosolic as well as mitochondrial translation. These findings may have implications during the complex life cycle of these parasites (Fig. 6) (Kulkarni et al., 2020, in preparation for submission into Nature Communications).



**Fig. 6.** Queuosine modified tRNAs are preferentially imported into *T. brucei* mitochondrion to play an important role in organelar translation.

## The ZÍKOVÁ lab

### The role of ATP synthase structure in the biogenesis and bioenergetics of the unique *Trypanosoma brucei* mitochondrion

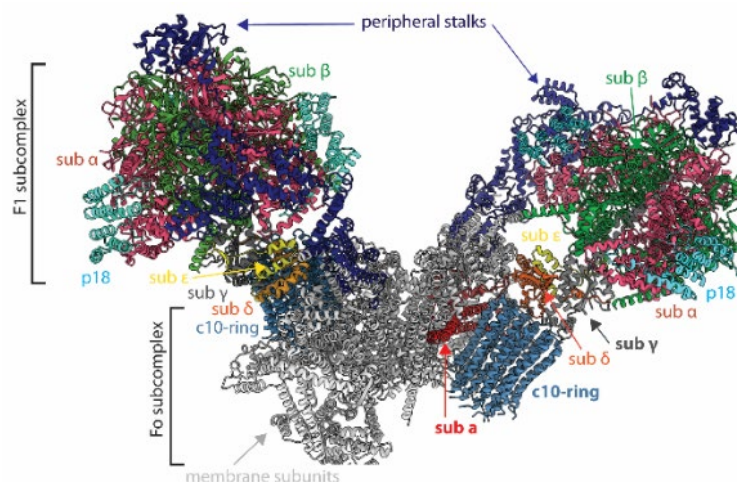
We employ *Trypanosoma brucei* as an excellent model system to understand regulation of ATP synthetic and ATP hydrolytic activities of mitochondrial F-ATP synthases as well as the role of F-ATP synthase dimer rows involved in biogenesis of mitochondrial cristae. As *T. brucei*, a digenetic parasite progresses through a complex life cycle, the activity of F-ATP synthase switches from ATP producing in the insect stage to ATP consuming in the bloodstream stage (BSF). Notably, the highly branched, cristae-containing mitochondrion transitions to a streamlined tubular and cristae-lacking organelle. The infectious BSF *T. brucei* fully depends on the hydrolytic activity of the F-ATP synthase and, thus, this complex is considered as a potential drug target. We showed that highly potent biphosphonates inhibit its ATPase activity, which contributes to the overall cytotoxicity of these inhibitors (Alkhladi et al., 2016, *Int. J. Parasitol. Drugs Drug Resist.* 6, 23-34).

To aid structure-based drug discovery, we developed purification protocol to extract F<sub>1</sub>-ATPase from *T. brucei* mitochondria (Gahura et al., 2019, *J. Vis. Exp.* 143, 10.3791/58334) and solved its crystal structure at 3.2 Å resolution (Montgomery et al., 2018, *PNAS* 115, 2102-2107). The enzyme does not have a conventional structure and differs from previously described complexes by the presence of a p18 subunit associated with the external surface of each of the three alpha-subunits. We further showed that while the p18 subunit is not involved in the catalytic mechanism, it plays an essential role in the assembly of the F<sub>1</sub>-ATPase complex (Gahura et al., 2018, *FEBS J.* 285, 614-628).

In eukaryotes, the ATPase activity of the F-ATP synthases is regulated by a small peptide called Inhibitory factor 1 (IF1) to prevent futile ATP hydrolysis when electron transport chain (ETC) becomes dysfunctional. We identified a homolog of this gene in the *T. brucei* genome, characterized in detail its F<sub>1</sub>-ATPase binding and inhibition properties (Gahura et al., 2018, *FEBS J.* 285, 4413-4423). Furthermore, we showed that its expression is developmentally regulated between the life cycle stages and forced *TbIF1* expression in the BSF parasites caused a drop in the mitochondrial membrane potential followed by death of the parasite (Panicucci et al., 2017 *PLoS Negl. Trop. Dis.* 11, e0005552). In summary, our data validate F-ATPase as a drug target providing protein structure at the atomic resolution and mode of inhibition *in vivo*. Using a high binding affinity of *TbIF1* to the F-ATPases, we purified F-ATP synthase dimers in the presence of lipids and subjected to cryo-EM structure determination. The final atomic model contains 25 subunits, of which 17 are phylum-specific (Fig 7, Gahura et al., manuscript in preparation). We performed an RNAi screen in which we individually silenced expression of all F-ATP synthase subunits. We identified a single subunit, Tb9, which is responsible for dimer formation. This cell line represents an excellent tool to explore the role of F-ATP synthase dimers in discoidal cristae biogenesis. We also explored several subunits in detail showing that the Tb2 subunit is part of the peripheral stalk and is responsible for the membrane attachment of the whole complex (Šubrtová et al., 2015, *PLoS Pathog.* 11, e1004660), while Tb1 subunit is membrane-bound and plays a role in the stability of the proton pore (Hierro-Yap et al, in preparation). Furthermore, we examined the functional and structural link



between F-ATP synthase and ATP/ADP carrier (**Gnipová et al., 2015, *Eukaryot. Cell* 14, 297-310**).



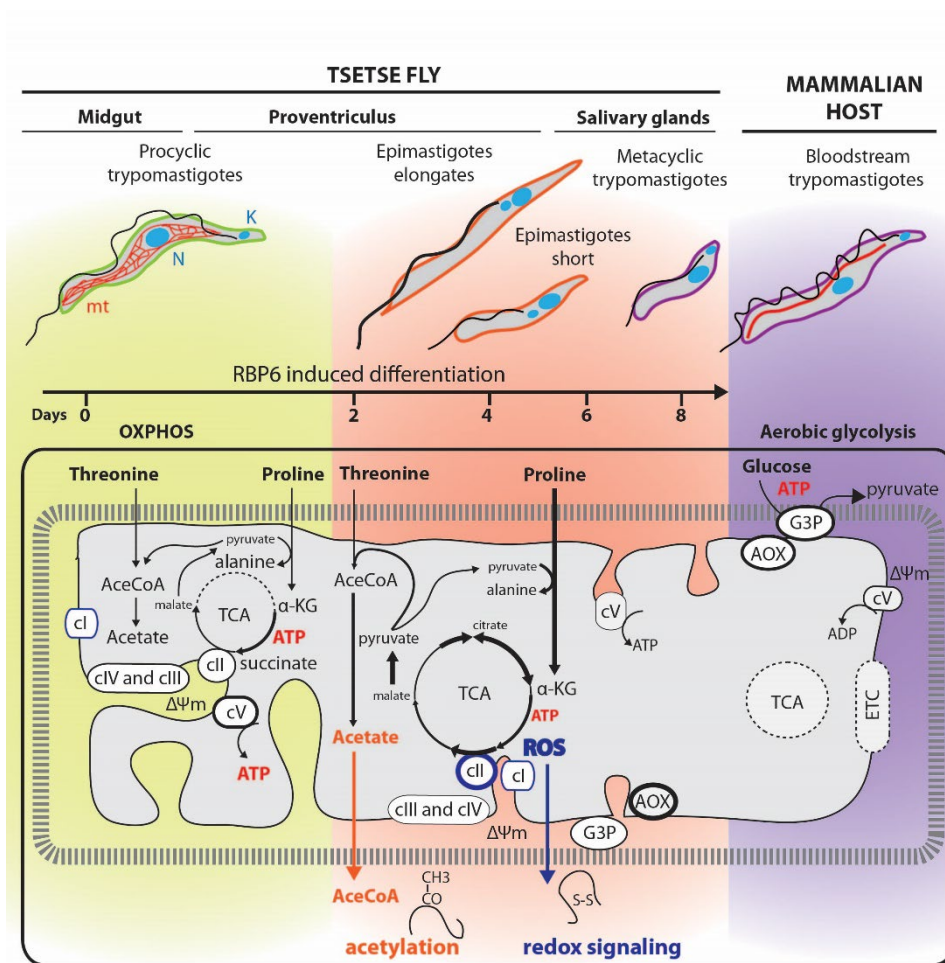
**Fig. 7.** Atomic model of the complete *T. brucei* F<sub>1</sub>F<sub>0</sub> ATP synthase dimer. The 2-Mda complex contains 25 different proteins.

#### Mitochondrial metabolic rewiring and signaling mechanisms that drive cellular differentiation of *T. brucei*

Mitochondrial metabolic remodeling is a hallmark of the *T. brucei* digenetic life cycle since the insect stage utilizes a cost-effective oxidative phosphorylation to generate ATP, while the BSF cell switches to aerobic glycolysis. Molecular mechanisms behind this striking rewiring are elusive. We performed multi-omics and cell-based profiling of cells undergoing *in vitro* induced differentiation to show that changes in expression of ETC components led to increased levels of reactive oxygen species (ROS) which acted as signal molecules driving forward parasite's developmental progression (**Fig. 8, Doleželová et al., 2020, *PLoS Biol.*, in press**).

While the *T. brucei* BSF mitochondrion is very much reduced in its activity and size, we showed, by analyzing publicly available cellular proteomes, that the mitoproteomes of the BSF and the insect form are very similar at the qualitative level (**Zíková et al., 2017, *PLoS Pathog.* 13, e1006679**). This implies that the metabolic potential of this organelle is much higher than previously thought and some of the “forgotten” pathways might be used when the parasite exploits various host niches, e.g., skin or adipose tissues. For example, we showed that under certain nutrient conditions, the bloodstream form mitochondrion employs substrate phosphorylation to produce its own ATP, breaking long-standing dogma about this not-so-dormant organelle (Taleva et al., in preparation). The metabolic flexibility also enables the parasite to quickly adjust to various stressful exo- and endo-stimuli induced by genetic alterations (**Procházková et al., 2018, *Sci Rep.* 8, 5135**) or chemically by drugs (**Zoltner et al., 2020, *J. Biol. Chem.* in press**).



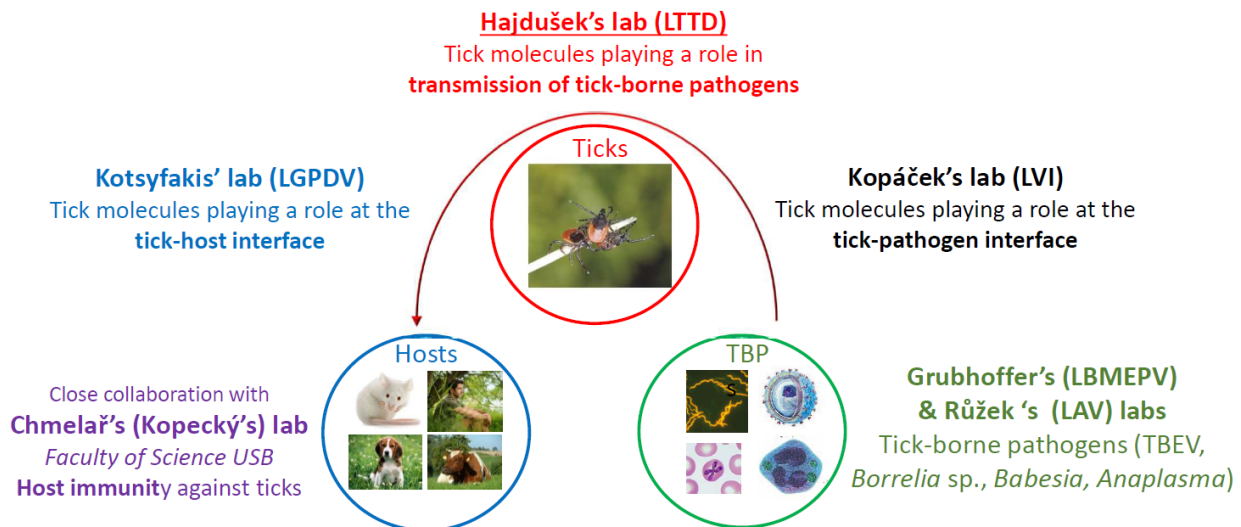


**Fig. 8.** Scheme of remarkable metabolic rewiring during the developmental progression of *Trypanosoma brucei*.

### Acyclic nucleoside phosphonates as potential inhibitors of purine phosphoribosyltransferases in human trypanosomatid parasites

Trypanosomatid parasites (e.g., *T. brucei*, *Leishmania* spp.) do not possess the *de novo* purine synthesis pathway, thus they fully rely on the purine salvage pathway (PSP) for their nucleotide generation. In collaboration with chemists, we designed, synthesized and tested hundreds of acyclic nucleoside phosphonates (ANPs), inhibitors of purine (6-oxo and amino) phosphoribosyltransferases (PRTase). To provide validation of this approach as a drug target, we have RNAi silenced the three 6-oxopurine PRTase isoforms in the infectious stage of *T. brucei*, demonstrating that the combined activity of these enzymes is critical for the parasite's viability (**Doleželová et al., 2018, *PLoS Negl. Trop. Dis.* 12, e0006301**). We also determined crystal structures of all three 6-oxopurine PRTs in complex with several ANPs (**Téran et al., 2019, *FEBS J.* 286, 4721-4736**; **Téran et al., 2016, *Sci. Rep.* 6, 35894**). We are extending our studies to amino-purine PRTs, with an aim to develop inhibitors that strongly impede the essential PSP.

## Research activity and characterisation of the main scientific results



**Fig. 1:** Overview of Budweiser Tick and TBP research at the Institute of Parasitology (BC CAS).

Note: The LVI and LTDD labs were joined in 2020 given the close inter-connections of research topics and overlap of scientific and technical staff.

The reported research activity of the T&TBP team will follow counter-clock-wise the pathogen-vector-host triangle shown in **Fig. 1** and will be arranged in the following chapters:

1. Tick-borne pathogens (TBP)
2. Tick - TBP interactions
3. Tick molecular physiology and systems biology
4. Tick-host interactions
5. Towards applications

Remark: For the cited references, see the complete list of team 17 publications and other outputs. The selected TOP publications are highlighted in bold.

### 1. Tick-borne pathogens

#### 1.1. Tick-borne encephalitis virus (TBEV)

Tick-borne encephalitis (TBE) is an illness caused by tick-borne encephalitis virus (TBEV) which is often limited to a febrile illness, but it may lead to very aggressive downstream neurological manifestations. The disease is prevalent in forested areas of Europe and northeastern Asia and represents the most important tick-borne viral infection on the Eurasian continent.

#### Insights on the pathogenesis of tick-borne encephalitis

We determined the structure of TBEV at near-atomic resolution using cryo-EM (**Fig. 2**). We also determined cryo-EM structures of the native TBEV virion and its complex with Fab fragments of a neutralizing antibody. We found that unlike most of the previously studied flavivirus-neutralizing antibodies, the Fab fragments do not lock the E-proteins in the native-like arrangement, but they interfere with the process of virus-induced membrane fusion (**Fuzik et al., 2018, Nat. Commun. 9, 436**).

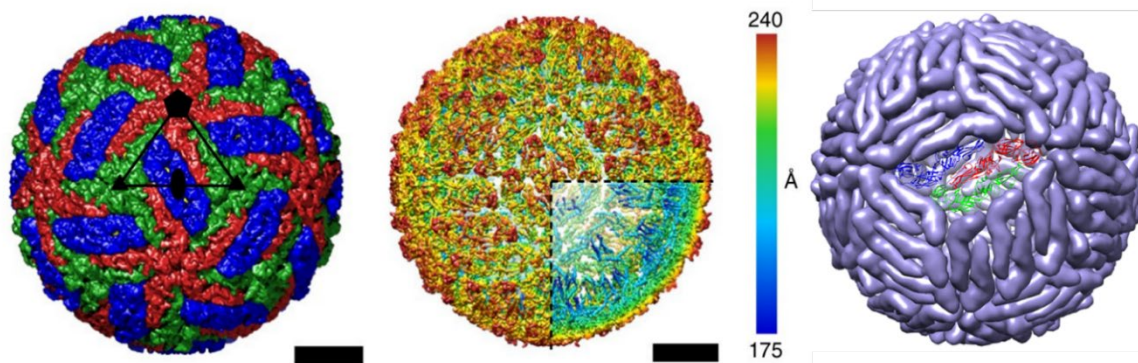


Fig. 2: The structure of TBEV particles was determined at a near-atomic resolution using cryoEM (from Fuzik et al., 2018, Nat. Commun. 9, 436.).

To uncover mutations in the TBEV genome which mediate virus pathogenicity, we provide a platform for analysis of TBEV evolution and the evolution of its antigenic properties since the first TBEV emergence in Europe, reporting the largest number of full genome sequences of patient-derived European TBEV strains to date (Formanova et al., 2015, Ticks Tick-Borne Dis. 6, 38-46).

The sensitivity of the host to TBEV infection is determined by several factors including the host genetic background. Our linkage analysis revealed a novel suggestive survival-controlling locus on mouse chromosome 7 linked to the genetic marker D7Nds5 (44.2 Mb) (Palus et al., 2018, BMC Neurosci. 19, 39).

The mechanism of blood-brain barrier (BBB) breakdown during TBE, as well as TBEV entry mechanism into the brain is unclear. We developed an innovative in vitro BBB model, and using this model we discovered that the virus crosses the BBB via a transcellular pathway, without compromising the integrity of the cell monolayer. The results indicate that human brain microvascular endothelial cells forming the BBB may support TBEV entry into the brain without altering BBB integrity (Palus et al., 2017, Virology, 507, 110-122).

Infection of brain cells with TBEV was investigated using diverse approaches. We used a multiplexed Luminex system to measure multiple cytokines/chemokines and growth factors in mouse serum samples and brain tissue, in human neuroblastoma cells (SK-N-SH) and primary cortical astrocytes (HBCA), which were infected with a highly pathogenic TBEV strain. Our results indicate that both neurons and astrocytes are potential sources of pro-inflammatory cytokines in TBEV-infected brain tissue. Infected/activated astrocytes produce cytokines/chemokines that stimulate the innate neuronal immune response, thus limiting virus replication and increasing the survival of infected neurons (Pokorna Formanova et al., 2019, J. Neuroinflammation, 16, 205). Similar results were obtained also with human astrocytes infected with Zika virus (Stefanik et al., 2018, BMC Neurosci. 19, 5). Morphological changes in TBEV-infected human primary neurons were investigated using electron tomography. This study was the first to characterize the 3D topographical organization of membranous whorls and autophagic vacuoles in TBEV-infected human neurons (Bily et al., 2015, Sci. Rep. 5, 10745).

Novel biomarkers associated with TBEV infection in the brain were identified; these novel inflammatory markers have important implications for TBE pathogenesis (Pokorna Formanova et al., 2019, J. Neuroinflammation, 16, 205; Palus et al., 2015, J. Med. Virol. 87, 885-892).

Additional results provide novel insights into the response of human neuronal cells to TBEV infection and the antiviral effects of type I and III IFN, contributing to understanding of the pathogenic effects upon infection (Selinger et al., 2017, J. Gen. Virol. 98, 2043-2060). To determine whether TBEV interacts with this host process in its natural target cells, we analyzed de novo protein synthesis in a human cell line derived from cerebellar medulloblastoma, observing a significant decrease in the rate of host protein synthesis, including the known interferon-stimulated gene viperin. In addition, we demonstrated that TBEV specifically targets the transcription of RNA polymerase I. These new findings further increase our understanding of TBEV interactions with a key target host cell type (**Selinger et al., 2019, PLOS Neglect. Trop. Dis. 9, e0007745**).

### 1.2. *Borrelia burgdorferi* sensu lato:

Lyme disease (LD) is the most common vector-borne zoonotic disease in Europe that is caused by certain species of spirochetes from the *B. burgdorferi* sensu lato (s.l.) complex, mainly by *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto (s.s.).

Studying host complement sensitivity or resistance of *Borrelia* species is an ideal model to understand the contribution of vertebrate host factors to the pathogen demographic processes (Ticha et al., 2016, Vector-Borne Zoonotic Dis. 16, 13-19).

Survival of spirochetes, in a hostile environment such as the presence of antibiotic pressure is achieved by changes in the level of bacterial gene expression leading to the establishment of a persistent subpopulation (persisters) that remains viable and able to reversibly convert into motile bacterial forms once again in a favorable growth environment, thus causing disease relapse (Rudenko et al., 2019, Parasit. Vectors, 12, 237). We confirmed this hypothesis by isolating live replicative spirochetes from patients with persistent signs of LD after extended antibiotic treatment (Rudenko et al., 2016, Clin. Microbiol. Infect. 22, 267.e9-15). Multilocus sequence analysis of isolated spirochetes confirmed the causative agent of infection as *B. burgdorferi* s.s. and *B. bissettii* (first isolation of live *B. bissettii* ever), validating the involvement of the latter species in human Lyme disease worldwide (Golovchenko et al., 2016, Parasit. Vectors, 9, 68). To understand the response of the LD spirochete exposed to stressful conditions and to assess their viability, we used a previously developed correlative cryo-fluorescence and cryo-scanning microscopy technique (**Strnad et al., 2015, Sci. Rep. 5, 18029**) for examining cell viability without the necessity to maintain suitable culture conditions during viability assays, thus focusing on rare and transient events. Spirochetes were found as able to survive the hostile environment pressure due to changes in their morphology, by switching to alternative forms, but keeping their viability as visualized by this technique (**Vancova et al., 2017, Front. Microbiol. 8, 596**).

### 1.3. *Babesia* spp.

Tick-transmitted apicomplexan parasites of the genus *Babesia* represent an important worldwide veterinary threat and an emerging zoonosis. During the past 5 years (spanning the Czech-French dual PhD program of Marie Jalovecká at our Institute and in the laboratory of Laurence Malandrin, ONRIS, Nantes, France), we have developed a *Babesia divergens* cultivation system in bovine erythrocyte cultures and established the unique laboratory mouse-tick-*Babesia microti* quantitative acquisition model. Next, we characterized molecular markers of *Babesia* gametocytes and tested several stimulants of gametocytogenesis, which are important for the sexual cycle of the parasite in the tick midgut and thus for the tick infection (Jalovecka et al., 2016, Parasit.

Vectors. 9, 439). Two comprehensive reviews were also published in the topic (Jalovecká et al., 2018, Front. Cell. Infect. Microbiol. 8, 248; **Jalovecká et al., 2019, Trends Parasitol. 35, 356-368**).

#### 1.4. Epidemiology of tick-borne diseases

Aiming at a disease risk prediction, our eco-epidemiological studies focused on the estimation of tick activity, prevalence of tick-borne pathogens and identification of key factors that determine these parameters. Host-seeking activity of ticks and tick-borne pathogen prevalence was found to be highly variable spatially. Vegetation cover appeared to be a crucial factor influencing the rate of the *I. ricinus* occurrence. The region of South Bohemia was confirmed to be TBE and Lyme disease (LD) high-risk area (Honig et al., 2015, Ticks Tick-Borne Dis. 6, 559-567). LD spirochetes detected in ticks displayed high variability in the representation of different genospecies, adding to LD-causing *Borrelia* spp. traditionally recognized in Europe other spirochete species of medical importance (namely *B. bissettii*, *B. spielmanii*, *B. bavariensis*, *B. lusitaniae*, *B. valaisiana*) (Daniels et al., 2016, Epidemiol. Mikrobiol. Imunol. 65, 183-193).

Europe-wide meta-analysis of the *B. burgdorferi* s.l. prevalence in areas undergoing current dynamic changes in tick/pathogen distribution (**Strnad et al., 2017, Apl. Environ. Microbiol. 83, e00609-17**), confirmed significant differences between European regions, with the highest infection rates detected in Central Europe. The most common genospecies were *B. afzelii* and *B. garinii*, despite a negative correlation of their prevalence rates. Collaborative work demonstrated that *I. ricinus* infected by *B. afzelii* via co-feeding can transmit the spirochete to vertebrate hosts (Belli et al., 2017, Sci. Rep. 7, 5006).

The broad-range survey of vector-borne pathogens revealed that *B. burgdorferi* s.l. spirochetes were the most common pathogen detected in *I. ricinus* in South Bohemia, followed by *Rickettsia* spp. (4.1%), *Anaplasma phagocytophilum* (2.8%) and *Babesia* spp. (1.4%). To offer easy public access to information about the risk of infection by tick-borne diseases, spatial models of tick abundance and probability of tick infection by *B. burgdorferi* s.l. and TBEV were constructed based on correlations with environmental variables (Honig et al., 2019, Int. J. Environ. Res. Public Health. 16, 1173). In the case of involvement of non-traditional tick vectors, our recent findings on spirochete-tick interactions suggest that, when compatible spirochete strains meet an appropriate tick population, maintenance and transmission of pathogens may occur (**Rudenko et al., 2016, Emerg. Microbes Infect. 5, e48**).

## **2. Tick-TBP interaction**

### 2.1. TBEV interaction with the tick vector

TBEV interaction with the tick vector was investigated using a systems biology approach to describe tick cell response to TBEV. Changes were revealed in the expression of genes and proteins involved in cell metabolism, transport, protein folding, nucleic acid processing, signaling, cell stress and immunity, revealing a complex response of tick cells to viral infection at both the transcriptome and proteome levels (Weisheit et al., 2015, Parasit. Vectors, 8, 599). To support the use of tick cell lines as a model system for the study of host-vector-pathogen interactions, mass spectrometry approach (MALDI-TOF MS) was applied to profiling of tick cell lines, revealing, for the first time, organ-specific MS signals (Loginov et al., 2019, Parasit. Vectors., 12, 212).



## 2.2. Tick immune system and its interaction with TBP

Tick-borne pathogens are capable of avoiding the tick innate immunity in order to persist, multiply and ultimately be transmitted to the host during tick feeding. We have long been focused on the tick complement-like system and the role it plays in the ticks' vectorial capacity. Earlier we found that ticks are exceptional organisms in the sense that they possess representatives of all major groups of the invertebrate thioester-protein family. Using the method of RNAi linked to a robust in-vitro phagocytic assay we gradually mapped the involvement of individual tick complement-like molecules in the phagocytosis of model microbes (*Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *B. afzelii*) by tick hemocytes (Urbanová et al., 2015, Dev. Comp. Immunol. 48, 55-64; Urbanová et al., 2017, Front. Cell. Infect. Microbiol. 7, 73). Similarly, we have functionally characterized the putative C3-convertase IrC2/Bf, a homolog of human factors B and C2 (Urbanová et al., 2018, Dev. Comp. Immunol. 79, 86-94). We have also deciphered the roles of tick fibrinogen-related lectins tagged as ixoderins, in opsonising model microbes for phagocytosis (**Honig Mondekova et al., 2017, Front. Cell. Infect. Microbiol. 8, 509**). Moreover, a tick-mouse transmission model to study borreliosis comprising the European tick *I. ricinus* and a local strain of *B. afzelii* has been developed (Validated technology, Production of SPF and infected ticks of genus of Ixodes and their use for testing antiparasitic agents, 2017). Using this model (see below), we have conclusively demonstrated in the above mentioned papers, that RNAi silencing of tick complement-like molecules and fibrinogen-related lectins, as well as blocking of pathogen phagocytosis by tick hemocytes, do not affect the transmission of *B. afzelii* from infected nymphs to naïve mice, suggesting that humoral and cellular factors within the tick haemocoel do not play a role in the transmission of LD spirochetes. We also investigated the evolution of the antimicrobial activity of *I. ricinus* defensins (Tonk et al., 2015, Dev. Comp. Immunol. 53, 358-365), and found that DefMT3, DefMT5, and DefMT6 showed in vitro antimicrobial activity against distantly related bacteria, broadening the scope of anti-microbial peptide (AMP) research and highlighting the overlooked ability of arthropod defensins to act against distantly-related microorganisms encountered by ticks during blood feeding or off-host periods.

We hypothesized that tick-pathogen associations evolved to form "intimate epigenetic relationships" similar to those described for *Theileria* spp. in which the pathogen induces transcriptional reprogramming in infected ticks (Cabezas-Cruz et al., 2017, Front. Cell. Infect. Microbiol. 7, 74). These phenotypic and genetic changes may have the potential to be transmitted to the next generation of ticks. As a result, the ecological associations between ticks, vertebrates, and pathogens would evolve to maximize pathogen circulation in these communities. This is also discussed when looking at identifying the molecular drivers involved in tick-pathogen interactions and vector-competence (de la Fuente et al., 2017, Front. Cell. Infect. Microbiol. 7, 114). (*Note: According to WOS, this review paper belongs to the top 1% of highly cited papers in microbiology*)

## 2.3. Transmission of TBP

Traditionally, most of the research on LD transmission and infectivity has been performed in the USA using *I. scapularis* nymphs, and *B. burgdorferi* s.s. spirochetes. In contrast, understanding the dynamics of Lyme borreliosis in Europe lags far behind the research carried out in the USA, mainly because of the existence of several different species in the *B. burgdorferi* s.l. complex that cause LD in Europe. Using our



Borrelia transmission model, we obtained data to fill this information gap and to bring a comprehensive view of *B. afzelii* transmission cycle. We show that the transmission cycle of *B. afzelii* differs from the model transmission cycle described for *B. burgdorferi*/*I. scapularis* in the USA and suggest that *B. afzelii* is probably not transmitted via salivary glands but most likely through the “midgut-to-mouthpart” route, thus supporting the original concept of *Borrelia* transmission proposed by Dr. W. Burgdorfer. We further demonstrate that spirochetes become infective in the tick midgut during the blood feeding course. Our data support the focus of our future research on the molecules involved in *Borrelia* interactions within the tick gut (**Pospíšilová et al., 2019, Infect. Immun. 87, e00896**).

Concerning the Anaplasma model, we have participated in the screening of *I. scapularis* transcriptomes and proteomes after infection with *A. phagocytophilum* (**Ayllón et al., 2015, PLOS Genetics, 11, t1005120**), and characterized several components of the tick RNA interference pathway which play a role in the infection of the tick cells (Ayllón et al., 2015, PLOS One, 10, e0133038). Moreover, we revealed the existence of a functionally distinct NLRC4 inflammasome involved in the recognition of the specific rickettsial agent by the vertebrate host (**Wang et al., 2016, PLoS Pathog. 12, e1005803**).

### 3. Tick molecular physiology and systems biology

#### 3.1. Blood meal processing in ticks

Ticks are completely dependent on the host blood as their exclusive source of nutrients. Tick adaptations to their extreme gluttony resulted in major physiological departures from their hosts that intuitively serve as potential targets for efficient tick control. Therefore, our long-term research has been focused on the specific physiological processes associated with blood meal processing in ticks. We have gradually deciphered the multi-enzyme network of cysteine and aspartic peptidases involved in intracellular digestion of hemoglobin. The artificial membrane feeding of *I. ricinus* females on hemoglobin-depleted serum (see below) made it possible to ascertain, that serum albumin is digested to amino acids by the same cadre of peptidases despite different trafficking of hemoglobin and albumin in tick digestive cells (Sojka et al., 2016, Ticks TickBorne Dis. 7, 604-613). We further revealed the existence of multiple isoenzymes of digestive peptidases (cathepsin L, B, C, legumain, and cathepsin D) involved in protein digestion. The long-term collaboration with the Institute of Organic Chemistry and Biochemistry, CAS, resulted in a comprehensive structural characterization of the *I. ricinus* cathepsin D (IrCD1) and the discovery of a novel structural mechanism of allosteric regulation of this enzyme, a research finding of high general interest (**Hanova et al., 2018, Cell Chem. Biol. 25, 318-329**).

Moreover, we characterized multiple genes encoding tick legumains (aspartic endopeptidases, AEs) found in the genome of the American hard tick *Ixodes scapularis*. The predominant form is an ortholog of the previously described IrAE1 which is up-regulated by blood-meal uptake of all tick stages (larvae, nymphs, adults). By contrast, expression of the other prevailing form, namely IrAE2, is relatively constitutive in all living stages including eggs (Hartmann et al., 2018, Int. J. Parasitol. 48, 167-178).

### 3.2. Heme and iron metabolism in ticks

Implementation of tick *in-vitro* membrane feeding in LVI allowed us to address so far experimentally inaccessible questions in tick physiology. Artificial feeding *I. ricinus* females on the full blood vs. hemoglobin-depleted serum (**Fig. 3**) revealed that serum-fed female ticks can successfully accomplish feeding and lay comparably large egg clutches. Genome-wide screening of *Ixodes* sp. confirmed that ticks are heme-auxotrophs not capable of endogenous heme biosynthesis. Moreover, ticks lack the gene encoding heme oxygenase, meaning that ticks cannot recycle iron from the degraded heme -as most other metazoans do. Further functional in-depth investigation using membrane feeding and RNAi revealed that hemoglobin from the blood diet is essential only as a source of heme for embryogenic development, but not an essential source of amino acids for synthesis of yolk proteins (vitellogenesis), and not a source of iron needed for tick metabolic demands (**Perner et al., 2016, elife, 5, 12318**).



**Fig. 3:** Image of engorged *I. ricinus* female ticks fed *in vitro* on the whole blood (dark) and hemoglobin-depleted serum (yellow).

This picture was used for the Elife homepage (Perner et al., 2016, elife, 5, 12318) and also was displayed as the Biomedical Picture of the Day (<http://bpod.mrc.ac.uk/archive/2016/6/19>)

To address specific adaptations to the hemoglobin-rich diet, we compared the midgut transcriptomes of genetically homogenous *I. ricinus* female siblings fed either on bovine blood or hemoglobin-depleted serum. This RNA-Seq analysis resulted in high quality midgut transcriptomes (**Perner et al., 2016, Sci. Reports., 6, 36695**) which are further exploited for follow-up studies. Surprisingly, the number of identified hemoglobin-responsive genes was rather low. One of the most hemoglobin-inducible transcripts encoded a specific delta-class glutathione S-transferase (tagged as IrGST1) that was further characterized as an intracellular heme scavenger (**Perner et al., 2018, Insect Biochem. Mol. Biol. 95, 44-54**). The comprehensive comparison of tick heme biology with other metazoan parasites was published as a review paper with the participation of highly recognized experts in the field (**Perner et al., 2019, Trends Parasitol. 32, 213-225, cover page**).

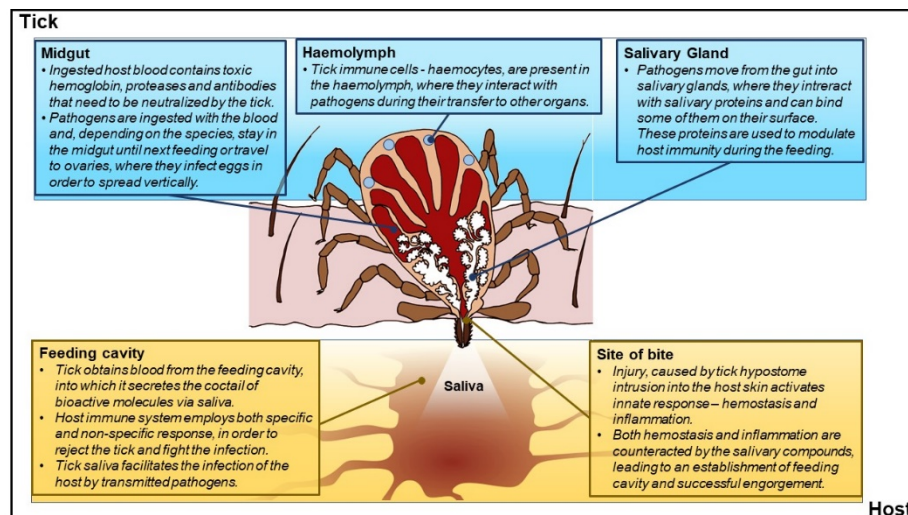
### 3.3. Systems Biology

The plentiful bioactive compounds produced by the tick salivary glands play a principal role in tick-host interactions. We used massive *de novo* sequencing to characterize the transcriptional dynamics of the salivary and midgut tissues of nymphal and adult *I. ricinus* at various time points after attachment on the vertebrate host (**Kotsyfakis et al., 2015, Sci. Rep. 5, 9103**).

We identified hemocyte transcripts from semi-engorged *I. ricinus* females by mass sequencing (NGS) of a hemocyte transcriptome and by annotating immune-related

transcripts based on their hemocyte abundance and/or their ubiquitous distribution (Kotsyfakis et al., 2015, PLoS Negl. Trop. Dis. 9, e0003754).

Using artificial membrane feeding, we could compare salivary gland transcriptomes (sialomes) from *I. ricinus* ticks fed naturally on laboratory rabbits with those from ticks membrane-fed on rabbit blood depleted of host immune components. For the first time we analyzed 18 individual female siblings (transcriptomes were coming from a single tick) which allowed us also to investigate the dynamics of sialome repertoire switches in response to the active host immune system during the early stages of tick feeding (Perner et al., 2018, PLOS Negl. Trop. Dis. 12, e0006410). Overall, the coding sequences deposited in GenBank significantly increased the publicly available gene/transcript dataset for the tick *I. ricinus* now supporting biochemical, pathophysiological, and translational studies in this model organism. Our systems biology approaches (Chmelař et al., 2016a, Trends Parasitol. 32, 242–254) represent a milestone in the field, and the application of quantitative proteomics to ticks with unknown genomes has provided deeper insights into the molecular mechanisms underlying tick-host-pathogen interactions (Fig. 4).



**Fig. 4: The complex interactions between ticks, hosts, and tick-borne pathogens.**

Pathogens transmitted via the “salivary-route” (e.g., *Borrelia burgdorferi* s.s., *Anaplasma* sp., *Babesia microti*) have to migrate through the tick midgut, interact with the tick innate immune system present in the hemolymph and infect tick salivary glands. Pathogens are then injected into the host along with tick salivary components that suppress the local host homeostatic responses, hereby facilitating infection of the host. Some TBPs (e.g., TBEV or *Babesia* sp.) can be transmitted to the progeny transovarially. A direct “gut-to-mouth” route has been recently proposed for the *Borellia afzelli* transmission by *I. ricinus*. Adapted from Chmelař et al., 2016a, Trends Parasitol. 32, 242–254.

## 4. Tick-host interactions

### 4.1. Tick saliva-host interaction

Given that tick salivary secretions are critical to the success of the tick transmission lifecycle, we reviewed the known interactions between tick saliva/salivary gland extracts and the vertebrate immune system (Kotál et al., 2015, J. Proteomics. 128, 58–68). This allowed us to promote the hypothesis that tick salivary protein family members display redundancy and pluripotency in their action on host homeostatic responses (Chmelař et al., 2016b, Trends Parasitol. 32, 368–377). This concept is observed in major groups of protease inhibitors secreted in tick saliva (Chmelař et al., 2017, Front. Cell. Infect. Microbiol. 7, 216). We described and functionally and

structurally characterized the novel immunomodulatory cysteine protease inhibitor - **Iristatin**, secreted by the salivary glands of feeding *I. ricinus* ticks (**Kotál et al., 2019, Cell. Mol. Life Sci. 76, 2003–2013**). Iristatin inhibited the proteolytic activity of host cathepsins, diminished pro-inflammatory cytokine production by different host immune cell populations, and inhibited OVA antigen-induced CD4<sup>+</sup> T cell proliferation and leukocyte recruitment *in vivo*. These pluripotent actions may be exploitable as an immunotherapeutic.

We also described novel arthropod miRNAs in *I. ricinus* salivary glands and saliva. *De novo* prediction yielded 67 miRNAs, of which 35 were not present in miRbase, potentially of exosomal origin. These data represent the first direct evidence of tick miRNA-mediated regulation of vertebrate host gene expression at the tick-host interface (**Hackenberg et al., 2017, RNA, 23, 1259–1269**), providing candidate miRNAs for drug discovery efforts.

Finally, we showed that tick bites induce anti-alpha-gal antibodies in dogs which leads to meat allergies (Hodzic et al., 2019, Vaccines, 7, 114).

#### 4.2. Tripartite vector/pathogen/host interactions

Noncoding RNAs (ncRNAs) are now known to be (i) transmitted by disease vectors to possibly modulate vertebrate host responses and (ii) regulated in the vertebrate host by parasites to favour parasite survival. We provided an overview of the involvement of ncRNAs in the parasite-vector-host triad and their effect on host homeostasis (**Bensaoud et al., 2019, Trends Parasitol. 35, 715–724**). We also reviewed the role that nanovesicles play during pathogen spread, host colonization, and disease pathogenesis (Chávez et al., 2019, J. Cell Sci. 132, jcs224212), focusing on a potential role for arthropod exosomes in this tripartite interaction, especially as carriers of long non-coding (lnc)RNAs (Hackenberg and Kotsyfakis, 2018, Trends Parasitol. 34, 549-552).

We also conducted a comprehensive review of the overlooked field of the protein-carbohydrate interactions and the overall glycobiology of ticks and TBPs. The review focuses on glycosylated proteins as well as glycan-binding proteins in ticks and TBPs underscoring the interactions that allow the infection of ticks and mammalian hosts by various bacteria and TBEV (Vechtova et al., 2018, Parasit. Vectors, 11, 594).

### **5. Towards application**

#### 5.1. Development of novel countermeasures effective against TBEV and other arboviruses

There are no antiviral therapies for treating TBEV (or other arboviral) infection. A series of candidate molecules, especially nucleoside analogues, were tested for their ability to inhibit TBEV replication (**Eyer et al., 2015, Antimicrob. Agents Chemother. 59, 5483-5493**) or the Zika Virus (**Eyer et al., 2016, J. Infect. Dis. 214, 707-11**; Fernando et al., 2016, Mol. Biotechnol. 58, 801-806). Several molecules were identified to have a high antiviral activity and some of them, mainly 7-deaza-2'-C-methyladenosine, were active also in animal models of TBEV and West Nile Virus infection (**Eyer et al., 2019, Antimicrob. Agents Chemother. 63, e02093-18**). The mode of action was discovered and described in the case of methylated nucleoside analogues and galidesivir (BCX-4430) (**Eyer et al., 2019, J. Virol. 93, e00367-19**). TBEV resistance to antivirals was generally associated with a strong attenuation of the virus (Eyer et al., 2016, Antiviral Res. 133, 119-129; Eyer et al., 2017, Antiviral Res. 142, 63-67; Eyer et al., 2017, J.

Viol. 91, e01028-17; Valdés et al., 2017, Biochem. Biophys. Res. Commun. 492, 652-658; Hercik et al., 2017, Biochem. Biophys. Res. Commun. 492, 652-658; Haviernik et al., 2018, Viruses, 10, 184; Krol et al., 2019, Molecules, 24, 1129). A novel veterinary vaccine against TBEV was developed and commercialized (Salat et al., 2018, Vaccine, 36, 7257-7261)

## 5.2. Drug and anti-tick vaccine development

Two primary interests have driven research into tick salivary secretions: the search for suitable pathogen transmission blocking or "anti-tick" vaccine candidates and the search for novel therapeutics derived from tick salivary components for immune-mediated human pathologies such as allergic asthma (Klein et al., 2015, J. Immunol. 195, 621-631). We next reviewed the major tick salivary protein families exploitable in medical applications (Chmelař et al., 2019, Front. Physiol. 10, 812). We also reviewed the cutting-edge strategies towards new or improved vaccines against ticks and tick-borne diseases (de la Fuente et al., 2016, Parasite Immunol. 38, 754-769). In this review as well as in another comprehensive review article, we emphasize the rational approach based on deep knowledge of tick physiology that may lead to the identification of tick associated factors as candidate molecules in designing "anti-tick" vaccines targeting pathogen transmission (Rego et al., 2019, Parasit. Vectors, 12, 229). This rational target-based strategy focused on "attractive" tick molecules playing crucial roles in tick-specific physiological processes has also the potential for developing novel potent "anti-tick" preparations (acaricides) (Kopáček et al., 2018. Ectoparasites: Drug Discovery Against Moving Targets, Willey, pp. 139-165).

The use of Ferritin 2 (FER2) as a potential "anti-tick" vaccine has been internationally protected by our group (Patent EP2324053 - Ferritin 2 for the host immunization against ticks, 2015) and is currently being tested among other vaccine candidates against the cattle tick *Rhipicephalus microplus* in frame of the CATVAC consortium (Schetters et al., 2016, Parasit. Vectors, 9, 105). We have further focused on another 11 *in silico* identified genes encoding proteins potentially involved in tick iron and heme metabolism. Neither RNAi silencing nor experimental vaccination with selected recombinant proteins displayed a host protective potential against ticks, which is comparable to that of targeting tick FER2 (Hajdusek et al., 2016, **Ticks TickBorne Dis. 7, 565-572**). We have also tested vaccination potential of crude tick midgut and salivary gland extracts for cattle vaccination against ticks (Knorr et al., 2018, Front. Physiol. 5, 1696).

To develop a specific and efficient treatment of babesiosis, we focused on the rapidly developing topic of selective proteasome inhibition as a strategy to target protozoan parasites, including *Plasmodium spp.* In collaboration with the Skaggs School of Pharmacy and Pharmaceutical Sciences, UC San Diego, we investigated the suppressive effect of epoxyketone and boronic acid proteasome inhibitors on the growth of *B. divergens* in RBC cultures and in a *B. microti* infected mouse model (Jalovecká et al., 2018, **Int. J. Parasitol. - Drugs and Drug Resistance, 8, 394-402**).