Characteristics of main research directions investigated at the institute and the achievements 2010−2014

Institute	Biology Centre of the CAS, v. v. i.

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 1 January 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 Soil Biology, the Institute of Hydrobiology, and the Institute of Plant Molecular Biology. The foundation of the BC enabled 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 major institution in the Czech Republic devoted to bioecological research, especially in entomology, parasitology, hydrobiology, soil biology and plant molecular biology.

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 modelling, 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 preparation of the large research infrastructure SoWa (Soil and Water) and active participation of the BC in the newly proposed Strategy AV21 of The Czech Academy of Sciences (CAS), described in detail below. The **mission of the BC** is to acquire, advance and disseminate knowledge of the biology, evolution and ecology of free-living and parasitic organisms, with a special focus on insects, parasites, plants and their pathogens, and aquatic and soil organisms and ecosystems. The BC pursues this goal through **research, education and other activities at both the national and international levels**. The results obtained have an impact on agriculture, forestry, fisheries and human health, and contribute to the prevention and control of human, animal and plant diseases. The **BC's main strategy** is to focus on the **quality of research in selected fields of contemporary biology and ecology**. A **variety of organisms** (insects, ticks, parasitic protists, algae, etc.) are used as relatively simple and inexpensive models to unravel fundamental biological processes at the population, organismal and molecular levels.

The BC has **significant international project experience**. For example, V. Novotný from Institute of Entomology of the BC recently obtained funding for the prestigious **ERC Advanced grant "Diversity6continents"** (Proposal No. 669609). Examples of other large international projects include "Use of model organisms to resolve crucial biological problems on the path to innovations" (MODBIOLIN, No. 316304, duration 2012-2015), "Exploring the salivary transcriptome of *Ixodes ricinus*, the Lyme disease vector in Europe, and the potential role of its cystatins in pathogen transmission" (RICYSTVACANT2010, No. 268177, duration 2010-2014), "Modelling individual life histories and population dynamics of predatory aquatic insects" (AquaMod, No. 39543, duration 2010-2014), "Building up modern biotechnologies for agriculture" (MOBITAG, No. 229518, duration 2009-2012), and 10 projects from the ESF, FP7 and H2020 programs. The BC has its own **Project Department** that consists of skilled and

experienced project, financial and communication managers. Managers are well suited for building 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 as well as across the Czech Republic. We work to positively influence the lives of the population of the region and of 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. The **Technology Transfer Office** (TTO) was established 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 institutes' activities is the **organisation 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. Since 2010 the BC has intensively prepared a LIREDI project in environmental sciences called **"SoWa (Soil & Water - for comprehensive monitoring of soil and water ecosystems in the context of sustainable use of the landscape)"**. The goal of the SoWa is to serve the needs of the research community, both in the Czech Republic (CR) and at the international level. SoWa is focused on the investigations of complex interactions between soil and water ecosystems from the microscale to the catchment level, with particular emphasis on systems under anthropogenic pressure. Till 2014 SoWa was, as a promising project, a part of LIREDI's **Roadmap of the CR**. A new application of the redesigned SoWa project was accepted in 2014 and it became a high priority project that will be listed in the updated Roadmap of the CR in 2015. SoWa will be financially supported by the European Regional Development Fund (ERDF) through Ministry of Education, Youth and Sports (MEYS) of the CR in 2015 – 2022. The BC would like SoWa to become an important **part of ESFRI** in the next few years. Two other proposals of LIREDI have the BC as a partner institution: **RECETOX** – Research Centre for Environmental Chemistry and Eco-toxicology and **ELIXIR CZ**. Both projects are also to be supported by the ERDF and MEYS in 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 21st century"). The BC teams take part in 3 different programs of this Strategy, and the BC and its director M. Šimek serve as coordinators in the "**Life Diversity and Ecosystem Health"** program. The program includes 8 institutes in the CAS and tens of collaborating partners. The motto - "Understanding of biological diversity is essential not only for sustainable exploitation of organisms, biological processes and current ecosystem services but also for their protection and preservation for future generations" – summarizes the program's goals:

- Clarifying the processes of biodiversity and the origin of new species

- Understanding ecosystem structure and dynamics
- Identification of key mechanisms of co-evolution and inter-species relationships
- Understanding the dispersal dynamics of invasive and introduced species
- Understanding the nature of stress responses that ensure the survival of organisms
- Understanding the mobility and accumulation of environmentally significant trace elements
- Analysis of current landscape structure and human interaction with it

The results are applied in proposals focused on sustainable systems of plant protection, agriculture, forestry, fisheries and other fields related to ecosystem services. The output will also include both theoretical and practical approaches to environmental care, modern nature and landscape conservation, and other recommendations for the effective and sustainable use of natural resources by human society, providing a high quality of life as a result. An important part of the program will be communication with the general public and the education of all target groups.

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 19 research teams (departments). 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.

The Institute of Entomology
carries out basic and applied research in areas where insects are either the focus of inquiry or serve as suitable models for studying general biological problems. The Institute consists of **four departments (research teams)**, which are each divided into 4-5 laboratories.

BC Team No. 1: Molecular Biology and Genetics
The scientific program consists of several topics but is mainly focused on: (i) the role of adenosine signalling in insect growth and regulation of cellular metabolism, (ii) the role of the Notch signalling pathway in cell differentiation and proliferation, (iii) hormonal and genetic regulation of insect metamorphosis and ontogeny, (iv) molecular mechanisms of circadian rhythmicity and biological clocks in insects, and (v) the evolution, structure and function of insect chromosomes.
Team members contributed to the understanding of the regulation of energetic homeostasis in insect cells by adenosine, identified a *Drosophila* adenosine receptor, showed the importance of adenosine signalling for fly immunity, and adapted targeted mutagenesis using TALENs for insects. They further identified tissue-specific targets of the Notch signalling pathway in *Drosophila*, explaining many characteristics of Notch-induced human diseases. The discovery of a juvenile hormone (JH) receptor clarified the role of JH signalling in the regulation of insect development and in the decision between reproduction and diapause. Research on insect biological clocks revealed remarkable differences in the molecular regulation of circadian rhythmicity even among closely related species. The discovery of sex-chromosome-autosome fusion in the family Tortricidae highlighted the role of neo-sex chromosomes in the adaptive radiation of moths. They also helped to reveal the genetic basis of industrial melanism in the moth *Biston betularia*. Finally, the finding that the telomeres of most insects are maintained by telomerase suggests that they are associated with cell proliferation and aging as they are in humans.

BC Team No. 2: Biochemistry and Physiology
Research in this team is focused mainly on the following topics: (i) the role of adipokinetic hormones (AKHs) in antioxidative stress response, (ii) principles of insect diapause and cold tolerance, (iii) insect pest control and the impact of genetically modified plants on non-target

species, (iv) silk proteins and silk protease inhibitors, and (v) development of analytical methods for metabolomic studies.
Team members contributed to the understanding of insect responses to various stress factors. They demonstrated that AKHs control various anti-stress reactions including those which protect insects against oxidative stress. They also found that the amino acid proline represents a prominent metabolite required for cold acclimation and freeze tolerance, and that those phenomena can be intensified by simple laboratory manipulations. Pest control studies on flight dispersal and cellulose digestion in the bark beetle provided practically applicable results that were implemented in forest protection in the CR. Furthermore, field experiments with GMOs clearly showed that non-target arthropods were not influenced by *Cry* transgenes. Studies on genes encoding silk proteins revealed species specificity and diverse properties of sericins. The team contributed to the development of novel analytical methods that allow complex metabolite profiling in samples of animal origin.

BC Team No. 3: Biosystematics and Ecology
Researchers in this team deal mainly with the following topics: (i) theoretical behavioural and population ecology, (ii) taxonomy, ecology, and paleontology of aquatic insects, (iii) biosystematics, community ecology and conservation of relict ecosystems, (iv) biosystematics and zoogeography of aphids and their parasitoids, (v) taxonomy and ecology of entomopathogenic and entomoparasitic nematodes, and (vi) biological control of insect and mite pests.
Using theoretical approaches, team members showed that adaptive animal behaviour promotes species coexistence in food webs and influences other descriptors of biodiversity. Modelling of host-parasite interactions characterized parasites that are promising candidates for pest control. The aquatic insect group described a new fossil insect order, Coxoplecoptera, which is a missing link between apterous and pterygote insects. Studies on predator-prey relationships in aquatic insects characterized trophic interactions in freshwater food webs. Research of relict ecosystems showed that low-altitude, freezing talus slopes serve as paleorefugia for arthropods. Team members contributed to the knowledge of invasive aphids in Europe and demonstrated the adaptive potential of local parasitoids for their control. Competition experiments with entomopathogenic nematodes showed that symbiotic bacteria play a substantial role in interspecies dominance. In collaboration with a private company, a unique strain of the entomopathogenic fungus *Isaria fumosorosea* was patented as a biopesticide.

BC Team No. 4: Ecology and Conservation Biology
Research activities include the following topics: (i) tropical insect diversity and conservation research in tropical rainforests, (ii) plant-herbivore food webs in tropical rainforests, (iii) diversity and ecology of ants in tropical rainforests, (iv) biodiversity and restoration ecology in post-industrial habitats, (v) ecology and conservation of endangered xylophagous insects in Central Europe, and (vi) diversity and conservation of butterflies in the temperate zone of Europe.
The team produced widely accepted estimates of local and global diversity for tropical arthropods and participated in a survey of key threats faced by tropical reserves world-wide. It also assembled a matrix, comprising >8,000 plant-insect and insect-insect trophic interactions, which is among the largest datasets for tropical ecosystems. Their on-line “Ants of New Guinea” database supports the study of ant diversity and predation in rainforests. Research in post-industrial habitats (abandoned military training grounds, limestone quarries, and power plant ash deposits) showed that these unusual habitats are of high conservation value to arthropods. The team also documented a serious decline of floodplain forests in Central Europe despite their importance for endangered saproxylic beetles. Their mapping of distribution and abundance of temperate butterfly species identified causes of their decline and allowed them to propose active conservation measures.

The Institute of Plant Molecular Biology
carries out basic and some applied research in plant genomics, bioinformatics and molecular cytogenetics, analysis of structural genes of biosynthesis pathways, interaction of plants with pathogens, and photosynthesis. The Institute consists of **4 departments (teams)**.

BC Team No. 5: Molecular Cytogenetics
The team's major focus is to explore repetitive DNA sequences in plant genomes. This research direction gradually emerged about 10-15 years ago from our previous subject of interest, the physical mapping of plant genomes and chromosomes, when we recognized the crucial role of repetitive DNA in plant genome structure and evolution. Since then, we have been involved in the investigation of all main types of repetitive elements, including retrotransposons, DNA transposons and satellite DNA. In addition to the identification of novel element sequences and mapping their distribution in various plant genomes, our attention also turned to the structural and functional characterization of these elements and investigating their role in specific genome regions such as centromeres. Since tremendous sequence diversity and genomic abundance of plant repetitive DNA imposed serious limitations on their investigation using traditional sequencing techniques, we actively sought more efficient ways to target this genome fraction in all its complexity. We were among the first labs to adopt next generation sequencing (NGS) technologies for the characterization of repetitive DNA in eukaryotic genomes (Macas et al., *BMC Genomics* 8, 2007). This enabled whole new research directions to be considered, including global characterization of all repeat types within a single species, comparative analysis of repeated genome fractions between multiple species, and analyzing large numbers of non-model organisms. At the same time, the practical utility of NGS data was seriously hampered by a lack of appropriate bioinformatics tools for their analysis. These circumstances determined our research activities in the following years, prompting us to split our efforts between developing novel bioinformatics tools and applying these tools to investigate the composition and evolutionary dynamics of repetitive DNA in a wide range of species. Consequently, much of our current research is at the interface of computational biology, genomics and molecular cytogenetics.

BC Team No. 6: Molecular Genetics
Research includes the following interconnected topics: (i) molecular genomic analysis of the non-model plant *Humulus lupulus,* (ii) viroid (small circular pathogenic RNA) research, and (iii) analysis of plant nucleases. Hop genomic research started 15 years ago with the analysis of hop genomic markers important for breeding, and with the analysis of hop 7SLRNA it was shifted to the analysis of the organization of structural genes encoding the prenylflavonoid pathway and identification of regulatory elements and transcription factors (TFs) involved in co-regulation of hop lupulin. Recently, the TF network has been investigated and a new generation of markers important for practical research has been derived from the regulatory genes. Viroid research includes *Pospiviroids* infecting *Solanaceous* species, hops, fruit trees and ornamental plants. This topic was shifted from evolutionary changes of thermomutations to viroid-caused pathogenesis and mechanisms of RNA silencing (PTGS). Plant nucleases were cloned and their potential functions during viroid pollen transmission and viroid pathogenesis were analyzed. Nuclease cDNA cloning from tomato, hops and *Brassica* enabled *in vitro* production and use as anticancer agents to complement or replace nucleolytic enzymes of animal origin. Plant nucleases produced very few side effects in animals. We are now performing in-depth analyses on these enzymes.

BC Team No. 7: Plant Virology
Research has traditionally focused on diagnostics and molecular biology of plant viruses, phytoplasmas and bacteria. Complete genomic sequences were determined and comprehensive phylogenetic and serological analyses were performed for several viruses, including a novel Potyvirus from the garden lupine. The antiviral activity of acyclic nucleoside phosphonates, synthetized by the late prof. A. Holý (IOCHB AS CR), have been shown to significantly reduce titers of Cauliflower mosaic virus dsDNA in *Brassica* plants. J. Vlasák

(transferred from the Dept. of Gene Manipulations) was a collaborator on worldwide phylogenetic studies using molecular methods to resolve fungal taxonomy, and worked with researchers in the Institute of Entomology (BC) on the recombinant protein expression of modified endotoxins from *Bacillus thuringiensis* that were transferred into *Picea abies* to defend against the spruce bark beetle. Extensive research on phytoplasma diversity in fruit tree orchards based on multilocus gene analyses has led to the discovery of new infections and to the development of a microarray-based system which reliably detects phytoplasmas. While bacterial research was terminated upon the transfer of I. Mráz to the Faculty of Agriculture, USB, a fascinating and quite unexpected discovery of plant viruses in lichens started a new research direction in the department. Rhabdovirus and *Apple mosaic virus* (ApMV) were first detected in a number of samples from diverse geographical regions. After obtaining axenic cultures of a photobiont identified as *Trebouxia* sp., ApMV was repeatedly detected by RT-PCR. A novel single-stranded RNA virus from the Hypoviridae family was isolated from a phytopathogenic fungus, *Phomopsis longicolla*. The fungal isolate, harbouring the virus, was debilitated and showed reduced virulence in soybean. The complete genomic sequence was obtained and its phylogenetic position in the family was resolved.

BC Team No. 8: Photosynthesis
The major interests of the team are the structure, function and evolution of pigment-protein complexes in the thylakoid membranes of photosynthetic organisms. There is substantial diversity in light harvesting systems in both prokaryotic and eukaryotic photosynthesis. In the reporting period the team studied light harvesting systems of photosynthetic bacteria, cyanobacteria, and *Chromalveolate* algae. The methods used span from biochemical and bioinformatical to advanced biophysical and single particle electron microscopy imaging.

The Institute of Hydrobiology
performs complex limnological investigations on lakes and reservoirs. Its scope traditionally includes all important trophic levels from water chemistry to fish. Although primarily concerned with fundamental research, the institute's research portfolio also addresses the practical needs of those areas where there is no other institution capable of providing sound ecological service. Limnological research in the Institute profits from long-term monitored reference lakes : the Římov and Slapy reservoirs (>35 and >55 years of investigations, respectively), Bohemian Forest and Tatra mountain lakes (>25-30 years of investigations), and, recently, three newly created post-mining lakes. Thanks to the these investigations, the Czech post-mining lakes are among the most studied and their succession will provide valuable information for future planned "aquatic revitalizations". Another significant scientific outcome was the preparation of a nationwide methodology for assessing the ecological potential of heavily modified and artificial water bodies – Category lake, which has already achieved both national and international recognition and received official certification.The institute consists of **three teams**.

BC Team No. 9: Fish and Zooplankton Ecology
The main activities of the team are the following: (i) studies of the interactions of trophic state, fish and zooplankton, (ii) analyses of long-term changes in model reservoirs, (iii) genetic studies of the *Daphnia longispina* complex, (iv) studies of post mining lakes, (v) physio-ecological adaptations of *Daphnia*, (vi) fish community quantitative assessment in various stages of ontogeny, (vii) fish behaviour and its effect on lake ecosystems, (viii) hydroacoustics, and (ix) fisheries management.
The Zooplankton Ecology Group analyzed genetic differentiation in reservoir populations of *Daphnia longispina* over a 10 year period. A survey of microsporidian infection in *Daphnia* was performed on samples from 11 Czech reservoirs. Three new species were discovered. Low winter temperatures were found to cause smaller offspring and a low number of filtering setae in overwintering *Daphnia*. The Fish Ecology Unit rapidly developed quantitative sampling methods suitable for all important fish species and size classes in all important large, inland water habitats. Their advances in acoustics, gillnetting, trawling, purse seining, and point

sampling by electrofishing contributed to European standards and reached worldwide recognition. Methodological advances permitted the development of the first holistic models of inshore/offshore distribution and migrations for both juvenile and adult fish.

BC Team No. 10: Aquatic Microbial Ecology
The team is focused mainly on the following topics: (i) the dynamics of key bacterioplankton groups and their role in carbon flow to the grazer food chain, (ii) temporal and spatial distribution of major Betaproteobacteria groups, (iii) isolation, ecophysiology, and genomic characterization of bacteria, (iv) examination of the physiological status and activity of microbial cells, (v) phytoplankton spatial heterogeneity and dynamics, (vi) polyphasic taxonomy and secondary metabolite production in cyanobacteria, and (vii) studies of rootless aquatic carnivorous plants (in cooperation with team 11).
A combination of field studies, innovative experimental design, and molecular approaches have brought new insights into the distribution, activity and survival strategies of many important bacterial groups across lentic freshwater habitats. The team has detected groups of bacteria that play a prominent role in carbon flow from dissolved organic carbon to the grazer food chain via protists. Significant progress has been achieved in the isolation and genomic characterization of freshwater betaproteobacteria and bloom-forming cyanobacteria, leading to understanding of their phylogeny, function, and toxicological importance. Several important discoveries in phytoplankton ecophysiology and interactions with bacterioplankton were acheived through a combination of fluorescence approaches with quantitative image analysis. Classical limnological methods combined with an intensive sampling program allowed the team to address issues related to reservoir spatial heterogeneity and the dynamics of microorganisms, which are both significantly affected by high inflow episodes.

BC Team No. 11: Hydrochemistry and Ecosystem Modelling
This team is dealing with biogeochemistry and ecology of catchment-lake systems and is focused mainly on the following topics: (i) key factors of catchment impacts on water quality, (ii) effects of forest disturbance on element cycling, nutrient leaching from soil to surface water, and recovery from acidification, (iii) photochemistry of dissolved organic matter, (iv) phosphorus in sediments and biofilms, (v) life strategy of aquatic quillworts (*Isoëtes*) in acidified lakes, and (vi) effects of water level fluctuation on macrophytes in reservoirs.
The team summarized the history of anthropogenic input of important chemicals in the Vltava River catchment at Slapy for 1900-2010, using fifty-year data series of IHB analyses plus older historical data and new ways of analyses. The same team followed long-term rehabilitation processes in acidified mountain lakes, modified by a recent bark beetle infestation and forest die out with consequences for nutrient balance in the watershed and water quality. These results contributed significantly to global generalisations of acidification processes and predictions of post-acidification ecosystem recovery. A fascinating story about nearly extinct glacial relict quillworts (*Isoëtes*) in central European mountain lakes was published and extended. Nutrient enrichment and eutrophication still cause problems in the heavily populated Central European region, so significant attention was paid to the nutrient budget and the fate of organic compounds. Some of these studies have extensive socio-economic implications.

<u>The Institute of Soil Biology</u>
carries out basic and applied research in all important fields of contemporary soil biology and ecology in order to elucidate relationships between the structure and function of the decomposer food web and the role of soil biota in ecosystem functioning. The Institute consists **of two departments (research teams).** Temporary teams (often composed of members from both departments) are formed to carry out individual research projects.

BC Team No. 12: Soil Microbiology and Soil Chemistry
Research activities include the following topics: (i) the response of microbial communities to anthropogenic impact and environmental stress, (ii) interactions between soil and cave

microbiota, invertebrates, and plant roots, (iii) characterization of new species and important groups of soil microorganisms, (iv) fluxes of important greenhouse gases, and (v) applied research.
Team members contributed to the finding that microbial diversity is a key factor influencing invasion by alien (e.g. pathogenic) bacterial species in soil. Studies on the effects of intensive cattle husbandry on pasture soil showed significant enrichment of the soil microbiome by anaerobes, which may influence both greenhouse gas production and transmission of harmful genes. The contribution of fungi to N_2O emissions and anaerobic CH_4 oxidation in soils was confirmed. We also found that the addition of manure to soil increases bacterial resistance to antibiotics (ATB) regardless of the ATB content in the manure and soil, but not the ATB concentration, which significantly affected the persistence of genes responsible for ATB resistance. We also characterized the tetracycline (TET) resistome of the soil and of rapidly growing mycobacterial (RGM) isolates from hospitals and found that intrinsic efflux pumps may be more important for TET resistance than horizontally transferred genes in both soil and clinical RGM. Taxonomic research on algae and fungi resulted in the description of new species and the revisions of higher taxonomic units. A new method for monitoring infectious American foulbrood was designed and patented.

BC Team No. 13: Soil Zoology and Soil Microstructure
Research activities include the following main topics: (i) taxonomy and diversity of soil animals and structure and dynamics of their assemblages, (ii) development of soil biota in primary and secondary succession, (iii) the role of soil fauna in organic matter transformations and formation of soil microstructure, and (iv) ecophysiology of soil invertebrates.
Research on community ecology revealed regularities in the distribution and adaptation of soil fauna along various environmental gradients. Team members participated in the EU project SoilService, which showed the importance of soil biota in plant-soil interactions and emphasized the role of soil biota in provisioning ecosystem services. Complex studies at post-mining sites revealed that the bacterial channel dominated the soil food web in early stages of succession, while later on the fungal channel took over. The best predictor of the fungal-bacterial ratio was the thickness of the fermentation soil layer, which corresponded with changes in topsoil microstructure driven by organic matter input and the engineering effects of earthworms. In aforested sites, the effect of tree species on soil development was substantially mediated by soil fauna activity, which in turn affects soil chemistry and microbial processes. Research on post-coal mining sites located along the climatic gradient across the USA showed that simpler, root-feeding soil biota communities in short-grass prairies approach climax faster than more complex, mostly saprophagous communities under tallgrass prairies or forests. Extensive taxonomic work on soil fauna resulted in the description of several new species and the revision of higher taxonomic units.

The Institute of Parasitology
performs research on human and animal parasites at the organismal, cellular, and molecular levels. Its mission is to acquire, advance, and disseminate knowledge of the biology and host relationships of protists, helminths, and arthropods. The results obtained have contributed to the prevention and control of human and animal parasitic diseases and have had an impact on agriculture.We aim to be the leading institution researching parasitological topics in Central Europe, with a strong international reputation.

BC Team No. 14: Molecular Protistology
Composed of: (i) Laboratory of Molecular Biology of Protists – led by Julius Lukeš. Its primary interest is the functional analysis of proteins in *Trypanosoma brucei*, a rather competitive field. Most work is focused on mitochondrial proteins, but extensive research is also being performed on the biodiversity of kinetoplastids and the emergence of parasitism in protists. Furthermore, the lab maintains a strong evolutionary focus in its research projects. This includes mostly kinetoplastids and diplonemids, but to some extent extends to

alveolates. Until recently, the lab was very well funded and shall see several strong publications in 2015. (ii) Laboratory of Functional Biology of Protists – headed by Alena Zíková, who received strong funding from EMBO and the EU for investigating the mitochondrial metabolism of *T. brucei* and *Leishmania major.* The team focuses on the respiratory chain, investigated through the use of genetic and biochemical methods, with the aim to identify new drug targets for the devastating diseases caused by *T. brucei* and *L. major*. The lab went through a long publication hiatus, but this has ended and strong publications, including a description of the crystal structure of complex V in collaboration with Nobel Prize winner Prof. Walker, are forthcoming. (iii) Laboratory of RNA Biology of Protists – the second youngest lab of the institute is headed by Zdeněk Paris. The lab wants to capitalize on the burgeoning field of small RNA research, with a primary focus on tRNA modification, nuclear tRNA export, and the role of the only intron-containing tRNA in *T. brucei* which could expose these versatile parasites to new drug targets.

BC Team No. 15: Evolutionary Parasitology

The team is composed of: (i) Laboratory of Evolutionary Protistology – headed by Miroslav Oborník. It studies the molecular evolution of protists, mainly those involved in secondary endosymbiosis. A highly visible topic arose from research on the free-living relative of apicomplexan parasites, *Chromera velia*. Recently, however, the lab diversified its portfolio by venturing into various functions of organelles in diatoms, apicomplexans and euglenozoans. It has strong funding for the next several years. (ii) Laboratory of Molecular Phylogeny and Evolution of Parasites – a small, young lab headed by Jan Štefka. Its research is mainly focused on the molecular phylogenetic analysis of the origin, evolution and relationships of parasites. It investigates their co-evolution with hosts, biogeography and other bionomical features, including intraspecific variability and genealogy. Research in the lab is mostly computer-based; recently, however, the team also made an important contribution through an article in *Cell* based on experimental investigations of bacterial endosymbionts in insects (Filip Husník). (iii) Laboratory of Environmental Genomics – another young lab, established thanks to EU funding obtained 2 years ago and headed by Aleš Horák. His aim is to study the hidden microbial diversity of primarily aquatic, but also terrestrial ecosystems. An important branch of his research is participation in the Tara Oceans project, which gives him prime access to a unique and unexplored collection of samples. While the lab's interests concentrate on alveolate and excavate protists, their long-term aim is to be able to investigate any protist group. It will be challenging to get funding, but the lab recently participated in a key *Science* paper so the prospects are promising.

BC Team No. 16: Tick-borne Diseases

Composed of: (i) Laboratory of Molecular Ecology of Vectors and Pathogens – jointly headed by Libor Grubhoffer (currently rector of the University of South Bohemia) and Natalia Rudenko. It is a large lab with diverse and solid funding from Czech and EU sources. The team studies molecular and cellular factors involved in pathogen transmission by ticks, in particular Lyme borreliosis spirochetes and the tick-borne encephalitis virus. Furthermore, modern techniques of pathogen diagnosis for studying the molecular ecology of both causative agents have been recently expanded and are now a strong component of this lab‘s research. (ii) Laboratory of Arbovirology – headed by Daniel Růžek, who is currently employed part-time but within 1 year will be fully committed. The lab's main research focus is investigating vector-borne viral diseases using methods from molecular biology and molecular epidemiology. Another interest lies in the pathogenesis of viral diseases, in particular the pathology of the tick-borne encephalitis virus. The lab is strong in publication output, including book chapters. It will profit from the opening of dedicated lab spaces for work with high risk pathogens in 2015.

BC Team No. 17: Biology of Disease Vectors
Composed of: (i) Laboratory of Vector Immunology - a well-established lab studying ticks, primarily their immune system and relationship with transmitted agents. It is headed by Petr Kopáček, who uses a reverse genetics approach to assess the function of molecules involved in the innate immunity of the hard tick, its pathogen transmission, and blood digestion. The lab works on a potentially exploitable and patented anti-tick vaccine in collaboration with members of the business sector. (ii) Laboratory of Genomics and Proteomics of Disease Vectors – a relatively young lab led by Michalis Kotsyfakis, who entertains several research activities including proteomic analyses aiming to discover salivary or midgut effectors that determine arthropod vectorial capacity. The team also studies vertebrate proteolytic cascades regulated by arthropod salivary secretion at the sites of disease vector infestation, facilitating blood meal uptake and/or pathogen transmission. Another research focus is the pharmacological action of arthropod salivary proteins in the vertebrate host (hemostasis, vascular biology, and immunomodulation) and their potential for the treatment of human diseases (cancer, multiple sclerosis, and allergic asthma). Hence, the lab has found an interesting interface between basic research aimed at functional genomics and the application of these findings in applied medical research. (iii) Laboratory of Tick-Transmitted Diseases – a young lab co-headed by Ondřej Hajdůšek and Radek Šíma. Their research is focused on the molecular interaction between ticks and tick-transmitted pathogens. The lab was also involved in testing anti-tick vaccines and vaccines interfering with pathogen transmission. They have recently produced a complete transmission model for *Borrelia* infections, used for testing candidate tick genes implicated in tick-parasite interactions using RNA interference. Recently, they attempted to set up a system for testing *Babesia* and *Anaplasma* infections.

BC Team No. 18: Fish Parasitology
This team is composed of two closely collaborating groups: (i) Laboratory of Fish Protistology - led by Astrid Holzer, who was recently able to diversify the funding portfolio and outbalance long-term failure to obtain funding from the main Czech Science Foundation by acquiring EU funds and private funding from, for example, the largest fish and shrimp food producer in the world (Skretting, Norway). Major projects focus on myxozoan fish parasites and include classical molecular phylogeny and evolutionary studies, surveys on invasive species, and applied approaches with the ultimate aim of designing antiparasitic strategies for the aquaculture sector. Other economically important fish parasites, such as those that cause amoebic gill disease or blood flukes are subject to *in vitro* and *in vivo* approaches in order to develop in-feed treatments for affected fish stocks. (ii) Laboratory of Helminthology - led by Tomáš Scholz and is a consistently highly productive group focusing on the taxonomy and systematics of cestodes. In addition to traditional methodology they have ventured into molecular methods, enabling them to attract a new generation of researchers as well as solid funding. Moreover, they have gained the attention of foreign colleagues and the ability to collect in countries that are off-limits for American and Western European scientists, which has led to their participation in large international consortia. The lab is currently gaining strength in studies of the life cycles of trematodes and other groups.

BC Team No. 19: Opportunistic parasitic diseases
This team is composed of: (i) Laboratory of Medical and Veterinary Protistology – despite a long tradition of work on cryptosporidians and microsporidians, high quality research proved elusive. In a risky move, the head of the lab was removed and replaced by Martin Kváč, who succeeded in redefining the lab and its priorities, attracting students and funding. Ever since, productivity has increased via a strategic combination of molecular biology and classical parasitological experiments, made possible by an extensive in-house animal facility. The lab was also able to build important links with regional hospitals and is involved in diagnostics of protist-related human illnesses, description of unusual cases, and other clinical aspects of parasitology. Funding has been secured, the lab is now well recognized abroad, and students are readily recruited. (ii) Laboratory of Parasitic Therapy – the youngest lab is headed by

Kateřina Pomajbíková-Jirků, who has already secured excellent funding from international sources. This line of research is based on data about the potentially beneficial impact of intestinal parasites on human health. Research is therefore focused on the impact of gut eukaryotes on some immune-mediated diseases, with the aim to identify novel therapeutic approaches. It was demonstrated recently that eukaryote and bacterial communities inhabiting the human gut may positively influence the health status of individuals suffering from some autoimmune diseases, especially intestinal inflammations. The topic is fast evolving and it remains to be seen how successful it will be, but current research is very promising.

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Molecular Biology and Genetics

The team of Molecular Biology and Genetics (MBG) studies a variety of crucial biological processes at the molecular and genetic levels utilizing several different classical as well as novel model insect species. Insects are recognized as powerful models for fundamental biomedical research. Relative to experimental mammals, insects offer low-cost, ethically acceptable, and less complex yet biologically relevant alternative. The major topics of our team research interest include molecular and biochemical mechanisms of energy metabolism, immunity, cell growth, signaling and differentiation, genetic and hormonal pathways involved in the regulation of metazoan development, molecular and genetic dissection of circadian biological clock, seasonal timing and reproduction, and studies on the evolution and function of eukaryotic chromosomes.

Genetics provides us with causal evidence for roles of genes *in vivo* and thus unravels the fundamental principles of development and functioning of live organisms. Our team uses a variety of powerful genetic tools to enlighten the unknown functions of genes in the above mentioned relevant biological processes. We have successfully employed the dsRNA gene silencing approach in a variety of novel model insect species to elucidate the function of selected genes of interest. Moreover, our team has pioneered and improved the efficiency of several *in vivo* gene manipulation methods including the stable germ line transformation outside of *Drosophila* (the housefly, *Musca domestica*, the silkworm, *Bombyx mori*, the red flour beetle, *Tribolium castaneum*), and *in vivo* mutagenesis using zinc finger nucleases (ZFNs) and TAL-effector nucleases (TALENs), respectively.

Over the past several years, our team has also established a state-of-the-art biological imaging and microinjection facility with the multiline laser scanning confocal microscope (Olympus FV1000), 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), 2 fluorescence and DIC microscopes (Zeiss Axioplan 2) with high sensitivity cooled CCD cameras, automated microinjection system (Eppendorf Transjector), and sophisticated 2D, 3D, and 4D computer image analysis system (Imaris Bitplane) (Fig.1).

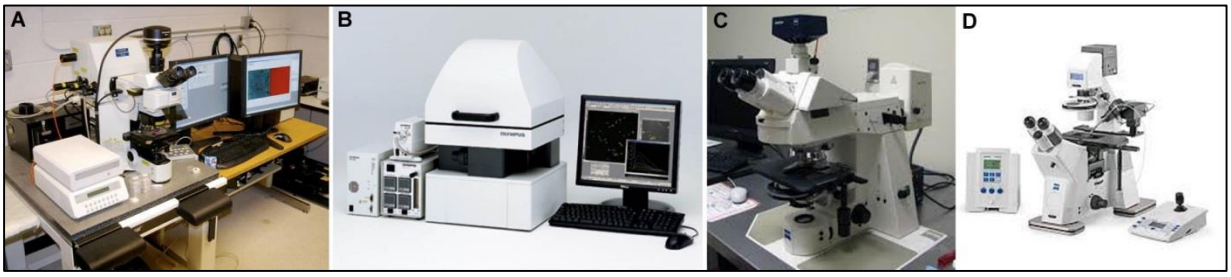


Fig.1. Biological imaging and microinjection facility. (**A**) Laser scanning confocal microscope Olympus FV1000. (**B**) Bioluminiscence imaging system Olympus LV200. (**C**) Fluorescence and DIC microscope Zeiss Axioplan 2. (**D**) Automated microinjection system Eppendorf Transjector.

The MBG team comprises of 15 key scientists who actively participated in research in the period of 2010-2014. Two new members joined our team in 2014 and represent a promise for

the future team development. The age structure of the MBG team shows a healthy distribution, including five senior research scientists (45-60 years), and a strong group of 10 junior and post-doctoral researchers (30-45 years). Importantly, 22 PhD students actively participated in the team research over the period of past five years, 6 of them already successfully completed the PhD program within this time frame. In the period of 2010-2014, we have published 55 original articles in impacted scientific periodicals including the most prestigious journals like *Science*, *Current Biology*, *PNAS*, and *Nature Neuroscience*, with the cumulative IF=374, which corresponds to an outstanding average publication IF=6.8 in the field of molecular insect science. During this period, the results of our team members were cited more than 3600 times.

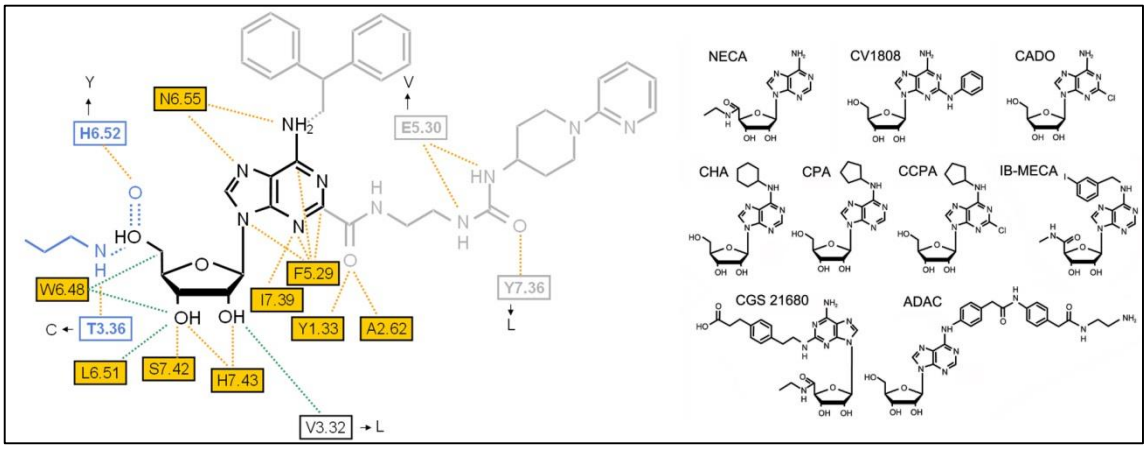
Formally, the MBG team is organized in four laboratories (Laboratory of Molecular Genetics, Laboratory of Developmental Genetics, Laboratory of Molecular Cytogenetics, and Laboratory of Molecular Chronobiology). However, many research topics and projects overlap among the laboratories and sometimes even between different teams of the Institute of Entomology. The MBG research team represents an international group with scientists, post-doctoral fellows, and students from the Czech Republic, Slovakia, Poland, Hungary, Great Britain, Portugal, Russia, Serbia, Argentina, India, Japan, and Taiwan, and it is involved in a number of intense and fruitful international scientific collaborations.

Since the MBG team performs mostly basic research (although we have produced two international commercial patents over the past five years) our funding comes predominantly from national and international government grant agencies and institutions. During the years 2010-2014, the team was awarded with 12 grants from the Grant Agency of the Czech Republic (GACR), four grants from the Grant Agency of The Czech Academy of Sciences (GAAV), two grants from European Union (EU-FP7), four grants from the Ministry of Education of the Czech Republic (MSMT), and one Marie Curie award. We have also successfully participated in two large EU infrastructural projects BIOGLOBE and MODBIOLIN.

The major research topics addressed by the MBG team in the period of last five years and the main achievements are summarized below.

Tight connection among energy metabolism, immunity and cell growth

Cells permanently adjust their levels of metabolic activity in response to energy needs. One of the most prominent signaling pathways involved in the regulation of energy homeostasis is adenosine (Ado) signaling pathway. Our research showed conservation of physiological responses regulated by extracellular Ado including the effects on fly immunity, cell survival, ATP synthesis (Fleischmannova et al. 2012, *Insect Biochem. Mol. Biol.* 42, 321-331), neural functions and synaptic plasticity (Knight et al. 2010, *J. Neurosci.* 30, 5047-5057). We characterized *Drosophila* adenosine receptor (AdoR), which belongs together with mammalian



AdoRs to a group of receptors coupled with G proteins. We found that *Drosophila* AdoR uses cAMP but not calcium as a second messenger system in *Drosophi*la cells. We also discovered several metabolically stable agonists and antagonists of *Drosophila* AdoR suitable for *in vivo* experiments. We showed that the treatment of flies with a full agonist 2-chloroadenosine in mimics the phenotype of AdoR overexpression *in vivo*, while the action of the antagonist, SCH58261, rescues flies from the lethal effect of AdoR overexpression (Kucerova et al. 2012, *J. Neurochem.* 121, 383-395) (Fig. 2). Our further investigation of adenosine signaling will clarify important aspects regarding the maintenance of tissue homeostasis at a cellular level, which play important roles in mechanisms involved in inflammatory responses and cancer.

Fig. 2. Interactions between AdoR and its agonists. (**Left**) AdoR binding site with bound adenosine (black lines), NECA (black and blue lines) and UK-432097 (black, blue and grey lines). Contact amino acid residues are shown in boxes. (**Right**) The list of AdoR agonists tested. The most efficacious agonists were C2-substituted adenine analogs, especially 2-chloroadenosine (CADO).

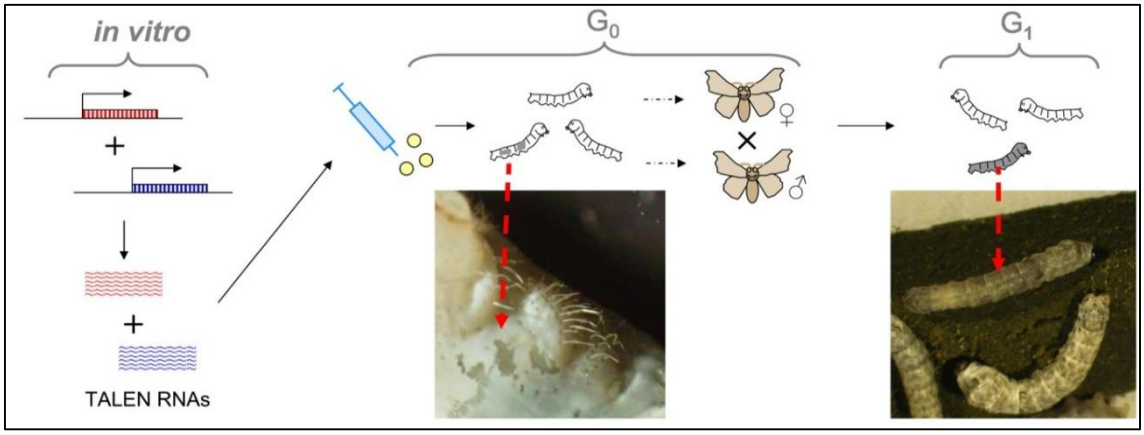
We have also prepared the infrastructure for functional analysis of *Drosophila* and *Bombyx* mutants in genes involved in the maintenance of energy homeostasis. The methods include respirometry and locomotor tests, as well as analysis of immune responses and heartbeat. Some of the results regarding our studies on locomotion and immune responses were already published (Kiss et al. 2013, *Gen. Comp. Endocrinol.* 191, 137-145; Arefin et al. 2014, *J Innate Immun.* 6, 192-204).

Improved method for the generation of mutants in flies and moths

Genetic analysis in insects has been limited until recently mainly to *Drosophila* because it was very difficult to induce mutants in desired genes in other species. To overcome this, we initiated a large collaborative project with two Japanese laboratories (Y. Takasu and T. Tamura, both from NIAS, Tsukuba) in order to establish targeted mutagenesis with engineered nucleases in *Bombyx mori*. While most of the embryo injections and crosses were done in Japan, our laboratory focused on construct cloning, testing of nucleases in yeast systems and mutant analyses.

Our first publication on engineered nucleases was finished in 2010 (Takasu et al. 2010, *Insect Biochem. Mol. Biol.* 40, 759-765). *Bombyx mori* thus became the first insect other than *Drosophila*, which was mutagenized by this approach. We used a gene called *BmBLOS2* responsible for color of larval epidermis. We showed that the custom-made nucleases based on zinc fingers (ZFNs) are able to induce germline mutations in *B. mori* genes of interest. The efficiency of mutagenesis was, however, still quite low. In spite of this, this report represented enormous methodological progress for *Bombyx* genetics and inspired many other teams.

It was very important for our further work to increase the efficiency of mutagenesis. This was made possible by the discovery of a simple mechanism of DNA-binding of bacterial proteins



called TAL-effector (the resulting chimeric nucleases are called TAL-effector nucleases, TALENs). We finished the first article on TALEN-type of engineered nucleases in the summer of 2012 (Sajwan et al. 2013, *Insect Biochem. Mol. Biol.* 43, 17-23) (Fig. 3). We compared the efficiency of our ZFNs with TALENs on the same targets using *Bombyx* germline as well as the yeast testing system. The efficiency of TALENs was still relatively low, since as it turned out later, the TAL-effector molecule needed some trimming in order to get its maximum efficiency. However, the success rate of the newly designed TALENs was higher than that of the ZFNs.

Fig. 3. Schematic diagram illustrating the procedure of *BmBLOS2* gene mutagenesis in *Bombyx mori* germline. The procedure includes synthesis of TALEN mRNA *in vitro*, microinjection of silkworm embryos and screening for mutants. *BmBLOS2* mosaic silkworms in G_0 display spots of transparent epidermis and some G_1 larvae are homozygous *oily* mutants.

In the following year (2013), we finished another paper on TALEN mutagenesis, in which we compared the efficiency of nucleases containing three different length variants of TAL-effector DNA binding domains (Takasu Y. et al. 2013, *PLoS ONE* 8, e73458). Our results showed that the use of truncated TALEN design using a framework lacking 152 N-terminal amino acids and having 63 C-terminal amino acid residues of the original TAL-effector) brought remarkably high efficiency - up to 77% of germline mutants in G_1. In 2014, we made several more improvements and described efficient crossing schemes and testing strategies needed for the successful use of this method in silkworms as well as in flies. The final protocol was also optimized for *Drosophila*, where it worked with similar efficiency using the same *pBlue-TAL* framework (Takasu Y. et al. 2014, *Methods* 69, 46-57).

Discovery of the juvenile hormone receptor

The sesquiterpenoid juvenile hormones (JHs) play vital roles in insects, crustaceans, and likely other arthropods that comprise most of the animal biomass and most of the species on our planet. The effect of JH on insect metamorphosis has been known since the 1930's, but the molecular action of JH remained enigmatic as no receptor for this hormone could be conclusively identified. We have demonstrated the JH receptor role for the transcription factor Methoprene-tolerant (Met). We determined specific amino acids required for high-affinity binding of Met to JH and for JH-dependent interaction of Met with partner proteins (Charles et al. 2011, *PNAS* 108, 21128-21133) (Fig. 4). Unlike nuclear receptors of steroid hormones, Met belongs to a distinct family of bHLH-PAS proteins and, like JH, is unique to arthropods. Our results have addressed a long-standing problem of the mechanism of action of JH and its widely used analog insecticides.

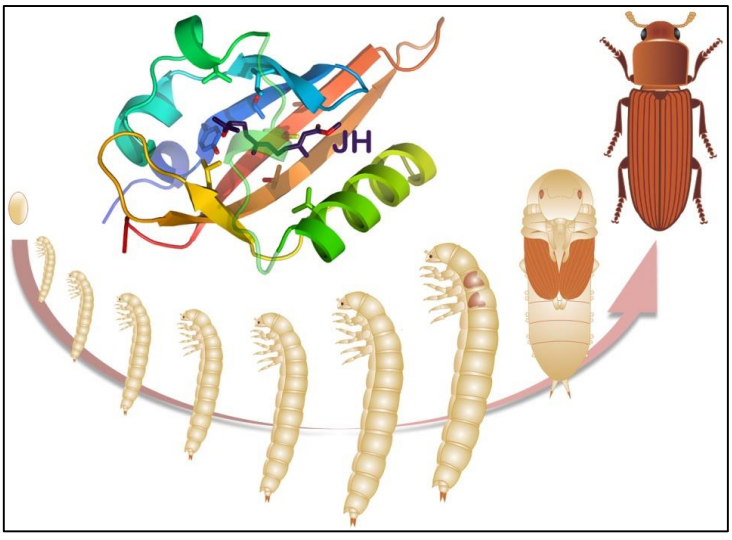


Fig. 4. Insect metamorphosis depends on juvenile hormone (JH). The unique chemical structure of JH is reflected in the unique nature of its intracellular receptor Met whose ligand-binding domain is formed by a PAS fold. Depicted is a structural model of the JH-binding pocket of Met from the red flour beetle, *Tribolium castaneum*. (Charles et al. 2011, *PNAS* 108, 21128-21133).

Hormonal regulation of insect development and oogenesis

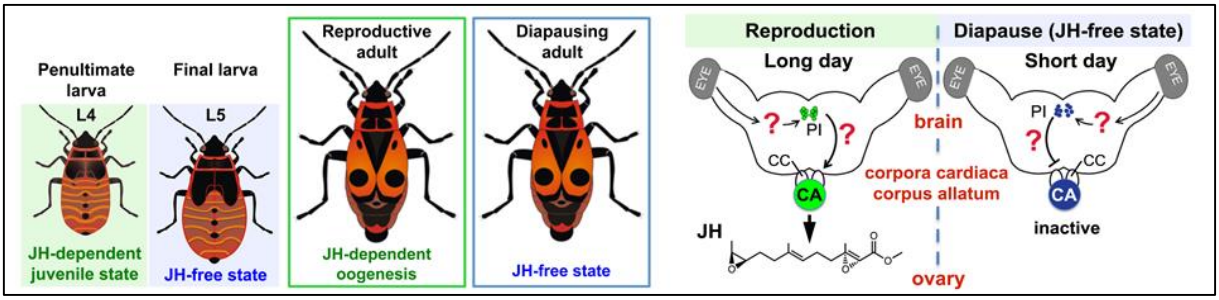
The main role of JH during insect development is to prevent transition of larvae to adults until the larvae have attained an appropriate stage. We have found that JH restricts metamorphosis through its receptor protein Met and its immediate target gene *Kr-h1* (Jindra et al. 2013, *Annu. Rev. Entomol.* **58**, 181-204). This anti-metamorphic core JH signaling is common to evolutionarily distant hemimetabolous and holometabolous species and possibly to all winged insects (Konopova et al. 2011, *PLoS ONE* **6**, e28728). Despite the traditional view that JH suppresses a premature onset of metamorphosis from the time of hatching, we have shown that JH signaling plays this essential role only at a late phase of development when larvae gain competence to metamorphose (Smykal et al. 2014, *Dev. Biol.* **390,** 221-230). Finally, we have established that the same JH receptor Met is required for JH to induce yolk protein synthesis and oogenesis in adult females (Bajgar et al. 2013, *PNAS* **110,** 4416-4421; Smykal et al. 2014, *Insect Biochem. Mol. Biol.* **45,** 69-76).

Function of human disease genes revealed in *Drosophila*

In collaboration with the University of Cologne (Germany), we employ *Drosophila* as a relevant *in vivo* model to study the function of conserved genes with unclear roles in human diseases. Thus, we have characterized a role for the bZIP transcription factor Atf3 in cell adhesion during development (Sekyrova et al. 2010, *Development* 137, 141-150). Our next study has shown that the single *Drosophila* Atf3 protein integrates functions of its two human homologs in maintaining immune and metabolic homeostasis, respectively (Rynes et al. 2012, *Mol. Cell. Biol.* **32**, 3949-3962). Finally, we have discovered that the *Drosophila ecdysoneless* (*ecd*) gene, known for its mutation causing steroid-hormone deficiency, encodes a novel pre-mRNA splicing factor. A human Ecd protein that has been implicated in tumor proliferation can functionally substitute for its *Drosophila* homolog (Claudius et al. 2014, *PLoS Genet.* 10, e1004287).

Mechanisms regulating insect diapause

In the Earth temperate regions, the shortening photoperiod informs many insect species to prepare for winter by inducing diapause. In spite of the fact that photoperiodic clocks are widespread in nature, their molecular architecture is rather elusive (Dolezel 2015, *Curr. Opin. Insect Sci.*, DOI: 10.1016/j.cois.2014.12.002). Using a combination of reverse genetic approaches, organ cultures and microsurgical operations, we have studied regulation of adult diapause of the linden bug, *Pyrrhocoris apterus*, and demonstrated that, at the organ-autonomous level of the insect gut, the decision between reproduction and diapause relies on the interaction between juvenile hormone (JH) signaling and circadian clock genes acting independently of the daily cycle. The JH receptor Methoprene-tolerant (Met) and the circadian proteins Clock and Cycle are all involved in a non-periodic, organ-autonomous feedback between Par domain protein 1 and Cryptochrome 2, which orchestrate the expression of downstream genes that marks the diapause vs. reproductive states of the gut (Bajgar et al. 2013, *PNAS* **110,** 4416-4421; Bajgar et al. 2013, *J. Insect Physiol.* **59**, 881-886) (Fig. 5). However,



the ovarian development and vitellogenin gene expression in the fat body require JH, Met and its binding partner Taiman (Tai) (Smykal et al. 2014, *Insect Biochem Mol. Biol.* **45,** 69-76).

Fig. 5. Juvenile hormone (JH) regulates several different life events in insects. (**Left**) JH is essential for maintaining larval development, while its absence triggers metamorphosis. In adults, JH is necessary for reproduction, while the absence of JH is a hallmark of adult diapause. **(Right)** Schematic illustration of the adult *P. apterus* brain regulating activity of *corpora allata* (the source of JH).

Chronobiology - comparative studies

Circadian biological clocks are found in most living organisms and their fundamental properties are highly conserved in vertebrates and invertebrates. Given the functional similarities of the circadian clocks among all metazoans, it was suggested that the molecular mechanisms underlying the clock function are conserved as well. Surprisingly, we found remarkable differences between the anatomical localization and molecular regulation of the circadian timing system even among closely related insect species (Závodská et al. 2012, *J. Biol. Rhythms*, **27**, 206-216; Kobelkova et al. 2015, *J. Biol. Rhythms*, DOI: 10.1177/0748730414568430). For instance, the German cockroach (*Blatella germanica*) has both mammalian and *Drosophila* type cryptochromes (CRYs), while the American cockroach (*Periplaneta americana*) has only the mammalian-type CRY. Interestingly, the specific interacting partner of the *Drosophila*-type CRY, the TIMELESS (TIM) protein, is present in both cockroach species. Yet, in another hemimetabolous insect model species, *Pyrrhocoris apterus*, we have found a unique combination of the mammalian CRY with TIM. Importantly, we can effectively knock down particular genes in all these insects to identify the specific role of each factor. This research involves international collaboration with How-Jing Lee (Taipei University, Taiwan) covered by our joint GACR grant (Fig. 6).

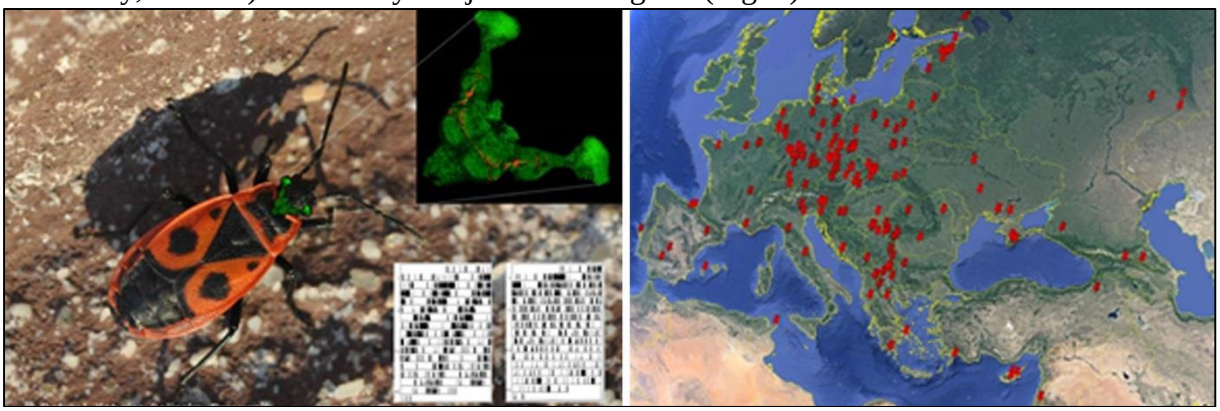


Fig. 6. (Left) *Pyrrhocoris apterus* serves as model for chronobiology and diapause research. Detail of its brain stained for the presence of Pigment Dispersing Factor (orange) is in the top right. Bottom right are two double plotted actograms of locomotor activity recorded in constant dark. Left actogram is a rhythmic animal (control), while the arrhythmicity on the right actogram was induced by depleting circadian factor Clock by means of RNA interference. **(Right) Geographical distribution of the origin of our *P. apterus* laboratory colonies.**

Dissecting the context specificity of Notch signaling

The well-conserved Notch signaling controls many diverse cellular decisions and is associated with diseases including cancer. Its outcome depends on cell type and the timing and length of the signal. For example, Notch is important for stem cell maintenance but can also promote cell differentiation and proliferation, or apoptosis. In collaboration with the University of Cambridge (UK), we investigate what gives Notch signaling this specificity. We have discovered that differentiation of a particular cell type in *Drosophila* blood requires interaction of Notch with a transcription factor Runx/Lozenge (Terriente-Felix et al. 2013, *Development*

140, 926-937). We also investigated the role of Notch signaling during the specification of neural stem cells (neuroblasts) in the *Drosophila* brain, showing that a Serrate-Notch-Canoe complex mediates essential interactions between glial and neuroepithelial cells (Pérez-Gómez et al. 2013, *J Cell Sci.* 126, 4873-4884). In another study, we employed *Drosophila* muscle precursor cells to show that even a short pulse of Notch activity (5 min) elicits a profound response. While identifying Notch target genes in this cell type, we found several feed-forward regulatory loops driven by early-response genes that time the response of delayed Notch targets (Housden et al. 2013, *PLoS Genet.* 9, e1003162). Finally, we have identified Notch targets in the *Drosophila* wing disc during hyperplasia. We described both autonomous and non-autonomous regulation of cell proliferation, growth and cell death, providing molecular explanation for many characteristics of Notch-induced hyperplasia (Djiane et al. 2013, *EMBO J.* 32, 60-71).

Evolution of sex chromosomes in Lepidoptera and their role in speciation

Moths and butterflies (Lepidoptera) have a WZ/ZZ sex chromosome system or its variations and represent the largest animal group with female heterogamety. In collaboration with W. Traut (Lübeck, Germany) and K. Sahara (Sapporo, Japan) we reviewed current knowledge of lepidopteran sex chromosomes in a book co-edited by member of our team (Marec et al. 2010, in Goldsmith and Marec, eds., *Molecular Biology and Genetics of the Lepidoptera*, CRC Press, pp. 49-63). In selected models, we examined molecular differentiation of sex chromosomes and performed synteny mapping of sex-linked genes to uncover general mechanisms of sex chromosome evolution (see below). In addition, we conducted the first comparative study of lepidopteran karyotypes, which showed a dynamic evolution of clusters of ribosomal RNA genes (major rDNA) in a representative sample of species. Results of this work suggest ectopic recombination as a primary motive force in rDNA repatterning (Nguyen et al. 2010, *Genetica* 138, 343–354).

In collaboration with colleagues from the University of Liverpool we performed comparative mapping of the peppered moth (*Biston betularia*) chromosomes with the silkworm (*Bombyx mori*). While our colleagues focused on linkage mapping, we performed analysis of the *B. betularia* karyotype and BAC-FISH mapping of sex-linked genes including the construction of the physical map of the Z chromosome (Van't Hof et al. 2013, *Heredity* 110, 283-295). Our study brought a strong evidence about an ancestral chromosome print in Macrolepidoptera (n=31) and added to accumulating evidence of unusual evolutionary stability of autosomes. The study also showed the first detail comparison of lepidopteran Z chromosomes (Fig. 7), pointing to a highly conserved synteny of Z-linked genes with only a few rearrangements of the gene order.

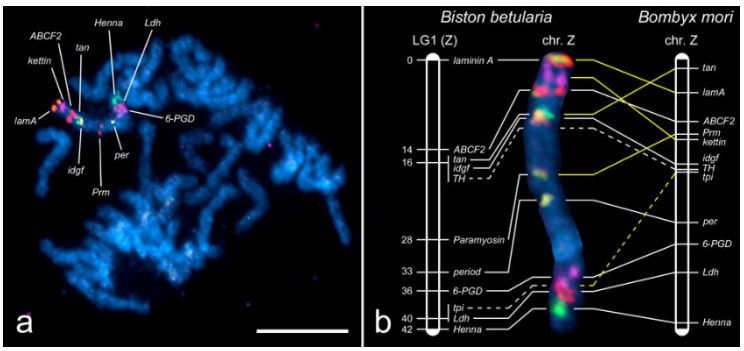


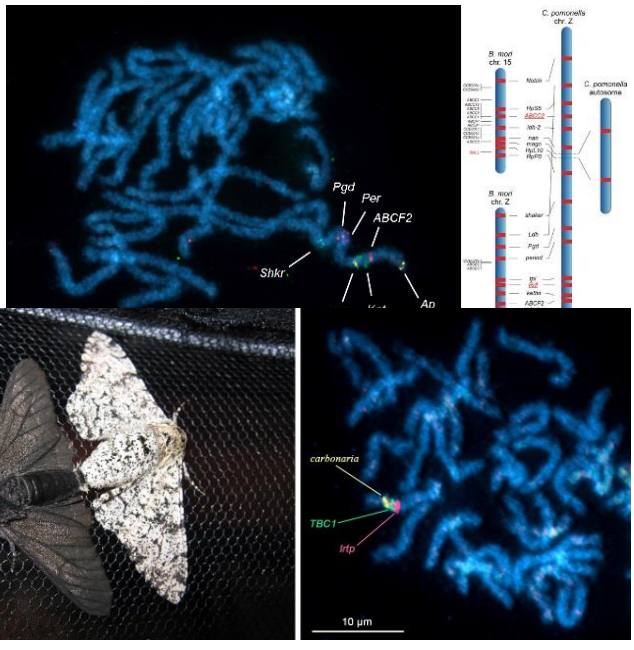
Fig. 7. BAC-FISH and linkage mapping of the Z chromosome in *B. betularia*. (**a**) Male pachytene with the ZZ bivalent identified by BAC probes. (**b**) Synteny maps of Z-linked loci in *B. betularia* and *B. mori*.

Our team performed mapping of the Z chromosome in the codling moth, *Cydia pomonella* (Tortricidae). Using BAC-FISH we showed that it is a neo-Z chromosome that arose by fusion between an ancestral Z and an autosome orthologous to chromosome 15 in *B. mori* (Fig. 8). We showed that the fusion originated in an ancestor of two tortricid subfamilies, Olethreutinae and Tortricinae (Nguyen et al. 2013, *PNAS* 110, 6931-6936; Šíchová et al 2013, *PLoS ONE* 8, e64520). The Z-autosome fusion brought two major genes for insecticide resistance and clusters of genes involved in detoxification of plant secondary metabolites under sex-linked inheritance, and thus significantly increased the adaptive potential of tortricids. We have also developed a two-colour TSA-FISH protocol with tyramide amplification of signals for single-copy gene mapping, which proved reliable for localization of genes in species with no genomic tools available (Carabajal Paladino et al. 2014, *BMC Genetics* 15, S15).

Fig. 8. Chromosomal mapping in *C. pomonella*. (**a**) BAC-FISH mapping of the Z. linked genes. (**b**) Map of the neo-Z chromosome compared to *B. mori* chromosomes Z and 15.

We also participated in the study on the origin of multiple sex chromosomes in geographical subspecies of wild silkmoths, *Samia cynthia* ssp. In the first step, largely conducted by colleagues from the Hokkaido University in Sapporo, FISH mapping of *S. cynthia* orthologs of *B. mori* genes enabled us to clearly show a step-by-step evolution of the neo-sex chromosomes by repeated autosome-sex chromosome fusions, likely facilitating divergence of *S. cynthia* ssp. towards speciation (Yoshido et al. 2011, *Heredity* 106, 614-624). Next we examined *S. cynthia* ssp. by FISH with W-chromosome specific painting probes, prepared by our team using laser microdissection. In *S. c. pryeri*, the W-probes revealed a unique W chromosome, much of which lacked homology in the genomes of other subspecies. Our findings suggest that the karyotype of *S. c. pryeri* with 2n=28/28 and the curious WZ system may represent an ancestral state of the *S. cynthia* species complex (Yoshido et al. 2013, *Chromosome Res.* 21: 149-164). In 2014, A. Yoshido joint our team, and we currently examine the role of neo-sex chromosomes in reproductive barriers between *S. cynthia* subspecies.

Genetic origin of industrial melanism in the peppered moth, *Biston betularia*



We participated in the study on industrial melanism, initiated by colleagues from the University of Liverpool. The rapid spread of a novel black form (*carbonaria*) of *B. betularia* in 19th-century Britain is a textbook example of how an altered environment may produce adaptation through genetic change. However, the genetic basis of the wild-type (*typica*) and *carbonaria* forms remained unknown. By linkage mapping and DNA analysis of population and museum samples our colleagues showed a common origin of all British *carbonaria* specimens and assigned the *carbonaria* locus to a linkage group orthologous to *B. mori* chromosome 17. We identified a homologous chromosome 17 of *B. betularia* and localized the *carbonaria* gene by BAC-FISH (Fig. 9). The results show that the *carbonaria* morph was seeded by a single recent mutation, which is controlled by a yet unknown

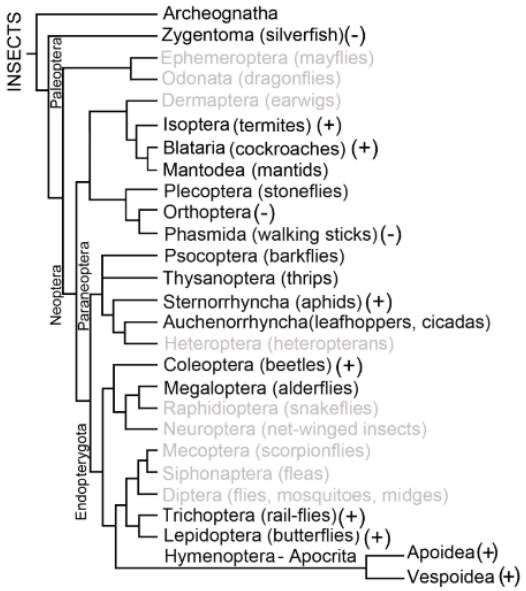
melanisation gene that maps to a 200-kb region of chromosome 17 (van't Hof et al. 2011, *Science* 332, 958-600).

Fig. 9. Mapping of the *carbonaria* locus in *Biston betularia*. (**Left**) Mating of *carbonaria* male and *typica* female. (**Right**) BAC-FISH localization of *carbonaria* on chromosome 17.

Structure and Function of Insect Telomeres

Telomeres, structures stabilizing chromosome ends, are maintained by mechanisms which keep their proper length. Short telomeres lead to cellular senescence and are one of key factors for cellular and organismal aging. The most common mechanism of telomere maintenance is based on the activity of telomerase that elongates telomeres by adding short DNA repeats to chromosome ends. In most arthropods, the telomeres are composed of TTAGG repeats. But due to frequent losses of the repeats in insects there was speculation that the TTAGG arrays might not be the true telomeric sequence but only a non-functional relict. We refuted this suspicion by showing TTAGG-specific telomerase activity in phylogenetically distant insect species (Fig. 10). We also quantified telomerase activity in the cockroach *Periplaneta americana* and showed that telomerase gradually declines during development (Korandová et al. 2014, *Chromosome Res.* 22, 495-503). Thus, the telomerase activity of insects seems to be, like in humans, associated with cell proliferation and aging.

Fig. 10. Distribution of the $(TTAGG)_n$ telomere motif and TTAGG-specific telomerase activity in insects. TTAGG repeats were found in the orders marked black. The orders in grey did not show TTAGG. Telomerase activity was found in the tested orders marked (+).



Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Biochemistry and Physiology

The exploration of insect responses to various stress factors represents the core unifying research theme of our Department. Our major aim is to bring new knowledge on mechanisms that help the insect organism to cope either with physiological stress (exercise, lack of energy, toxins, oxidative stress) or with harsh environmental factors (especially low temperature during overwintering). We focus on studying the ways of hormonal control of anti-stress reactions, the regulation of entry into overwintering diapause and the principles of cold tolerance. In addition to numerous "classical" methods of biochemistry and physiology, the major strength of our group lies in wide implementation of sophisticated metabolomic analyses using originally developed methodology and well equipped Laboratory of analytical biochemistry. This laboratory is serving the needs of many other researchers within and outside the Biology Centre. All basic methods of molecular biology are exploited and the methods of comparative analysis of transcriptome on custom cDNA microchips are now well established. Some of our research lines aim to application of theoretical knowledge into the practical field. We study the stress responses elicited by insecticides or natural toxins. Overwintering is being analyzed in detail in several insect pests. And we also continue in a research line with fruitful and long tradition: characterization and potential technological use of insect silk.

Our research was continuously supported by grants obtained mainly from the Czech Science foundation (12), the Ministry of Education, Youths and Sports (5), international territorial co-operation EFRE (2), and several other sources (5). The research on forest pests got support from the Forests of the Czech Republic (FCR), and from the National park Šumava. EU projects Bioglobe and Modbiolin allowed us to hire postdoctoral students and experienced researchers from abroad as well as to update the equipment of our laboratories.

Currently, the team consists of 13 researchers (2 foreigners, 4 in retirement age with part time equivalent) and 9 post-docs (4 foreigners). The numbers of students fluctuate: currently we have got 11 doctoral students and 14 undergraduates (5 of them foreigners). During the period 2010-2014, 7 PhD students successfully defended their doctoral theses. Four research groups cooperate within the team: Laboratory of Insect Physiology (4 researchers, 1 post-doc and 5 PhD students), Laboratory of Applied Insect Physiology (3 researchers, 2 post-docs and 4 PhD students), Laboratory of Insect Diapause (3 researchers, 3 post-docs and 4 PhD students), and Laboratory of Analytical Biochemistry (3 researchers, 3 post-docs and 2 PhD students). In 2010-2014, we have published 130 impact-factor papers (including two papers in PNAS), 26 other papers, 2 books, 15 book chapters and numerous Proceeding contributions. We have also produced 9 applied results and 1 patent. Publications of our Laboratory leaders were cited almost 3,000 times in the monitored period 2010-2014 (Kodrík, 458 times; Sehnal, 744 times; Košťál, 958 times; Šimek, 825 times)

Although each Laboratory follows its own principal research direction, all groups mutually collaborate, share equipment and methodological background, and submit joint grant projects. In addition, each group develops and maintains numerous cooperations with other groups in BC and especially with many abroad laboratories around the world. A brief description of major research directions follows:

Role of adipokinetic hormones in antioxidative stress response

Adipokinetic hormones (AKHs) are insect neuropeptides that control energy metabolism via the mobilization of stored nutrients, however, their activities also include stimulation of the heart, locomotor and immune systems, and even regulation of some reproductive processes. Thus, AKHs behave as typical stress hormones: they stimulate anti-stress reactions to eliminate or at least to reduce the impact of stress to insect organisms. In a series of publications, we demonstrated that AKHs are also involved in the activation of defensive mechanisms that protect insects against oxidative stress. Using our insect model species (the firebug *Pyrrhocoris apterus* and the cotton leafworm *Spodoptera littoralis*) we showed that the application of oxidative stressors (paraquat, tannic acid) increases the AKH level in both the central nervous system, where those hormones are synthesised, and also in the haemolymph. Further, AKHs mobilize anti-oxidative mechanisms that ameliorate damage incurred by oxidative stress such as increased protein carbonylation, decrease of reduced glutathione (GSH) level and impaired total antioxidant activity in haemolymph (Večeřa et al. 2012, *Comp. Biochem. Physiol. C* 155, 389-395). AKHs also efficiently modulate the activity of superoxide dismutase, catalase and glutathione-S-trasnferase, the principal enzymes involved in anti-oxidative stress protection of insect body (Bednářová et al. 2013, *Physiol. Entomol.* 38, 54-62; unpublished data). Our results suggest that the activity of these enzymes is controlled at the post-translational level rather than via regulation of expression of their genes; at least the catalase and superoxide dismutase mRNA expression was not affected after the AKH injection into the insect body. We have proposed several biochemical steps of possible mode of AKH in anti-oxidative stress response. Using *in vitro* assay we proved the importance of extra and intra-cellular Ca^{2+} stores as well as the involvement of protein kinase C (PKC) and cyclic adenosine 3',5'-monophosphate (cAMP) pathways in this process. Lipid peroxidation product (4-HNE) was significantly enhanced and membrane fluidity reduced in microsomal fractions of isolated brains (CNS) of *P. apterus* when treated with hydrogen peroxide (H_2O_2) as a stressor, whereas these biomarkers of oxidative stress were reduced to control levels when H_2O_2 was co-treated with AKH. The effects of mitigation of oxidative stress in isolated CNS by AKH were negated when these treatments were conducted in the presence of Ca^{2+} channel inhibitors ($CdCl_2$ and thapsigargin). Presence of either bisindolylmaliemide or chelyrythrine chloride (inhibitors of PKC) in the incubating medium also compromised the anti-oxidative function of AKH. However, supplementing the medium with either phorbol myristate acetate (PMA, an activator of PKC) or forskolin (an activator of cAMP) restored the protective effects of exogenous AKH treatment by reducing 4-HNE levels and increasing membrane fluidity to control levels. Taken together, our results strongly implicate the importance of both PKC and cAMP pathways in AKHs' anti-oxidative action by mobilizing both extra and intra-cellular stores of Ca^{2+}. This point seems to be unique, because parallel activation of both mentioned pathways has never been recorded in other AKH activities (Bednářová et. al. 2013, *Comp. Biochem. Physiol. C* 158, 142-149).

All these studies came fully from our projects and were done partly in our laboratory and partly in cooperation with our former post-doc Dr. Krishnan during stays of our students in his present lab in the USA (University of Mississippi). One set of experiments was done by our student in the Max Planck Institute in Jena, Germany.

Adipokinetic hormones enhance efficacy of insecticides

We examined the physiological consequences of adipokinetic hormone (AKH) application on insect models (*P. apterus, Tribolium castaneum*) treated by insecticides. For these studies we used five various insecticides (all neurotoxins) from different insecticidal groups. Co-application of them with AKH significantly increased insect mortality compared with application of the insecticides alone (Kodrík et al. 2010, *Pest Manag. Sci.* 66, 425-443). This

co-treatment was accompanied by substantial stimulation of general metabolism, as monitored by carbon dioxide production (Velki et al. 2011, *Gen. Comp. Endocrinol*. 172, 77-84). We suggest that the elevation of metabolism, which is probably accompanied with faster turnover of toxins, might be responsible for the higher mortality that results after AKH and insecticide co-application (Plavšin et al., *Comp. Biochem. Physiol. C*, in press). However, interactions of AKH with insecticides on the neuronal level cannot be completely excluded as well: recently, we showed that AKH genes are expressed also in brain neurones, and that the resulting peptides are transported from secretory granules localised in neuronal bodies into axons, where they could play a role in neuronal signalling (Kodrík et al., *Gen. Comp. Endocrinol*., in press). In summary, we are far from using the AKHs as biorationale pesticides at this stage, but any successful involvement of insect neurohormones in this matter may result in a welcome shift away from harmful insecticides, which is appealing both economically and with respect to environmental pollution.

This research was conducted entirely in our laboratory, including part carried out by two Croatian students (University of Osijek), who spent their Erasmus stays in our Institute.

Effect of adipokinetic hormone on insect digestion

We have recently described a novel adipokinetic hormone function in the control of insect gut activities. Our data indicate that the AKH stimulates food digestion by more intensive food turnover and perhaps by the stimulation of metabolite absorption; the activation of digestive enzymes seems to be species specific, both primary and secondary, or controlled by other mechanisms (Kodrík et al. 2012, *J. Insect Physiol*. 58, 194-204; unpublished data). Further, AKHs stimulate also salivary gland function: in *P. apterus* saliva we identified a very specific digestive enzyme, polygalacturonase (typical for herbivorous insects which use it to destroy plant cell walls), of which activity seems to be under AKH control: the transcription of polygalacturonase mRNA and its enzymatic activity were significantly increased after the AKH treatment (Vinokurov et al. 2014, *J. Insect Physiol*. 60, 58-67). We have also described a unique digestive strategy used by *P. apterus*, employing plant enzymes from the food (linden seeds) in digestive processes. The mechanism of AKH action in salivary glands is unknown, but likely involves cAMP (and excludes cGMP) as a second messenger, since the content of this compound doubled in this organ after the AKH treatment.

This research was largely done in our laboratory, except the polygalacturonase study that was partly done by our student in Dr. Krishnan lab in the USA (University of Mississippi).

Principles of insect diapause and cold tolerance

We continued our research focusing on the analysis of two broad adaptive complexes – diapause and cold tolerance, which evolved in insects in response to environmental seasonality. Diapause is a hormonally mediated developmental arrest that is usually accompanied with metabolic suppression. Cold tolerance typically develops after the entry into diapause, during the process of cold acclimation. These two adaptive complexes are often studied in separation. Nevertheless, we study them simultaneously as they are physiologically linked and together they allow insects to safely overwinter in temperate habitats and exploit seasonally fluctuating resources.

We analyzed physiological mechanisms of cold tolerance in diapausing cold-hardy insects such as *Pyrrhocoris apterus or Chymomyza costata, and also used the tropical chill sensitive insect model, Drosophila melanogaster, for comparative purposes*. In the larva of the drosophilid fly, *Chymomyza costata*, we examined the associations between the physiological and biochemical parameters of differently acclimated larvae and their freeze tolerance. Entering diapause was found to be essential and sufficient prerequisite for attaining high levels of survival after freezing and cryopreservation in liquid nitrogen, although cold acclimation

further improved this capacity. Profiling of 61 different metabolites identified proline as a prominent compound whose concentration increased from 20 to 147 mM during diapause transition and subsequent cold acclimation. We provided direct evidence for the essential role of proline in high freeze tolerance by increasing the levels of proline in the larval tissues by feeding larvae proline-augmented diets. Differential scanning calorimetry analysis suggested that high proline levels, in combination with relatively low content of osmotically active water and freeze dehydration, increased the propensity of the remaining unfrozen water to undergo a glass-like transition (vitrification) and thus facilitated the prevention of cryoinjury (Košťál et al. 2011, *PNAS* 108, 13041-13046). In the third-instar larvae of *D. melanogaster*, we found that long-term cold acclimation considerably improved cold tolerance measured as survival time at 0°C. Such physiological plasticity in Lt_{50} was accompanied with modification of the metabolomic profiles of the larvae. Accumulations of proline (up to 17.7 mM) and trehalose (up to 36.5 mM) were the two most prominent responses. In addition, restructuring of the glycerophospholipid composition of biological membranes was observed (Košťál et al. 2011, *PLoS One* 6, e25025). Thus, the larvae of *D. melanogaster* showed a mild metabolic potential for the accumulation of proline, which was exploited in our next study. We showed that surprisingly simple laboratory manipulations can change the chill susceptible larvae of *D. melanogaster* to the freeze tolerant insect. Synergy of two fundamental prerequisites was required: (a) shutdown of larval development by exposing larvae to low temperatures (dormancy, quiescence), and (b) incorporating the free amino acid proline in tissues by feeding larvae a proline-augmented diet (cryoprotection) (Košťál et al. 2012, *PNAS* 109, 3270-3274). Restructuring of lipid composition of biological membranes is considered as another important mechanism that helps coping with changing environmental and body temperatures. We contributed to research in this field significantly in the past and we reviewed current knowledge on adaptive changes in membrane structure and function in insects (Košťál 2010, *Low Temp. Biol. Insects*, book chapter). In addition, we extended the scope of classic lipidomic analysis and brought original results on seasonal changes in minor phospholipid classes such as lysophosopholipids, free fatty acids, phytosterols and tocopherols in heteropteran insect, *P. apterus*. Distinct seasonal patterns of sterol and tocopherol concentrations were observed showing a minimum in reproductively active bugs in summer and a maximum in diapausing, cold-acclimated bugs in winter. Possible adaptive meanings of such changes might include: preventing the unregulated transition of membrane lipids from functional liquid crystalline phase to non-functional gel phase; decreasing the rates of ion/solute leakage; silencing the activities of membrane bound enzymes and receptors; and counteracting the higher risk of oxidative damage to PUFA in winter membranes (Koštál et al. 2013, *J. Insect Physiol.* 59, 934-941).

Overwintering in insect pests

We exploited the accumulated basic knowledge on physiological principles of insect cold tolerance in the follow up research oriented on the analysis of overwintering in economically important insect pests. The bark beetles, especially *Ips typographus*, are generally regarded as most devastating pests of spruce and some other trees. Our results demonstrate a strong need to fill in the gaps in our knowledge of bark beetle phenology. The seasonal development of physiological features underlying gradual acquisition of relatively high cold tolerance in overwintering individuals was followed in detail in the bark beetles, *I. typographus* (Koštál et al. 2011, *J. Insect Physiol.* 57, 1136-1146) and *Pityogenes chalcographus* (Koštál et al. 2014, *J. Insect Physiol.* 63, 62-70). Applying field collected data from the study period 2008-2013 and the results regarding induction of imaginal diapause and sister brood establishment in *I. typographus* to the mathematical model of bark beetle population dynamics PHENIPS

considerably increased the preciseness of the model (Berec et al. 2013, *Forest Ecol. Manag.* 292, 1-9). Its use in forestry praxis is currently being prepared.

The codling moth (*Cydia pomonella*) is a major insect pest of apples and several other fruits. We conducted a detailed study of overwintering in the larvae of codling moth (Rozsypal et al. 2013, *PLoS One* 8, e61745). We focused on seasonal changes in cold tolerance expressed as either increasing the capacity for supercooling or tolerance of freezing. The whole complex of physiological parameters was measured to provide explanatory base for observed phenotypic plasticity: hydration; osmolality of body fluids; activity of thermal hysteresis factors; relative proportion of bulk and bound water fractions; membrane phospholipid composition; amount and composition of storage fat; metabolic rate; metabolomic composition with special attention to potential cryoprotectants, etc. The physiological limits of cold tolerance that were measured at individual level in laboratory were compared with survival success in the field experiments. Such results will serve for improving the predictions of winter survival of pest populations in the field.

In collaboration with Spanish colleagues, we described unusual type of larval diapause and its hormonal control in the moth *Sesamia nonagrioides* that causes severe losses to maize and some other crops in the Mediterranean, and it is likely to expand to the Central Europe. We suggested that similar diapause may also occur in related pest species living in Africa and Asia (Perez-Hedo et al. 2010, *Mol. Cell. Endocrinol.* 70, 519-529). This information facilitates the timing of insecticide application and may be used in designing pest control by modified agrotechniques.

Control of pest species and biorationale insecticides

In 2012 and 2013, we performed a large scale field experiment on active dispersal of bark beetles. The project was supported by EU, National Park Šumava and Forests of the Czech Republic. We compared the flight distance in the adults of the spruce bark beetle in two localities of different population density and concentrated also on protective measures (trap trees vs. pheromone traps vs. poisoned trap trees) against this pest (Montano et al., *J. Appl. Ecol*., in press). In a parallel study we proved that bark beetles actively digest cellulose with their own cellulolytic system, which is in a sharp contrast with the previously published theories about symbiotic microorganisms. The methodology used in this study was published together with our foreign partners (Kasson et al. 2013, *Fungal Gen. Biol*. 56, 147-157). As a part of collaboration with FCR, we summarized practically applicable results in six research reports and compared our findings with the traditionally applied protective measures and valid legislation, clearly demonstrating higher efficiency and lower cost of protective measures applied according to the above mentioned results. Therefore, some of the results were implemented to the internal methodology of forest protection against bark beetles in the Czech Republic. One study was published together with the Laboratory of Molecular Genetics of our Institute (Viktorinová et al. 2011, *Arch. Insect Biochem. Physiol*. 77, 179-198). At the request of our foreign partners we performed limited studies on the phenology and its external (environmental) and internal (neuro-hormonal) control in diverse pest species. In the mosquito *Aedes aegypti*, the transmitter of malaria, we participated in research demonstrating fluctuations in its sensitivity to insecticides (Yang et al. 2010, *J. Insect Physiol*. 56, 1219-1223). The mechanism has not been elucidated but the phenomenon is of considerable practical importance. Changes in the reproduction potential of the locust *Schistocerca gregaria* are important for the switch from its inoffensive solitary to the devastating gregarious phase. We showed that the lack of mating due to low population density plays considerable role (Wang and Sehnal 2013, *Chinese Sci. Bull*. 58, 3244-3247). Our studies on direct pest control focused on the environment-friendly methods: biological control by entomopathogenic nematodes and fungi, application of insect growth regulators, and use of "genetically modified" (GM) plants

(see also next paragraph). A combination of two different kinds of insect regulators (nonsteroidal ecdysone agonist RH-5992 and chitin biosynthesis inhibitor lufenuron) was tested in *Spodoptera littoralis* (Gelbič et al. 2011, *Centr. Eur. J. Biol*. 6, 861-869).

Daily and seasonal activities of different species of mosquitoes were studied in the South Moravia within the 2010-14 period (Šebesta et al. 2012, *Centr. Europ. J. Biol*. 7, 288-289) and later used for assays to test the effects of tebufenozide, a nonsteroidal agonist of ecdysone, on the egg development and reproduction in *Culex quinquefasciatus*. It was found that doses below the LC50 (1.29 ng/l H_2O) induce vitellogenesis in the females that subsequently lay eggs autogenically (without blood feeding). The number of eggs laid in each clutch was lower compared with the anautogenous clutch, and also gradual degeneration of the ovaries was observed in the treated mosquitoes (Gelbič and Rozsypalová 2012, *Physiol. Entomol*. 37, 119-126,).

Genetically modified plants and their impact on insect populations

More than 10 years ago, the plantation of GM crops began to spread overseas but was objected by many Europeans who feared of unexpected environmental damage. We decided to involve to verification of this concern by studying insect communities in plots planted with a GM and non-GM but otherwise related cultivars. Results were published in a series of papers. Insect-resistant cultivars contained one or more genes derived from *Bacillus thuringiensis* and expressing insecticidal Cry proteins. Epigeic spiders were examined in plots planted for 3 years with GM maize MON 88017 (tolerance to glyphosate and resistance to western corn rootworm, *Diabrotica virgifera virgifera*, due to expression of Cry3Bb), its near isogenic non-GM cultivar treated or not treated with an insecticide and two unrelated maize cultivars (Svobodová et al. 2013, *J. Appl. Entomol.* 137, 56-67). Similarly, the non-target plant-dwelling insects were not influenced by the Cry1Ab expression (Habuštová et al. 2014, *J. Appl. Entomol*. 138, 164-172). Similarly, neither abundance nor species richness of the ground-dwelling arthropods were influenced by the presence and expression of a *Cry* transgene (Skoková-Habuštová et al., *J. Appl. Entomol*, in press).

B. thuringiensis is widely used in spray and its *Cry* genes are introduced to diverse crops. However, some insect taxa are resistant to all Cry proteins that have been tested. This appears to be true for the bark beetles (Scolytidae). We prepared more than 20 recombinant modifications of Cry3Aa and tested them first on *Tribolium castaneum*. The natural Cry3Aa was the best, followed by the recombinant Cry3Aa (Mostafa et al. 2013, *J. Appl. Entomol*. 137, 684-692).

Use of silk protease inhibitors in plant protection

Prior to the evaluation period we discovered a gene expressed in the silk glands of *Galleria mellonella* and encoding a protease inhibitor (called SPI2) active on some fungal proteases. Transgenic potatoes containing *SPI2* gene were prepared and tested in collaboration with plant molecular biologists and potato pathologists. Unfortunately, the resistance to the late blight, *Phytophthora infestans*, was weak and unstable (Navrátil et al. 2012, *IOBC/WPRS Bulletin* 73, 61-67; Kodrík et al. 2013, *Appl. Biochem. Biotechnol*. 171, 209-224).

Silk is an interesting natural material that has not been fully exploited (Sehnal 2010, Insect Biotechnology, book chapter, Springer). Our studies contributed to the generalization that the core of silk fiber, which is called fibroin, is typically composed of 3 types of proteins. The fiber is enveloped by several sticky proteins that are derived in *Bombyx mori* from 3 genes. We showed that both fibroins and sericins are to great extent species specific and harbor diverse properties (Žurovec et al. 2013, *Biomacromolecules* **14**, 1859-1866). On the basis of *B. mori* sericin 2 we proposed and patented peptides with good adhesiveness to different substrates (Kludkiewicz et al. 2010, Patent CZ 302255). In addition to these structural proteins, the silk

contains proteins of little known functions. We have identified several categories of such proteins, for example serpins (serine protease inhibitors) (Yonemura et al. 2012, *Insect Biochem. Mol. Biol*. 42, 371-380).

Novel methods for metabolomic studies

We expanded our methodological background and developed several novel analytical platforms for metabolomic analyses. Within the glycomics platform we developed a new method of processing of saccharides and polyols which includes oximation of carbonyl groups and silylation of the present hydroxyls, prior to subsequent GC-MS analysis. Within the lipidomics platform we developed novel HPLC-MS approach to study insect phospholipids and fatty acids (Tomčala et al. 2010, *Comp. Biochem. Phys. B* 156, 26-37; Zahradníčková et al. 2014, *J. Sep. Sci*. 37, 2062-2068). Furthermore, we investigated a class of cyclic peptides by HPLC-MS, mainly beauverolides, beauvericin, cyclosporines, enniatines and leucinostatines produced by economically important entomopathogenous fungi. This research was contracted by the world leading bioinsecticide manufactures Futureco Bioscience S.A., Olerdola, Barcelona, Spain and Laverlam Corp, Butte, MT, USA. We also studied in depth a novel analytical method for acid and basic metabolites which enables to profile more than 200 polar metabolites, mainly amino acids, small peptides, organic acids and biogenic amines, involved in central metabolism and related pathways. Particular steps of the sample preparation workflow were investigated in depth with a research team of the Faculty Hospital Ostrava, Institute of Clinical Biochemistry, Czech Republic (Hušek et al. 2012, *J. Pharm. Biomed*. Anal. 67-68, 159-162; Švagera et al. **2012**, *Anal. Bioanal. Chem.* 402, **2953-2963).** The methodology enables concurrent measurements of the metabolite extracts by GC-MS as well as LC-MS. The latter method was employed for the analysis of the metabolite dipeptide proline-hydroxyproline in urine (Cimlová et al. 2012, *J. Mass Spectrom*. 47, 294-302). Within the analysis of sterols and steroids metabolites we discovered that fluoroalkyl chloroformates under anhydrous conditions derivatize aliphatic and alicyclic hydroxyls efficiently as classical silylation procedures. This completely new methodology was developed and reported for GC-MS analysis of sterols, neuroactive steroids and tocopherols (Řimnáčová et al. 2014, *J. Chromatogr. A* 1339, 154-167).

An increased demand to study highly polar, ionic metabolites stimulated a development of an HPLC-MS method on hydrophilic (HILIC) stationary phases**.** We accomplished quantitative analysis of adenosine, inosine and their phosphates in mutant larvae of *Drosophila melanogaster* and measured increased levels of extracellular adenosine in adenosine deaminase deficient flies (Žuberová et al. 2010, *Dis. Model. Mech.* 3, 773-784), and performed comparative nucleoside analyses in different *Drosophila* cell lines (Žurovec et al. 2012, *Insect Biochem. Mol. Biol.* 42, 321-331).

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Biosystematics and Ecology

Biosystematics and Ecology carries out ecological and taxonomical research. The strength of the team is in its combination of theoretical, experimental, and empirical approaches to address a variety of ecological questions. This research is backed up by strong taxonomic background in several groups (Coleoptera, Lepidoptera, Hymenoptera, Ephemeroptera, Sternorrhyncha, Arachnida and entomopathogenic Nematoda) combined with molecular approaches. The team also builds on the long-term data series on community dynamics of phototactic Lepidoptera, tri-trophic associations between plants, aphids and parasitoids, and a unique dataset on mayfly, stonefly and caddisfly communities in running waters across the Czech Republic. Some research is also applied to biological control and Integrated Pest Management.

In the period 2010-2014, the team included 15 researchers and 7 postdocs. In addition, several students at various stages of their higher education participated in the research of the team. Team members published 140 articles in ISI-ranked journals, two monographs (Bauernfeind & Soldán 2012, *The mayflies of Europe (Ephemeroptera)*, Apollo Books; Spitzer and Bufková, 2013, *Peatlands of Šumava*, Národní park Šumava), 87 other publications, one US patent, and one industrial design. Many of these outputs resulted from intensive international collaboration. Members of the team (co-)organized two international conferences in the Czech Republic. In the monitored period 2010-2014 articles (co-) authored by members of the team were cited over 3000 times. The team has been funded by several grants from the Czech Ministry of Education, the Grant Agency of the Czech Republic, and one Marie Curie European Reintegration Grant. Four postdocs were financed by the Bioglobe/Modbiolin projects. During the period 2010-2014 three PhD students successfully defended their doctoral thesis.

Biosystematics and Ecology consists of five labs:

Theoretical Ecology (currently 2 researchers, 1 postdoc, 1 foreign postdoc financed from the Bioglobe project, 6 doctoral students) focuses on applications of mathematical tools to understand key mechanisms that promote biodiversity, limit pest species impacts, support persistence of threatened species, and exploit economically important species in a sustainable way. Two senior researchers teach regular courses at the Faculty of Science, University of South Bohemia at Ceske Budejovice and Faculty of Science, Masaryk University at Brno. The lab has developed international cooperation with several researchers and institutes worldwide (e.g. Mathematical Bioscience Institute, Columbus, USA; Wilfred Laurier University, Waterloo, Canada; EAWAG, Kastanienbaum, Switzerland; Valparaiso University, Valparaiso, USA; U.S. Forest Service, Morgantown, USA; University of Georgia, Athens, USA etc.). One international conference (15th ISDG symposium) was co-organized in the Czech Republic.

Ecology of Aquatic Insects and Relict Ecosystems (currently 7 researchers, 1 postdoc, and 1 PhD student) focuses on life histories, population dynamics and community assembly of aquatic insects and other invertebrates. These taxa serve as model groups to answer fundamental

questions on the role of selected biotic and abiotic factors on interactions between individuals and populations and on community assembly. Three researchers regularly teach at the Faculty of Science, University of South Bohemia, and several undergraduate students are expected to start their PhDs in late 2015. The laboratory has ongoing international cooperation with researchers and institutes in Czech Republic and across Europe (e.g., Staatliches Museum für Naturkunde Stuttgart, Germany; University of Sheffield, UK; University of Bergen, Norway; Norwegian University of Science and Technology, Trondheim, Norway; Museum of Zoology, Lausanne, Switzerland; Museum of Natural History, Wien, Austria; University of Pécs, Hungary; Hungarian Academy of Sciences, Debrecen, Hungary; Jagellonian University, Kraków, Poland; Technical University in Zvolen, Slovakia; Institute of Biodiversity and Ecosystem Research, Sofia, Bulgaria; Masaryk University, Brno, Czech Republic).

Aphidology (currently 2 researchers) carries research on aphids and their parasitoids worldwide. The work integrates the research in biosystematics, distribution, host range, genetics and phylogeny (using molecular approaches), ecosystems and agroecosystems, up to ecological approaches in pest aphid management. Extensive world-wide collaboration network includes e.g., University of Beograd, Serbia, University of Nis, Serbia, University of Lleida, Spain; Univ. de Barcelona, Spain; Benaki Institute of Phytopathology, Greece; University of Zabol, Iran; University of Malta, Malta; University of Batna, Algeria; University of Vilnius, Lithuania; Seoul National University, Korea; Komensky university, Slovakia, etc. The two researchers have acted as external consultants of doctoral and postdoctoral students at some of these institutes.

Entomopathogenic Nematodes (currently 2 researchers, 2 postdocs, 1 foreign postdoc financed from the Modbiolin project, 5 undergraduate students) focuses on nematodes that belong to the families Steinernematidae and Heterorhabditidae. High efficiency and possibility of mass production in artificial media make EPNs a widely used bioagents in the biological pest control. One researcher teaches regular courses at the Faculty of Science, University of South Bohemia at Ceske Budejovice. Long term collaboration has continued with University of Florida, Gainesville, USA; the A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia; John Paul II Catholic University of Lublin, Poland; China Agricultural University, Beijing, China.

Experimental Ecology (currently 1 researcher, 1 foreign postdoc financed from the Bioglobe project, 1 PhD student) focuses on antagonistic interactions among arthropods that can be used in biological/biorational methods of pest control. Main interest is ecology and behaviour of model predatory mite species (Phytoseiidae) and some phytophagous mites (Tetranychidae, Eriophyidae), acaro/entomopathogenic fungi and plant-herbivore interactions. The laboratory also collaborates with other institutes from the Czech Republic and abroad (Crop Research Institute Prague, Czech Rep.; MTT Jokioinen, Finland, National Research Center Cairo, Egypt). One international conference (IOBC working group) was organized in the Czech Republic.

The main research themes and results for period 2010-2014 are summarized below.

Theoretical behavioral/evolutionary ecology. At the behavioral level we use techniques of the game theory and adaptive dynamics to develop models of species spatial distribution. The Habitat Selection Game that we introduced earlier seeks the evolutionarily stable (i.e., resistant to mutant invasions) distribution of a single or multiple species in a heterogeneous habitats consisting of several patches. Although we proved earlier that the IFD is evolutionarily stable provided patch payoffs are negatively density dependent, it turns out (Cressman & Krivan 2010,

Oikos 119, 1231-1242) that when population abundance changes in time and the resident population distribution follows the IFD at each population abundance, individuals that play another strategy can coexist with residents. In other words, a polymorphism with respect of patch use can evolve once population dynamics are considered. The single species distribution theory was extended for two interacting species that are either competing, or in predator-prey relation (Cressman & Krivan 2013, *J. Math. Biol.* 67, 329-358). Evolution of mobility in predator-prey systems was studied in Xu et al. (2014, *Disc. Cont. Dyn. Syst. B* 19, 3397-3432). Game theory was introduced to the optimal foraging by Cressman et al. (*PLoS ONE* 9, e88773). Here we introduced decision trees to build models of optimal foraging with time constraints. A classical example of such a model is the diet choice model of the optimal foraging theory. This model was used as the starting point and we showed how more general models that include e.g. prey recognition time or simultaneous encounter with two prey types can be built. Game theoretical approach based on adaptive dynamics was also used to resolve an apparent paradox concerning sexually transmitted parasites promoting mating success in their hosts. Seemingly profitable strategy for a parasite has been observed only rarely. It turns out that such a strategy is often too costly for the parasite or its host to be frequently found in nature (Berec & Maxin 2014, *J. Theor. Biol.* 342, 47-61).

Theoretical population ecology. At the population level we have studied the impact of Allee effects on population and community dynamics, as well as of a variety of parasites on dynamics of their hosts. Allee effects occur when individual animals profit from presence of their conspecifics. If strong enough they trigger a critical population density, the Allee threshold, below which the population is doomed to extinction, a phenomenon of critical importance in applied ecology. Regarding specifically pest control efforts, if a tactic is applied that pushes the population density below the Allee threshold pest eradication becomes a viable option. We have developed this idea in a number of articles, including both theoretical ones (Berec 2010, *Bull. Math. Biol.* 72, 94-121; Pavlova et al. 2010, *J. Theor. Biol.* 264, 787-798; Pavlova & Berec 2012, *Theor. Ecol.* 5, 533-544; Berec & Mrkvicka 2013, *Oikos* 122, 845-856; Krivan 2014, *J. Math. Biol.* 69, 1497-1513) and ones with highly applicable potential (Tobin et al. 2011, *Ecol. Lett.* 14, 615-624; Blackwood et al. 2012, *Proc. R. Soc. B* 279, 2807-2815; Epanchin-Niell et al. 2012, *Ecol. Lett.* 15, 803-812). These articles convincingly demonstrate that a variety of tactics can be used for species eradication, operating over a reasonable timeframe and at a reasonable cost. From the theoretical perspective we showed that Allee effects can make the population and community dynamics much more complex compared with when absent.

Parasite models. Parasites have recently been considered a viable option for controlling a variety of alien animal populations. As with more classical biocontrol approaches, theoretical ecology has become an inevitable tool in this area, since it helps to avoid unpromising (ineffective and/or costly) yet potential control tactics. We have considered two hitherto unexplored aspects of host-parasite dynamics to understand their effect on host population dynamics. In particular, we have been interested in an added value of increased life expectancy of hosts sterilized by the parasite, since increased lifespan and hence increased potential of the infected individuals to spread the disease might further enhance pest control effectiveness (Berec & Maxin 2012, *J. Math. Biol.* 64, 1281-1311). We found the effect quite significant, especially for relatively short-lived species in which the efficiency of sterilization was relatively high. The second aspect not considered so far in modelling host-parasite interactions can be shortly stated as follow: for animals in which mating and giving birth are tightly coupled and which are attacked by a sterilizing, sexually transmitted disease, mating mediates both reproduction and parasite transmission. Hence, to model dynamics of such interactions, a structural consistency between the processes of reproduction and pathogen transmission needs

to be accounted for. We show that highly sterilizing, sexually transmitted pathogens trigger bistability in the host population, such that the host population can end up in two extreme alternative states, disease-free persistence and pathogen-driven extinction, depending on its initial state (Berec & Maxin 2013, *Bull. Math. Biol.* 75, 258-273). In general, both our results characterize pathogens that are promising candidates for an effective pest control and that might possibly be engineered if not occurring naturally.

Food webs. Species in isolated complex food webs are prone to extinction due to strong interactions, such as competition or predation. The decrease of species coexistence with food web complexity (often expressed as food web connectance) is one of the major results of theoretical ecology. Some recent works suggested that adaptive foraging can reverse this trend. In Berec et al. (2010, *J. Theor. Biol.* 266, 211-218) we showed that for the classical search image model and the diet choice model the adaptive foraging increases the number of coexisting species but it does not reverse the negative trend between the number of species that survive and the food web connectance. It has been shown that in di- and tri-trophic food webs adaptive foraging relaxes apparent competition and increases species biodiversity (Krivan 2014, *J. Theor. Biol.* 343, 127-137). The topology of food webs is frequently described using the niche model. A modified version of the niche model that allows to control the strength of size-dependence of predator-prey links was developed (Klecka 2014, *PloS ONE*, e99355). This empirically motivated extension of the niche model captures structure of real food webs more realistically when compared to the classical niche model.

Taxonomy, ecology and palaeontology of mayflies and related taxa. Our taxonomic work focused on mayflies (Ephemeroptera) and builds on the unique collection and expertise of T. Soldán, who represents one of the leading personalities in the study of this group world-wide. We focused on disentangling sytematic relationships and applying innovative approaches (morphometric matrices, molecular data) in combination with classical morphogical studies to resolve complex taxonomic problems. This effort resulted in a number of papers dealing with a variety of mayfly taxa (e.g. Sroka *et al*. 2010, *Zootaxa* 2490, 16-32; Sroka 2012, *Aquat. Ins*ects 34, 23-53).

The book „*The mayflies of Europe (Ephemeroptera)*" by Bauernfeind and Soldán (2012, Apollo Books, Denmark) summarized all available information on the ecology, taxonomy and biogeography of almost 300 mayfly species on 800 pages; it will be the main reference on the group in the future. Data acquired by a team lead by T. Soldán further resulted in collaborative studies of long-term changes in aquatic insect assemblages and identification of the main drivers and threats to biodiversity loss in Czech running waters (e.g., Bojková *et al*. 2012, *Freshw. Biol*. 57, 2550-2567; Bojková *et al*. 2014, *Insect Conserv. Divers*. 7, 252-262). Another long-term collaborative project summarized the diversity, long-term changes and recovery dynamics of aquatic insects in Bohemian Forest glacial lakes (Soldán *et al*. 2012, *Silva Gabreta* 18, 123-283).

Our expertise on the morphology of extant basal pterygotes was used in collaborative research of presumably related fossil taxa. The collaboration led to the descriptions of new insect orders Carbotriplurida (Staniczek *et al*. 2014, *Syst. Entomol*. 39: 619-632), the putative link between apterygote Zygentoma and all pterygote insects, and Coxoplecoptera (Staniczek *et al*. 2011, *Insect Syst. Evol*. 42, 101-138). Sroka et. al. (2014, *J. Syst. Palaeont*., early online) provided the first cladistic analysis of all major fossil paleopterous insect lineages. These studies are of crucial importance for a proper understanding of the phylogeny of the entire order Insecta.

Life histories and trophic interactions of aquatic insects. In 2010, we have started to develop this topic as a new line of research, with emphasis on predatory insects in standing waters. Using laboratory experiments with common predatory freshwater insects (diving beetles, dragonfly and damselfly larvae and water bugs) and their prey, we found that the structuring of trophic interactions in standing waters is non-random and gives rise to highly interconnected food webs with partly separated benthic and pelagic modules (Klecka & Boukal 2012, *PLoS ONE* 7, e37741). Moreover, we provided the first quantitative evidence showing that underlying predator-prey body size allometries and predation strength are modified by additional traits such as predator and prey microhabitat use, foraging strategies and morphology of predators, and vulnerability of prey (Klecka & Boukal 2013, *J. Anim. Ecol*. 82, 1031-1041). These results indicated that the inclusion of commonly available data on species traits could substantially increase biological realism of food web descriptions, which rely predominately on body size data. We used additional evidence from other taxa to outline a general trait-based framework that can characterize trophic interaction strengths in freshwater food webs (Boukal 2014, *J. Limnol*. 73, 171-185). We have also begun to study the role of abiotic conditions such as temperature and habitat complexity in trophic interactions (Klecka & Boukal 2014, *Oecologia* 176, 183-191) and plasticity of life histories using laboratory and mesocosm experiments.
Paleorefugia. The study of relict ecosystems benefited from high geological, geomorfological and biotic diversity of the Czech Republic. This creates local Central European refugia for invertebrates with the main distributional area the boreal forests or tundra; typical habitats include peat bogs, talus slopes and sandstone labyrinths (Růžička 2011, *Polish J. Ecol.* 59: 367–380). Long-term research on the ecology of Lepidoptera of peat bogs was summarized in the book „*Peatlands of Šumava*" (Spitzer & Bufková 2013, Administration of the Šumava National Park and Protected Landscape Area, Vimperk). We also elucidated larval ecology of many peatbog moths, especially those associated with *Ledum palustre* and *Vaccinium uliginosum*, and described their parasitoids (Lozan *et al*. 2011, *Entomol. Fenn.* 21, 243–253; Lozan, Spitzer & Jaroš 2012, *J. Insect Conserv*. 16, 391–397; Spitzer & Jaroš 2014, *SHILAP Rev. Lepid*. 42, 319–327); we found that typical peat-bog moth species are parasitized by widely distributed, generalist parasitoids. We also showed that low-altitude, freezing talus slopes can serve as palaeorefugia of boreo-alpine bryophytes, pteridophytes and arthropods (Růžička al. 2012, *J. Nat. Hist.* 46, 2145–2157) and developed a theory on the repeated and reversible evolution of troglomorphism in spiders during Quaternary climatic cycles (Růžička et al. 2013, *Int. J. Speleol*. 42, 133–140).

Invasive aphid and parasitoid species. Research on invasive species in Europe centered on the occurrence, ecological adaptation and prognosis of the Russian wheat aphid, *Diuraphis noxia* in the Czech Republic on the world background (Liu et al. 2010, *J. Econ. Entomol.* 103, 958-965; Havelka et al. 2013, *J. appl. Ent.* 138, 273-280; Novotná et al. 2011, *Genetica* 139, 281-289). Also, other invasive species have been targeted: *Impatiens glandulifera* (Starý et al. 2014, *J. Insect Sci.* 14, 1-6; Starý et al. 2014, *J. Entomol. Res. Soc.* 16, 33-43), *Aphis illinoisensis* (Havelka et al. 2011, *Arch. Biol. Sci* 63, 269-274), the Greenidene aphids (Starý et al. 2010, *Ann. Entomol. Soc. Amer*. 103, 307-321) and *Brachycaudus divaricatae* (Bašilova et al. 2012, *Biologia* 67, 959-965). Adaptations of invasive parasitoid species in Europe were studied on *Lysiphlebus testaceipes* (Mitrović et al. 2013, *Biological Control* 66, 150-158; Žikič et al. 2015, *NW J. of Zoology* 11, 97-101), *Lysiphlebus orientalis* (Petrović et al. 2014, *Bull. Ent. Res.* 103, 451-457; Tomanović et al. 2014, *Bull. Ent. Res.* 104:552-565). Also, parasitoid of invasive pests on soybean in the USA were targeted (Starý et al. 2010, *J. Hym. Res.* 19,179-186).

Aphid and parasitoid biosystematics. Integrative contributions to the biosystematics of parasitoids including DNA analyses were made (Stanković et al. 2015, *Eur. J. Entomol.* 12,

165-174; Petrović et al. 2014, *Ann. Entomol. Soc. Am.* 107, 1027-1032; Rakhshani et al. 2015, *Zootaxa* 3905, 474-488). Complex biosystematic research on aphids and parasitoids has been applied to the aphid fauna and its practical importance in Lithuania (Bašilova et al. 2012, *Biologia* 67, 959-965). Biosystematic research on aphids and parasitoids also covered the tropics of Thailand (Starý et al. 2010, *Zootaxa* 2498, 47-52.), Cost Rica (Zamora et al., 12010, *Psyche* 278643; Zamora et al. 2011, *J. Ent. Soc.* 13, 107-115), India (Rakhshani et al. 2012, *Zootaxa* 3397, 45-54), and the Mediterranean up to Central Asia (Stary et al. 2014, *Arthr. fauna of the UAE* 5, 407-425; Nazari et al. 2012, *Acta ent. Mus. Nat. Pragae* 54, 559-584; Mossadegh et al. 2011, *Asian J. Biol. Sci*. 4, 175-181; Laamari et al. 2012, *African Entomology* 20,161-170; Barahoei et al. 2014, *J. Crop Prot.* 3, 199-232; Mitrovski-Bogdanovic et al. 2014, *Zool. Anzeiger* 253, 270-282). Determination of the use of DNA barcoding in the identification of conifer woolly aphids (Sterrnorhyncha, Adelgidae, *Gilletteella* species complex) contributed to clarifying the species identity and biology of pest aphids in forestry (Zurovcová et al. 2010, *Eur. J. Ent.* 107, 147-156). The whole group of *Cinara* aphids associated prevailingly with conifers has been studied in detail, integrating the taxonomic- molecular approaches.

Tritrophic associations of plants, aphids and parasitoids on crops in various areas were studied at several locations: Turkey (Satar et a. 2014, *J. Insect Sci.* 14, Article Number 178), SE Europe (Kavalieratos et al. 2010, *Ann. Ent. Soc. Am* 103, 307-321; Kavallieratos et al. 2013, *Ann. Entomol. Soc. Am.* 106, 294-309; Pike et al., 2011, *Zootaxa* 2802, 58-62; Kos et al. 2012, *Zootaxa* 3456, 36-50), Iran (Rakhshani et al. 2012, *J. Insect Sci*.12, Article 143; Alikhani et al. 2013, *Biologia* 68, 966-973; Nayari et al. 2012, *Acta ent. Mus. Nat. Pragae* 54, 559-584). Nature conservancy studies targeted tritrophic associations on *Aconitum* in the Czech republic (Havelka et al. 2014, *Bull. of Insectology* 67, 57-61), in Serbia and Slovenia (Petrović et al. 2011, *Zootaxa* 2895, 58-64; Kos et al. 2012, *Zootaxa* 3456, 36-60), and in the wetlands (Tomanović et al. 2012, *Ann. soc. entomol. Fr.* 42, 189-198). The dataset on plant-aphid-parasitoid tri-trophic associations in the Czech Republic was mathematically analyzed (Melián et al. 2015, *Am. Nat.* 185, 157-168).

Biocontrol and IPM using parasitoids. Several parasitoid biocontrol agents were re-classified on grounds of taxonomy, ecology ,distribution and the molecular approaches (Tomanović et al., 2014, *Bull. ent. Res.* 104, 552-565; Mitrović et al. 2013, *Biological Control* 66, 150-158) The adaptation of the local parasitoids of newly invasive aphids manifested significant role of the local parasitoids as pre/emptive biological control in ecosystems and agroecosystems/urban agglomerations (Havelka et al. 2011, *Arch. Biol. Sci*. 63, 269-274). Research on aphids and parasitoids contributed to the IPM on alfalfa in Spain (Pons et al. 2011, *J. Pest Sci.* 84, 437-445; Pons et al. 2013, *BioControl* 58, 733-744). Aphids and their associated parasitoids have been determined as a useful group/model association in the research on ecosystems and agroecosystems in ecological/friendly pest management as well as the nature conservation efforts.

Taxonomy of entomopathogenic and entomoparasitic nematodes. Description and redescription of entomopathogenic nematode species is important in biological control of insect pests. Three isolates of entomopathogenic nematode were isolated by baiting soil samples from the Mendi area, Western Wollega, Ethiopia and described as *S. ethiopiense* sp. n. (Tamiru et al. 2011, *Nematology* 14, 741-757). In another study, a new entomopathogenic nematode, *Steinernema huense* sp. n. (Fig 1), belonging to the *carpocapsae* group, was recovered in Bach Ma National Park (Thua Thien Hue province), Vietnam (Ke Long Phan et al. 2014, *Nematology* 16, 761-775). Another new steinernematid nematode, *Steinernema poinari* sp. n., was recovered in three localities of southwest Bohemia, Czech Republic (Mráček et al. 2014, *Zootaxa* 3760, 336–350).

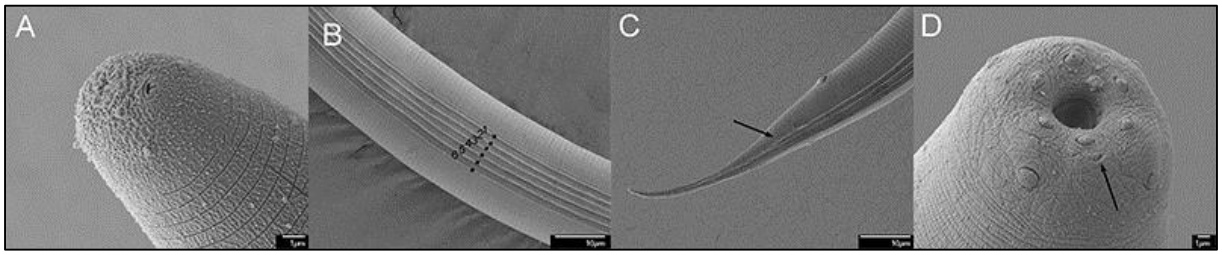


Fig 1. *Steinernema huense* sp. n. SEM of infective juvenile, male and female. A-C: Infective juvenile; A: Head region with four papillae and amphid openings; B: Lateral field in mid-body; C: Lateral field in tail region with phasmid opening; D: First generation male, Head with labial and cephalic papillae.

A population of *Oscheius chongmingensis* was isolated from a soil sample collected from an alfalfa field in the vicinity of Hailar, Inner Mongolia, and was designated as the Tumian strain In our re-description of this nematode (Liu et al. 2012, *Nematology* 14, 139-149) we added important morphology and molecular characters and studied its entomopathogenicity. In cooperation with Polish fellows several new morphological characters were found in steinernematids. Our observations based on scanning electron microscope (ScEM) analysis revealed the presence and location of the phasmids on first generation males of *Steinernema arenarium* (isolate BYS), *S. carpocapsae* (RaP) and *S. feltiae* (RaP). The phasmids are situated at the end of the tail, usually close to the base of the last pair of caudal papillae or centrally among the penultimate and the ultimate couples. No apertures at the end of spicule tip were observed (Skrzypek et.al. 2012, *Russ. J. Nematol*. 20, 119-126). In the light of current morphological knowledge, the *S. carpocapsae* morphology was not satisfactorily described, some important characters of this species are missing and no thorough molecular characterisation of this species has been performed. *S carpocapsae* represents a type of the '*carpocapsae*' group and thus our objective was to investigate important morphological and molecular characters of this species (Mráček et.al. 2014, *Russ. J. Nematol*. 22, 109-120). Both studied genetic markers effectively characterize *S. carpocapsae* as a species, showing very low interspecific variation.

Competition of entomopathogenic nematodes. Previous research has shown that in the competition within one insect host, nematode *S. affine* strongly dominates over *S. kraussei.* In another study, various aspects of the coexistence of these nematodes were studied in the field and laboratory (Půža & Mráček 2010, *Appl. Soil Ecol.* 45, 65-70). Competition experiments showed a strong dominance of *S. affine* in all tested insects. Generally it seems that both species share the same ecological niche and thus the avoidance of competition with the latter species seems to be a crucial factor for *S. kraussei*. Patchy distribution and implicit differences in horizontal distribution probably markedly contribute to the coexistence of both species. This study suggested an important role of the symbiotic bacteria in the EPN competition. Thus we isolated the bacteria and prepared axenic larvae of both strains, and we reared the larvae of both strains separately and in mixture on single or mixed symbiotic bacteria (Půža et al., 2013 *IOBC-WPRS Bulletin* 90, 273-276). While *S. affine* is able to develop, mature and produce viable progeny on the symbiont of *S. kraussei,* the later developed and reproduced well only on its own symbiont, however, its growth on the symbiont of *S. affine* was minimal. These experiments explained the previously observed dominance of *S. affine* over *S. kraussei*.

Scavenging of EPNs. EPNs have been shown to invade also insect cadavers. We assessed the scavenging of EPNs by exposing of the living and freeze-killed natural and laboratory hosts,

with different susceptibility to *Steinernema affine* and *S. kraussei* (Půža & Mráček 2010, J. *Invertebr. Pathol.* 104, 1-3). Both nematodes colonised both living and dead *G. mellonella* and *B. germanica*. Living carabid beetles and wireworms were resistant to the infection, however, both nematodes were able to colonise and multiply in their dead bodies (Fig. 2). We demonstrated that by scavenging, EPNs can utilise cadavers of insects that are naturally resistant to EPN infection, and so broaden their host range.

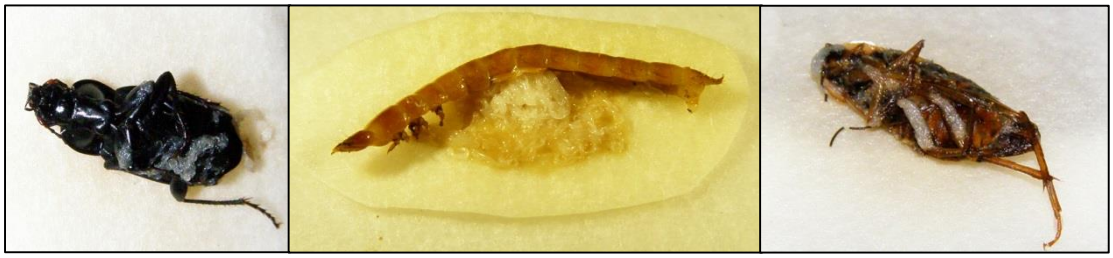


Fig 2. Cadavers of *P. cupreus*, wireworm and *B. germanica* with growing *S. affine*

Effect of pesticides on EPNs. The influence of pesticides on the viability and infectivity of EPNs *Steinernema feltiae, S. arenarium* and *S. kraussei* was determined in water solutions of 13 pesticides. The highest negative influence on infectivity and viability was seen in oxamyl, sulphur and also mancozeb and fenitrothion (Nermuť and Mráček 2010, Russ. J. Nematol. 18, 141-148).

Ecology of slug parasitic nematodes. P. hermaphrodita is an important parasite of noxious slugs, but some aspects of its life history have not yet been described satisfactorily. We studied the effect of intraspecific competition on the development and reproduction of this nematode in a series of laboratory experiments. We demonstrated that intraspecific competition negatively affects the yield and quality of dauer juveniles of the slug parasitic nematode *P. hermaphrodita*, but it seems that this nematode can partly prevent overcrowding by avoiding occupied sites. (Nermuť et al. 2012, *B. Sci. Technol*. 22, 1389-1397). It is known, that soil types affects survival of various nematodes. We observed persistence of *P. hermaphrodita* in four different soils (Nermuť 2012, *Russ. J. Nematol*. 20, 61-64). Overall, we demonstrated that *P. hermaphrodita* is well able to detect host associated volatile cues and move in mineral substrate (Nermuť et al. 2012, *Biol. Control* 61, 201–206). The ability of this nematode to exploit various nutritional sources is probably mediated by the diverse species composition of bacterial associates (Nermuť et al. 2014., *B. Sci. Technol*., 24, 1026-1038).

Biological control of insect and mite pests. Chemical control of insect and mite pests shows its limits due to increasing resistance to pesticides and also due to public concerns about residues in food and negative impacts on environment. Among others, this leads to changes in the European regulatory environment and a considerable growth of the biopesticide industry in recent years which is expected to continue its growth momentum to reach approximately US $1.371 billion by 2017. Mycoinsecticides based on various entomopathogenic fungi species have a big potential for sustainable pest control. In collaboration with a private company we developed and patented a unique strain of entomopathogenic fungus *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*) (Prenerová E. et al. 2013, Strain of entomopathogenic fungus *Isaria fumosorosea* CCM 8367 (CCEFO.011.PFR) and the method for controlling insect and mite pests. *US Patent No. US8,574,566 B2*). The strain was isolated from *Cameraria ohridella*, a serious invasive pest of horse chestnut trees in Europe, and is currently deposited under number CCM 8367 as a patent culture in the Czech Collection of Microorganisms in

Brno. It is extremely virulent towards major agricultural pests and pests with importance for communities (Fig. 3). The organism is safe towards non-target insects. *Isaria fumosorosea* has been approved by US and EU legislation as a biopesticide. The running trials confirm further strong effects against numerous invasive pests (Hussein et al. 2011, *IOBC/WPRS Bulletin* 66, 241-244; Zemek et al. 2012, *Comm. Agric. Appl. Biol. Sci.* 77, 685-690; Zemek et al. 2012, *Acta Fytotech. Zootech*. 15: 79-80; Hussein et al. 2013, *Arch. Phytopathol. Plant Prot.* 46, 1307-1319).

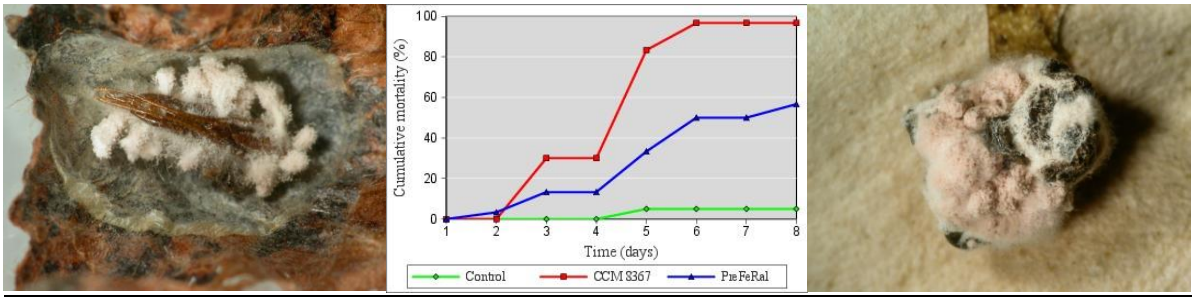


Fig. 3 *Cameraria ohridella* pupa infected by *Isaria fumosorosea* strain CCM 8367 (left), comparison of efficacy of CCM 8367 with commercial strain Apopka (active ingredient of mycoinsecticide PreFeRal) against Colorado potato beetle (in the middle) and mycosis on a Colorado potato beetle last-instar larva (right).

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Ecology and Conservation Biology

The Ecology and Conservation Biology (ECB) team studies fundamental ecological and phylogenetic mechanisms generating and maintaining biodiversity on ecosystem, landscape, regional and continental scales, surveys insect biodiversity patterns from communities to continents, and devises practical measures for the conservation of this biodiversity. This research spans from populations through communities to insect macroecology and phylogeny, using molecular methods, laboratory ecophysiological experiments, field surveys and ecological experiments, as well as modeling. Our studies include a wide range of insect taxa from all trophic levels, as well as host vegetation and vertebrate predators. Geographically, we are focused both on temperate and tropical ecosystems, with centers of our activity in Europe, Asia (Japan, Malaysia, Thailand), America (Panama, Ecuador), Africa (Cameroon, Gabon), and the Pacific (New Guinea).

Our team includes 11 key researchers active in 2010-2014, as well as 5 new members who have joined our team in late 2014 and will contribute to its future development. The team's age structure is that of a healthy growing population, comprising four senior researchers (45-61 years: Novotný, Lepš, Konvička, and Basset), a strong group of 12 junior and post-doctoral researchers (30-40 years), and a broad base of 32 PhD students who have participated in research over the past five years. In 2010-2014, we have published 127 impact-factor papers with combined IF = 399.5, including 4 papers in *Science*, *Nature* and *PNAS*. The work of our team members was cited 1,836 times (WoS) during that period of time, i.e. more than one citation a day.

The team is structured in four laboratories (Tropical ecology, Temperate biodiversity, Ecology of saproxylic insects, and Ecology and evolution of social insects), but many research themes and projects involve multiple labs. The team is international, as it includes researchers and students from the Czech Republic, UK, Switzerland, Germany, New Zealand, Ireland, France, Poland and Papua New Guinea, and is engaged in broad international collaboration (>15 active partners from 5 continents, including Harvard, Cambridge, Oxford, Sussex and Tokyo Universities, Imperial College, University of Minnesota and the Smithsonian Institution), including participation in the Center for Tropical Forest Science (CTFS) and the Stability of Altered Forest Ecosystems (SAFE) international consortia.

The team constituted, together with the University of South Bohemia partners, the Center of Excellence for Global Study of Biodiversity and Function of Forest Ecosystems, funded by the EU and the Czech Government (2011-2014), and is presently leading the Center for Tropical Biology (2014-2018), a 4-member consortium of Czech institutions, supported by the Grant Agency of the Czech Republic (GACR). The team has been funded in 2010-4 by several GACR and GAAV grants, the Czech Ministry of Education (Kontakt) grants, the Czech Nature Conservancy contracts, single Technological Agency (TACR), Marie Curie, and National Geographic Society grants, and a contract with the Imperial College, whilst the team members also participated as co-PIs on several overseas grants from the US National Science Foundation, the UK Darwin Initiative, the Australian Research Council, and others.

Our team has build important scientific infrastructure, including large-scale databases on tropical plant-herbivore food webs (>250,000 records), on the ecology and molecular taxonomy of tropical ants and moths (>30,000 DNA barcodes), on detailed distribution of moths and butterflies in the Czech Republic (>1,500,000 records), and on plant distribution within a 50-ha rainforest dynamics plot in New Guinea (>250,000 records).

As summarised below, this information have been used to analyse insect diversity on multiple scales, using observational and experimental ecological methods as well as phylogenetic approaches, and focusing on both fundamental ecological enquiry and practical conservation.

Restoration ecology: surprising biodiversity value of unusual post-industrial habitats

Our research documented important biodiversity value of such unusual habitats as abandoned military training grounds, limestone quarries, coal mine spoil dumps and even power plant ash deposits. For instance, we have discovered that ash deposits from coal powered plants play a crucial role as secondary refugees of arthropods specialized to continental drift sand dunes (Tropek et al. 2013, *Biol. Cons.* 162, 60-64; Tropek et al. 2014, *Ecol. Eng.* 73, 45-52). We documented several critically endangered psammophilous species, some of them considered nationally extinct, dependent on these ash deposits. This discovery opened a whole new area of research, showing that ash dumps, traditionally viewed as a major environmental problem, are crucial novel habitats for highly threatened species (Fig. 1). Likewise, we showed that abandoned military training grounds have a high conservation value for insects (Čížek et al. 2013, *PLOS One* 8, e5312) or birds (Reif et al. 2011, *Biodiv. Cons.* 20, 3645-3662).

We have also studied the value of natural succession, versus various industrial restoration techniques, in post-industrial habitats. Tropek et al. demonstrated startling differences in biodiversity between technically and spontaneously restored surfaces, favouring the latter, in limestone quarries (2010, *J. Appl. Ecol.* 47, 139-147) and on coal mining spoil dumps (2012, *Ecol. Eng.* 43, 13-18, 2012). These and other studies (Tropek & Konvička 2011, *Biol. Cons.* 144, 1299; Harabiš et al. 2013, *Ecol. Eng.* 55, 51-61) proved that technical reclamation is much less effective than spontaneous succession. Our results were well received by the scientific community (Tropek et al. 2010 highlighted as an Editor's choice in the *J. Appl. Ecol.*), and practical conservation where our results were noted by the national conservation authorities and already implemented in several plans for restoration of post-mining sites.

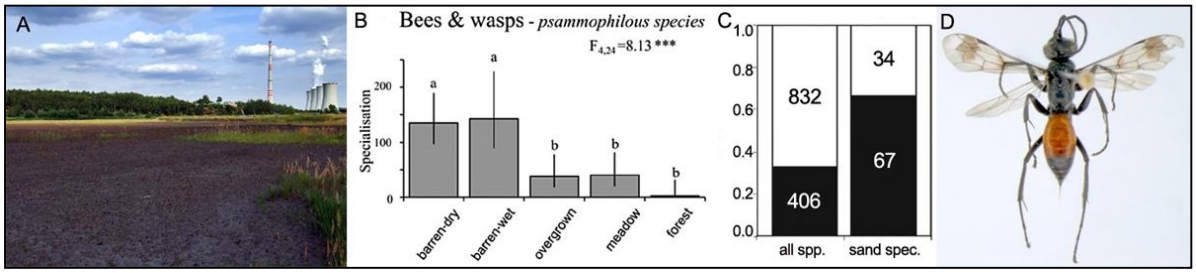


Fig. 1. Prime estate for insect conservation: ash deposit of the Chvaletice coal power station (A), important for drift sand specialists (B: barren areas hosting most specialized bee and wasps species) which are amongst the most endangered insect guilds (C: the proportions of regionally extinct and critically endangered bee and wasp species [black bars] in complete fauna and drift sand specialists in the Czech Republic). The Chvaletice site hosts also four species previously thought to be nationally extinct, incl. *Arachnospila westerlundi* (D).

Effects of habitat change on endangered xylophagous insects in Central Europe

The open woodlands are amongst the most diverse terrestrial habitats of temperate zone. We have documented and quantified their serious decline in 14,000 ha of floodplain forests over past >70 years using aerial photographs (Miklín & Cizek 2014, *J. Nat. Cons.* **22**, 35-41). These habitats are essential part of the regional biodiversity hotspot and host numerous endangered species. We compared vertical distribution of saproxylic beetles in open and closed-canopy

forest (Vodka & Cizek 2013, *Forest Ecol. Manag*. **304**, 33-41). We also studied habitat preference, population dynamics and ecology of *Rosalia alpina* and *Cerambyx cerdo*, iconic species of insect conservation, using capture-mark-recapture and molecular methods (Drag et et al. 2011, *PLoS One* **6**, e21345; Albert et al. 2012, *Eur. J. Entomol.* 109, 553-559; Drag & Cizek 2015, *Cons. Gen. in press*). Active management practice, such as pollarding, substantially increase the formation of key dead-wood microhabitats, such as tree hollows, compensating for habitat loss of many endangered insect species including e.g. the EU priority species *Osmoderma barnabita* (Šebek et al. 2013, *PLoS One* **8**, e60456) (Fig. 2).

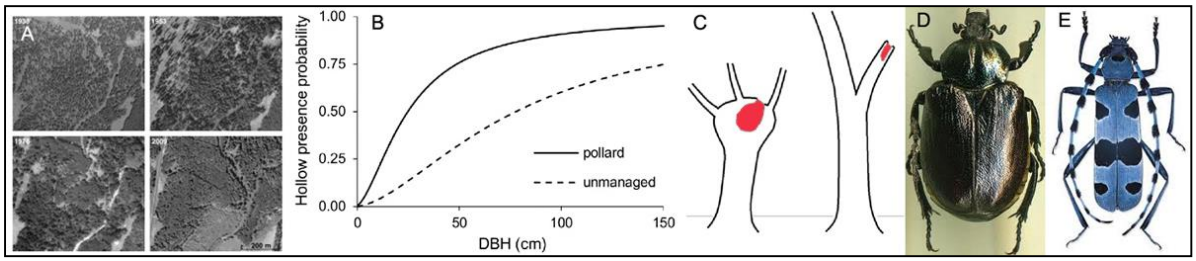


Figure 2. Loss of open woodlands in floodplains of lower Dyje and Morava rivers in SE Czech Republic between 1938 and 2009 illustrated by aerial photographs (A). Pollarding could compensate for this habitat loss by increasing the frequency of hollows (B); the hollows in pollard and unmanaged trees are shown in red (C). Hollows are important for many endangered insect species, including *Osmoderma barnabita* (D); *Rosalia alpina* is another endangered species dependent on old trees (E).

Conservation research and conservation action in tropical rainforests

Our research contributes to rainforest conservation by (i) addressing biological questions of conservation relevance, (ii) developing practical conservation approaches for rainforests owned by indigenous people, and (iii) monitoring the biodiversity status of rainforests (Fig. 3). In particular, we have studied the effect of rainforest fragmentation on bird diversity to assess biodiversity value of small, village-operated rainforest reserves in New Guinea (Sam et al. 2014, *J. Field Ornit.* 85, 152–167). Further, we have explored and implemented direct-payment schemes for indigenous communities, using conservation royalties to promote rainforest reserves (Novotny 2010, *Biotropica* 42, 546-549), and are one of the internationally recognized leaders in tropical conservation capacity building, using paraecologists recruited from indigenous communities (Novotny et al. 2012, pp. 154-7 in Lowland et al., *Methods in Forest Canopy Research*, Univ. California Press). We have also participated, for Papua New Guinea, in a highly collaborative survey of the status and key threads faced by tropical reserves world-wide (Laurance et al. 2012, *Nature* 489, 290-294).

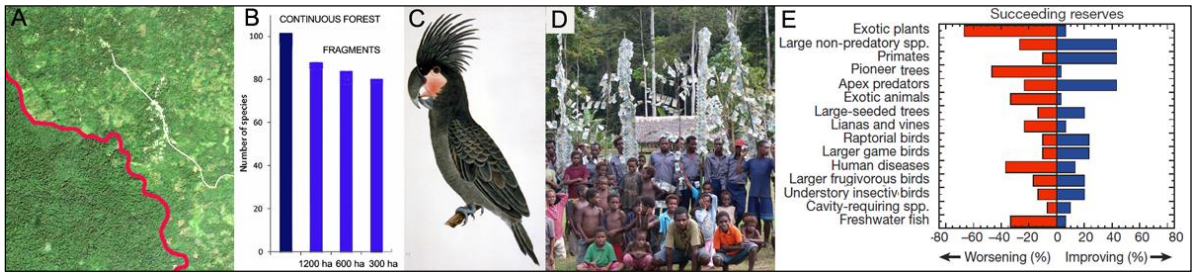


Figure 3. Tropical conservation: Border between village rainforest reserve and agricultural landscape in New Guinea (A), bird diversity retained in village reserves of different sizes compared to continuous forest (B), Palm Cockatoo (*Probosciger aterrimus*), one of the species susceptible to forest fragmentation (C), a social experiment with conservation royalties paid to village reserve owners (D) and global analysis of quality trends in tropical reserves, including our report on New Guinea (E).

Dimensions of tropical insect diversity

Our work has provided some of the most accurate estimates of local and global diversity for tropical arthropods. A recent collaborative study in Panama (Basset et al. 2012, *Science* 338,

1481-1484) used spatially and temporally replicated design for 20 different sampling methods to asses total diversity of arthropods in 6,000 ha of lowland forest, estimated at 25,000 species (Fig. 4). This was the first such estimate provided for a tropical forest.

Our long-term study of plant-insect interactions in New Guinea provided factual basis to challenge the widely held assumption that herbivore species coexistence in the tropics is a consequence of finely divided plant resources and consequently reduced global estimates of arthropod diversity from 30 to ~6 million species (Hamilton *et al.* 2010, *Amer. Nat.* 176, 90-95; Hamilton *et al.* 2013, *Oecologia* 171, 357-365), now widely accepted by the research community.

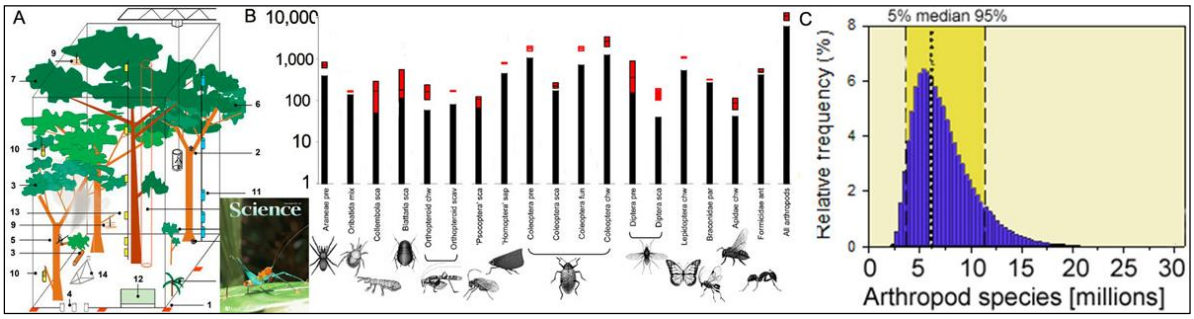


Fig. 4. The first comprehensive survey of arthropod diversity in a tropical forest included replicated design using 20 sampling methods in a rainforest in Panama (A); the number of species sampled (black) and extrapolated for 6,000 ha of the forest (red) totals 25,000 species across arthropod orders (B); on the global scale, our estimate of arthropod diversity ranged from 4 to 11 million species (C).

Global patterns in insect host specificity: key to explain hyper-diversity in the tropics?

Extreme diversity of insects in the tropics requires explanation. One of the hypothesis suggests that insect herbivores not only have more host plant species available on tropical vegetation, but also divide these resources more finely between species, and are more specialized than temperate zone insects. This suggestion has been controversial, but a recent comprehensive analysis, that involved seven authors from our team, showed globally increasing host specificity from temperate to tropical ecosystems for insect herbivores, explaining thus partly the increased tropical diversity in insects (Forister et al. 2015, *PNAS* 112, 442-447). The study also introduced new quantitative description of host specificity, based on the discrete truncated Pareto distribution (Fig. 5).

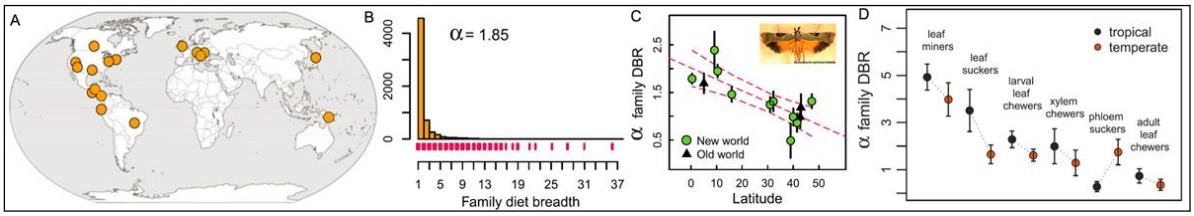


Fig. 5. Global patterns of herbivore host specificity, sampled from 5 continents (A) can be described by α parameter of truncated Pareto distribution (for host plant families in B); we found increasing host specificity of Lepidoptera from temperate to tropical forests (C), and similar pattern dominated tropical-temperate contrasts in different herbivore guilds (D).

Herbivores and their plants: niche theory does not fit plant-herbivore food webs

We have assembled host records for >250,000 herbivorous insects including ~2,500 species and 11 feeding guilds in PNG (Novotny et al. 2010, *J. Anim. Ecol.* 79, 1193-1203). This matrix, comprising >8,000 plant-insect and insect-insect trophic interactions, is among the largest data sets for tropical ecosystems and, although incomplete, "likely to be as good as it gets", as

commented in the Editor's choice feature on our paper (Lewinsohn 2010, *J. Anim. Ecol.* 79, 1143-5). We used this data set for to the first estimate of the dimensions of a rainforest plant-herbivore food web at ~200 plant species hosting ~9,500 herbivore species involved in ~50,000 distinct trophic interactions.

We tested the predictions of niche theory, postulating that more specialized herbivore guilds should be able to reach higher diversity per plant species as they could divide the available resources more finely. However, we found exactly the opposite pattern of negative correlation between host specificity and species diversity among six folivorous guilds (Fig. 6), suggesting other mechanisms important in community assembly, including the effects of both host and herbivore phylogeny (Novotny et al. 2012, *Am. Nat.* 171, 351-362).

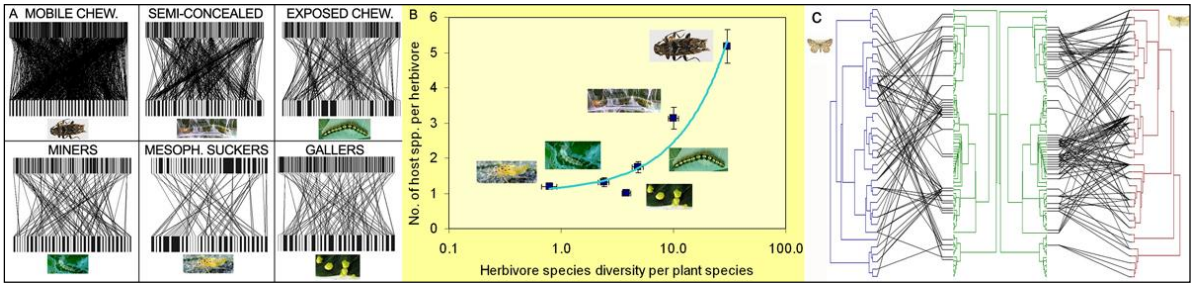


Fig. 6. Plant-herbivore food webs in tropical forests vary in structure among guilds (A); in particular, host specificity of herbivores is negatively correlated with their diversity per plant species (B), at variance with the predictions of niche theory. The food web can be partially structured by phylogeny, illustrated here for plant species (green) and geometrid (left) and pyralid (right) moths (C).

Testing the Ehrlich & Raven's coevolutionary theory: Complicated relationship between the phylogeny, chemistry and ecology of plants and their herbivorous communities

In the course of evolution, plants have acquired a wide range of defenses against herbivores. Ehrlich & Raven´s coevolutionary theory suggests that diversification of defensive traits is driven by strong impact of novel traits on insect herbivores. We tested the theory using willows in Central Europe, studying their phylogeny, secondary chemistry and folivorous insects. We found that predictably, the content of salicylates, a novel group of defensive metabolites in willows, was correlated with low diversity and high host specificity of herbivores. Despite these effects, salicylates were lost in some *Salix* lineages. This was probably because salicylates were costly, as they reached up to 22% of dry leaf mass, but did not decrease the overall abundance of herbivores (Fig. 7). The lineages lacking salicylates may thus benefit from their loss and redirect energy into other defenses or growth. The balance between costs and benefits of defensive traits is thus not necessarily in favor of novel compounds as predicted by the Ehrlich´s & Raven´s theory (Volf et al. 2015 *J. Anim. Ecol.* in press, *Ent. Exp. Appl.* in press). We are replicating this study using *Ficus*, another species-rich genus of trees, and their insects in New Guinea.

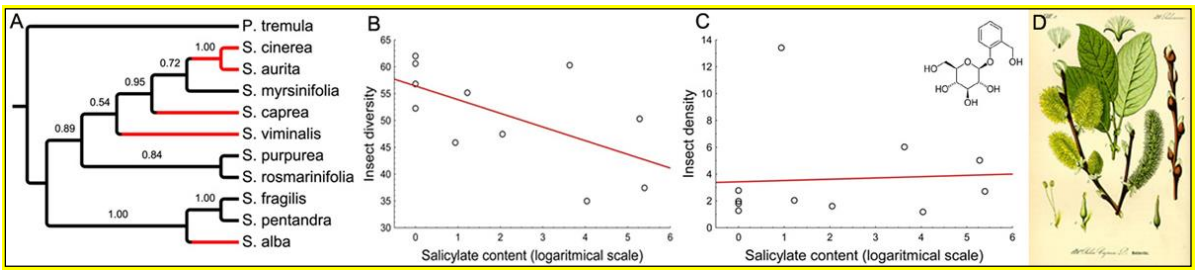


Fig. 7. Phylogeny of 10 *Salix* species (A, loss of salicylates in red). The relationships between salicylate content and the diversity (B) and density (C) of their folivorous insect communities (each point

representing one *Salix* species) shows that the loss of salicylates does not increase herbivore pressure; *S. caprea* (D), one of the species that lost salicylates.

Mechanisms structuring population genetics in tropical insect herbivores: host plants or geography?

Our surveys within a 500x150 km of lowland rainforest documented low beta diversity of herbivorous insects, but detected genetic differentiation of their populations, based on mtDNA analysis. Approximately half of Lepidoptera species exhibited geographic differentiation of populations independent from host plant species, such as in *P. disjuncta* (Fig. 8) specialized on a single host, while one third of species were structured in response to their host plant species. The remaining species, e.g., *A. pusilla*, showed a uniform genetic structure within the entire study area (Craft et al. 2010, *PNAS* 107, 5041-5046).

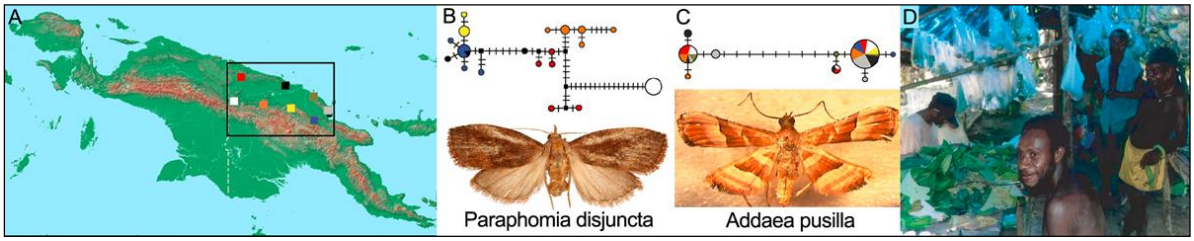


Fig. 8. Genetic diversification of Lepidoptera in a 500 x 150 km rainforest area in New Guinea (A) was explained by geography and by host plant species. The mtDNA haplotype networks (colours represent the geographic origin of specimens, as shown in A, diameter is relative to the number of specimens, and lines denote genetic distances between haplotypes; each mark represents a single nucleotide substitution) are shown for *P. disjuncta* (B) exhibiting geographic differentiation, and *A. pusilla* (C) unresponsive to geography. The specimens were reared in field camps as in (D).

Ants: bottom-up effects of vegetation on the communities in tropical rainforests

Our team is running on-line database Ants of New Guinea (http://www.newguineants.org/) that is the best resource on ant taxonomy for that region. Building on this foundation, we have analysed ecological determinants of increasing ant diversity in the course of rainforest succession, from secondary to primary rainforest vegetation (Klimes et al. 2012, *J. Anim. Ecol.* 81, 1103–1112). This study was the Editor's choice, and C. Parr (*J. Anim. Ecol.* 81, 937-9) commented that "... Klimes et al. raise the bar by addressing the deceptively simple, yet inherently complex, question of why species richness is lower in secondary forests. Using the first plot-scale inventory of arboreal ant nests, combined with an innovative rarefaction technique, they ... highlight the contribution of beta diversity to the higher richness in primary forest." This study also described 5 ant species (Klimes & McArthur 2014, *Myrmec. News*, 20, 141-158) and showed that the species diversity of foraging ants is mostly generated by tourist ants coming from the surrounding trees rather than locally nesting ants, highlighting thus the importance of ant nest sampling (Klimeš et al. 2015, *PLoS ONE* in press) (Fig. 9).

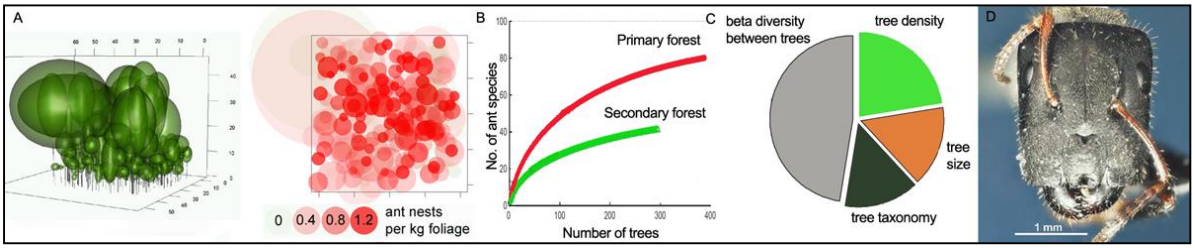


Fig. 9. Complete census of ant nests in primary (A) and secondary rainforests documented differences in diversity (B) that were explained by beta diversity in ant communities between trees and the structural differences of vegetation (tree size and density), rather than differences in tree diversity between the forests (C); *Camponotus wanangus* is a newly described species from the study (D).

Ants: key predators of herbivorous insects?

The role of ants as predators in rainforest communities was studies experimentally, as we reduced the density of all arboreal ants within 0.05 ha primary and secondary forest plots by 80% for 10 months (Klimes et al. 2011, *Ecol. Entomol.* 36, 94–103), showing surprisingly low impact on insect herbivores. We are following up on this discovery using experimental exclosures of ants, birds, and bats to quantify relative effects of different predators, while also assessing the effects of parasitoids.

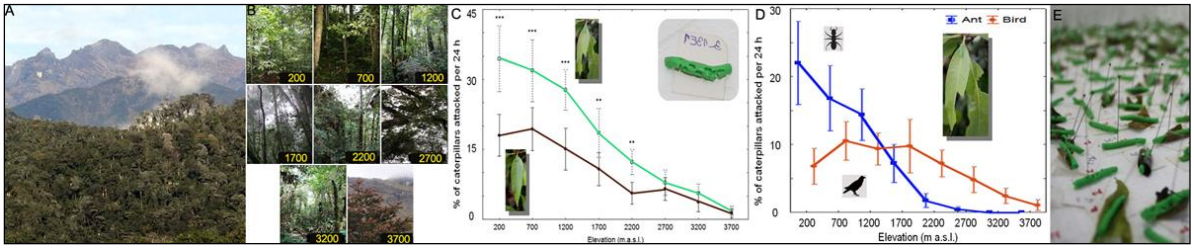


Fig. 10. Predation along altitudinal gradient at Mt. Wilhelm (A) comprising complete altitudinal range of rainforests from 200 to 3700 m asl. (B) decreased with altitude, and was higher on damaged than intact leaves (C); ants were dominant predators in lowlands, while birds at higher elevations (D), as measured by attack on artificial caterpillars (E).

We used artificial caterpillars as baits (Low et al. 2014, *Ent. Exp. Appl.* 152, 120-126) to monitor predation rate along a complete altitudinal rainforest gradient in New Guinea (Sam et al. 2014, *Ecography* 37, doi: 10.1111/ecog.00979) and found that predation pressure decreases towards higher elevations. This seemingly simple pattern is a combination of rapidly decreasing predation by ants with elevation, and bird predation that is constant from lowlands to mid-elevations, then decreases. The dominant predators are thus ants in lowland forests, and birds at higher elevations (Fig. 10). Further, we have documented higher predation on artificially damaged leaves, both by ants and birds. This suggests both chemical and visual clues used by predators to focus on damaged plants.

Ant communities: experimental and phylogenetic approaches to explaining their composition

Microcosms in epiphytic ferns of SE Asia represent an ideal experimental system for community assembly studies (Fig. 11). We tested various combinations of ant species competing within epiphytic ferns, both from observation of species co-occurrences against random models, and in laboratory experiments. We found that similar-sized ant species rarely shared the same fern. We then tested whether this result was due to competition between similar-sized species by introducing pairs of ant colonies into ferns in the laboratory. When the ants were similar in size there was strong competition, with one colony ejecting the other from the fern, while ants of different body sizes co-existed peacefully (Fayle et al., 2015, *Ecol. Letters., DOI: 10.1111/ele.12403*).

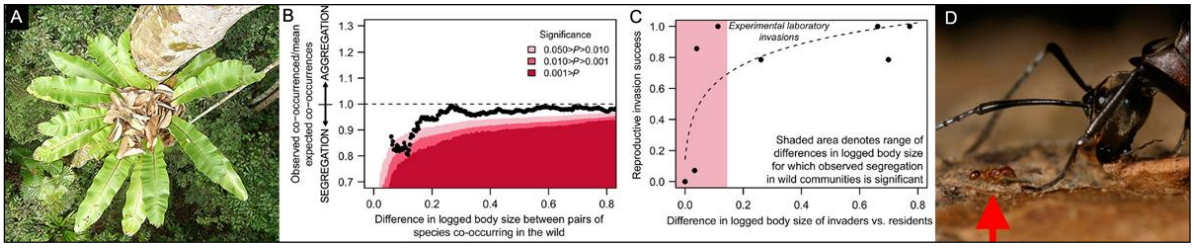


Fig. 11. Ants colonizing epiphytic *Asplenium* ferns in Borneo (A) form communities with non-random segregation of species according to their body sizes (B), the pattern confirmed also by laboratory experiments with ant invasions, that are more successful for species of diverging body sizes (C); the range of body sizes in *Asplenium*-colonizing ants is significant (D).

Another study (Machac et al. 2011, *Ecography* 34, 364-371) combined community data with phylogeny and examined patterns of ant diversity along climatic gradients in three temperate montane systems. We found that communities at low-elevation sites are structured by interspecific competition but at high-elevation sites primarily by environmental filtering caused by low temperatures. These results highlight the potential role of niche constraints, temperature, and competition in shaping broad-scale diversity gradients.

Ant diversity in New Guinea and beyond: exploring their biogeographical dynamics

We have taken advantage of our detailed understanding of New Guinea ant taxonomy and our extensive samples from the region and joined broad international collaboration to test the hypothesis that the origin of the South Pacific island fauna was primarily in New Guinea, the Philippines, and the Indo-Malay archipelago (Clouse *et al* 2014, *Cladistic* DOI: 10.1111/cla.12099). Using species of the ant genus *Camponotus*, we performed a series of phylogenetic analyses and ancestral area reconstructions. Surprisingly, the Pacific members of this group comprise several clades with different biogeographical histories, with an important role for Australia as a source of Pacific colonizations (Fig. 12).

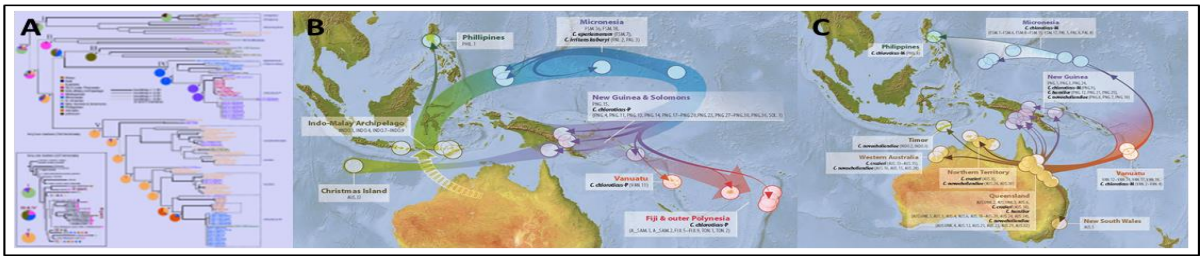


Fig. 12. The phylogeny of Melanesian *Camponotus* ants revealed a complex biogeographic history and unexpected dispersal patterns across Indo-Australia (A). One lineage colonized New Guinea and east Melanesia from Indonesian region through Micronesia (B), while the other lineage originated in north Australia, and remarkably diversified across New Guinea and Micronesian islands (C).

Tri-trophic food webs in tropical forests: plants, herbivores and parasitoids

We have assembled tri-trophic plant-herbivore-parasitoid webs from tropical forest, using molecular tools to detect parasitoids (Hrcek *et al.* 2011, *Mol. Ecol. Res.* 11, 786-794) and identify their hosts (Miller et al. 2013, *Proc. Entomol. Soc. Wash.*, 115, 107-109). We have documented food webs dominated by relatively specialized parasitoids attacking less specialized caterpillar hosts, in a reversal of patterns known previously for highly specialized miners attacked by generalist parasitoids (Hrcek et al. 2013, *Oecologia* 173, 521-532). We documented differential preferences for individual herbivore guilds by each parasitoid family, generating thus complex host specificity patterns in parasitoids. Tropical parasitoid fauna is virtually unknown taxonomically; our studies also described new species (e.g., Quicke et al. 2012, *J. Hym. Res.* 28, 85-121; 2013, *J. Hym. Res.* 31, 65-78) (Fig. 13).

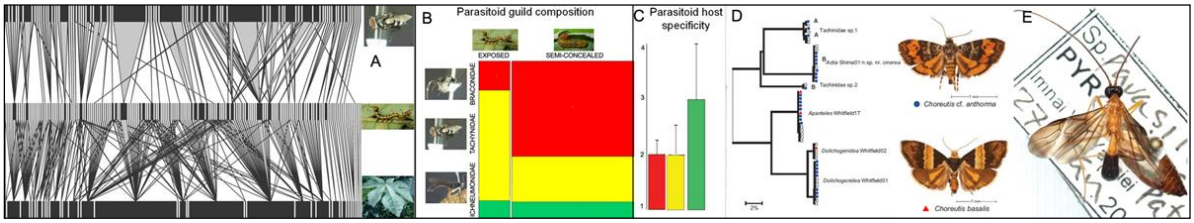


Figure 13. Tri-trophic food web between 38 tree species, 11,621 caterpillars from 267 species, and 1,523 parasitoids from 166 species (A), taxonomic composition of parasitoids feeding on exposed and semi-concealed host guilds (B), and their differential host specificity (C), molecular identification of parasitoids dissected from two host species (D), and a newly described *Colastomion maclayi* (E).

Mutualistic networks: plants and pollinators

We studied pollination systems in Cameroonian Highlands and described the first co-evolutionary system of hovering sunbirds and native plants (Janeček et al. 2011, *Oikos* 120, 178-183; an Editor's choice in *Science*). We also examined different roles of individual flower visitors in these pollination systems and found relatively high pollinator specialisation in systems expected to be generalised (Tropek et al. 2014, *Afr. Zool.* 48, 392-394, Padyšáková et al. 2013, *PLoS One* 8, e59299, Bartoš et al. 2012, *S. Afr. J. Bot.* 78, 63-74) (Fig. 14).

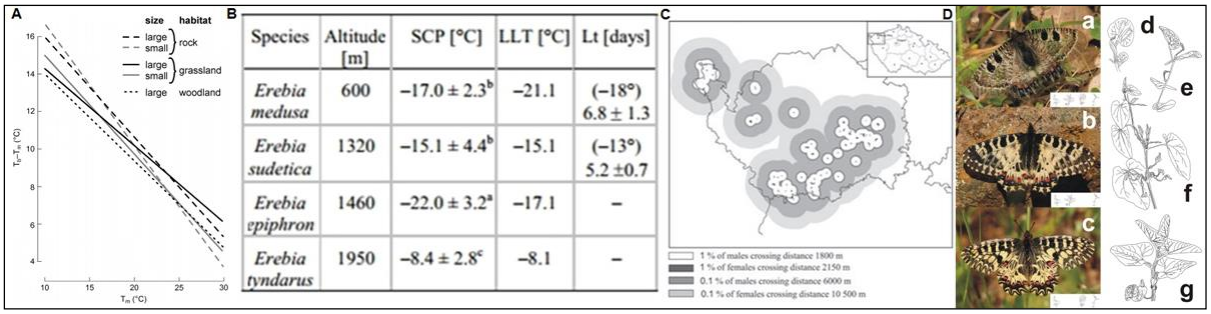
Fig. 14 Physiological traits including nectar composition (A) characterise the first African system involving a specialized bird pollinator (*Cyanomitra oritis*) hovering over a native plant (*Impatiens sakerana*; B). The interactions between a plant species (*Hypoestes aristata*) and its flower visitors exemplify a pollination system with high number of visitors, yet highly specialised (C).

Butterflies as a model for conservation research, and the subject of conservation

We used butterflies as a model group for temperate insect conservation. We determined population parameters of endangered *Euphydryas aurinia* (Zimmermann et al. 2010, *Ecol. Entomol.* 36, 499-510; 2011, *Eur. J. Entomol.* 108, 243-254), demonstrated inverse mobility-density relationship among butterfly species (Konvicka et al. 2012, *Pop. Ecol.* 54, 91-101), and quantified dispersal for several co-occurring species (Fric et al. 2010, *Ecol. Res.* 25, 543-552). We identified ecological requirements for endangered *Chazara briseis* (Kadlec et al, 2010, *Anim. Cons.* 13, 172-183) and *Erebia aethiops* (Slamova et al. 2011, *J. Insect Behav.* 24, 230-246) (Fig. 15).

Inspired by climate change debates, we studied factors determining low-altitude distribution boundaries for montane insects (Vrba et al. 2012, *Cryoletters* 33, 251-258; 2014, *Cryoletters* 35, 247-254; 2014, *J. Entomol. Sci.* 49, 63-69). We found reverse altitudinal clines in cold hardiness of these species, making them dependent on predictable snow cover during larval diapause, whereas related lowland species could tolerate deep frosts. Kleckova et al. (2014, *J. Therm. Biol.* 41, 50-58) showed pivotal role of behavioural thermoregulation in adult thermal requirements for alpine and lowland *Erebia* butterflies,

Expanding from observational to experimental studies, and from passive to active conservation measures, we studied effects of grassland management on insects. We demonstrated positive effects of patchy mowing on butterflies (Cizek et al. 2012, *J. Insect Cons.* 16, 215-226) and moths (Sumpich & Konvicka, 2012, *J. Insect Cons.* 67, 973-987). This resulted in practical guidelines on reserve management (Jarosik et al., 2011, *Biol. Cons.* 144: 490-499). Slancarova et al. (2014, *J. Insect. Cons.* 18, 1-12) demonstrated positive effects of landscape heterogeneity on fauna of insular reserves.



Species	Altitude [m]	SCP [°C]	LLT [°C]	Lt [days]
Erebia medusa	600	−17.0 ± 2.3[b]	−21.1	(−18°) 6.8 ± 1.3
Erebia sudetica	1320	−15.1 ± 4.4[b]	−15.1	(−13°) 5.2 ±0.7
Erebia epiphron	1460	−22.0 ± 3.2[a]	−17.1	–
Erebia tyndarus	1950	−8.4 ± 2.8[c]	−8.1	–

Fig. 15. A: Species-dependent relationship of microhabitat temperature excess with temperature for 5 *Erebia* spp. differing in body size and habitat. B: Supercooling point, lower lethal temperature after 24 h and lethal time at selected temperature. C: Connections among *Euphydryas aurinia* colonies, predicted from IPF values. D: *Aristolochia*-feeding Papilionids (**a** – *Archon apollinus*, **b** – *Zerynthia cerisy*, **c** – *Z. polyxena*) and their host plants (**d** – *Aristolochia pallida*, **e** – *A. rotunda*, **f** – *A. clematitis*, **g** – *A. hirta*); the small inserts show hosts utilized by the respective species at the study site.

Butterflies across continents: origins and invasions

Two species, *Phengaris teleius* and *P. nausithous*, originated from a common ancestor probably in Central Asia. *P. teleius* populations diversified mainly in Asia whereas its distribution in Europe is of recent origin. In contrast, the radiation center of its sister species, *P. nausithous*, lies in Europe. Both species exhibit deep intra-specific mitochondrial divergences between *Wolbachia*-infected and non-infected populations (Fig. 16). The divergence is larger than the values usually used for definition of cryptic species, but it is not paralleled by divergence in nuclear genes, suggesting complicated picture of separate mtDNA and nuclear DNA divergences (Ritter et al. 2013, *PLoS One* 8, e78107).

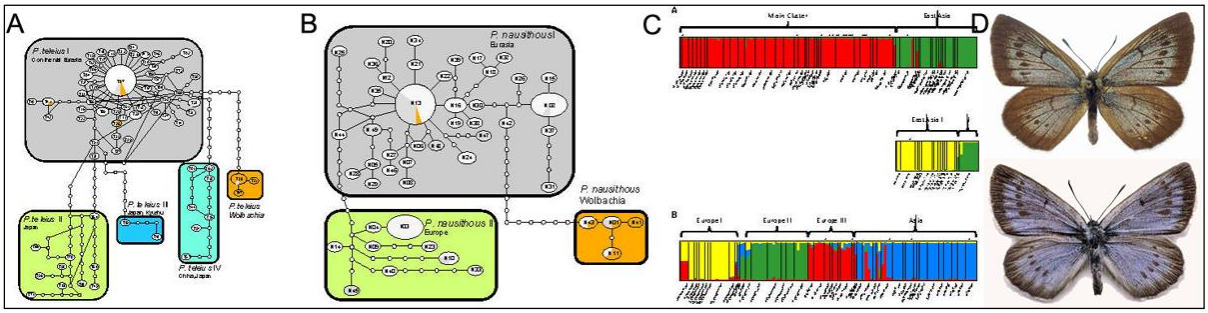


Fig 16. Haplotype networks of mitochondrial gene COI of *Phengaris teleius* (A) and *P. nausithous* (B). In both species clades infected by *Wolbachia* differ from the non-infected populations so that they could be mistaken for sympatric cryptic species. This is not supported by nuclear genes, showing the usual geographic pattern (C); *P. nasithous* (D, top) and *P. teleius* (D, bottom).

Plant functional traits and community assembly rules: observations and experiments

We have studied the mechanisms of community assembly, particularly its composition in terms of species traits in several grassland systems. We studied changes in trait composition in response to environmental conditions as well as to experimental treatments (such as removal of the dominat species, mowing, or fertilizing), as well as the relationships between species traits and the dynamics of individual populations (Fig. 17). The publication (Majekova et al. 2014, *Ecology* 95, 2369-2374) was the Editor's choice in the Sep 2014 issue.

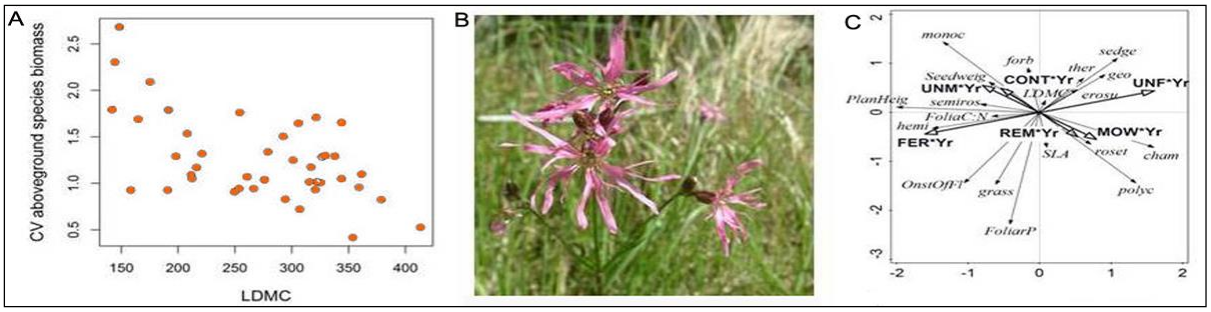


Fig. 17. Relationship between the LDMC (Leaf dry matter content) and species temporal variability (coefficient of variation of plant biomass) (A) in *Lychnis floss-cuculi* (B), a species with extremely low LDMC and high temporal variability. Ordination diagram (C) visualizes vegetation response in trait composition to fertilization, mowing and dominant species removal.

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Molecular cytogenetics

2.1 Background information

The major focus of our team is to explore repetitive DNA sequences in plant genomes. This research direction gradually emerged about 10-15 years ago from our previous subject of interest, the physical mapping of plant genomes and chromosomes, by recognizing the crucial role of repetitive DNA in plant genome structure and evolution. Since then, we were involved in the investigation of all main types of repetitive elements, including retrotransposons, DNA transposons and satellite DNA. In addition to identification of novel element sequences and mapping their distribution in various plant genomes, our attention also turned to structural and functional characterization of these elements as well as investigating their role in specific genome regions such as centromeres. Since tremendous sequence diversity and genomic abundance of plant repetitive DNA imposed serious limitations on its investigation using traditional sequencing techniques, we were actively seeking more efficient ways to target this genome fraction in its entire complexity. Thus, following the invention of next generation sequencing (NGS) technologies, we were among the first labs that adopted this approach for the characterization of repetitive DNA in eukaryotic genomes (Macas et al., *BMC Genomics* 8, 2007). The introduction of NGS-based approaches enabled whole new research directions to be considered, including global characterization of all repeat types within a single species, comparative analysis of repeated genome fractions between multiple species, and performing this analysis for large numbers of non-model organisms. At the same time, the practical utility of NGS data was seriously hampered by a lack of appropriate bioinformatics tools for their analysis. These circumstances determined our research activities in the following years, prompting us to split our efforts between developing novel bioinformatics tools and applying these tools to investigating composition and evolutionary dynamics of repetitive DNA in a wide range of species. Consequently, much of our current research is at the interface of computational biology, genomics and molecular cytogenetics.

2.2 Research activities 2010-2014

2.2.1 Development of bioinformatics tools for analyzing repetitive DNA from NGS data

The rationale behind most approaches utilizing NGS for repeat characterization in complex genomes is that due to the presence of multiple copies of repeats even low-pass sequencing (equivalent to genome coverage of 0.01-0.5x) provides sufficient representation of most repeat families. However, individual approaches for repeat analysis from this type of data differ in their applicability, some of them being simply based on read comparison to assembled genomes or to repeat databases derived from model organisms, which limits their use in taxonomically distant species. Thus, we aimed at developing a universal tool for *de novo* identification and classification of repeats that could also provide information about structure and variability of repetitive elements in spite of the limitations imposed by the absence of genome assembly and very short lengths of NGS reads. We introduced a similarity-based clustering approach and later improved it by transforming read similarities into a virtual graph, resulting in partitioning

of genomic NGS data into groups of frequently overlapping reads representing individual families of repetitive elements (Novak et al., *BMC Bioinformatics*, 2010). Since cluster sizes are directly proportional to genomic abundance of corresponding repeats, this analysis is utilized to obtain quantitative information about genomic proportions of different types of repeats. This basic analysis was subsequently augmented by introducing additional programs aiding in repeat classification, the study of their variability and comparative analysis between species, which resulted in assembling a computational pipeline named *RepeatExplorer* (Novak et al., *Bioinformatics*, 2013). We have also established a computer cluster and a public front-end web server for running the pipeline (http://www.repeatexplorer.org) which is gaining popularity as a unique tool to study repetitive DNA especially in non-model species (there are currently over 400 registered scientists running >10,000 jobs/year; our papers describing the method and the server have been cited 58 times). Although the pipeline was primarily designed for analyzing plant genomes, we have also successfully used it in collaboration with the lab of D. Ray (Mississippi State Univ., USA) for repeat analysis in bat (Pagan et al., *Genome Biol. Evol.*, 2012) and crocodillian genomes (unpublished). Prompted by the demand from the user community, we organize a practical workshop on using *RepeatExplorer* combined with a mini-conference focused on repetitive DNA research which takes place annually at our institute (http://w3lamc.umbr.cas.cz/repeatexplorer/?page_id=14).

2.2.2 Sequence composition and evolutionary dynamics of repetitive DNA in selected species

Since its first application to genomic NGS data from pea (*Pisum sativum*) and soybean (*Glycine max*) (Novak et al., 2010), we have used the graph-based clustering approach to reveal repeat composition in species selected for their contrasting genome sizes or those anticipated to undergo significant changes in their repeat composition as a consequence of (allo-)polyploidization. Due to early availability of NGS data from our collaborating group of J. Dolezel (Institute of Experimental Botany, Olomouc) we selected bananas (genus *Musa*) as representatives of small plant genomes, resulting in two consecutive studies focusing on banana (*M. acuminata*) alone (Hribova et al., *BMC Plant Biol.*, 2010) and on a comparative analysis of repeats in a broader range of two genera including five *Musa* and one *Ensete* species (Novak et al., *PLoS ONE*, 2014). The lilly genus *Fritillaria* was selected as a representative of extremely large genomes, which are about 70-fold larger than banana and over 10-fold larger than the pea genome. We constructed fosmid libraries from two *Fritillaria* species which led to identification of the most abundant retroelements and one satellite repeat (Ambrozova et al., *Ann.Bot*., 2011) and later participated in the generation of NGS data from these species and their clustering analysis (to be published in 2015). A number of additional species with intermediate-sized genomes from genera of *Orobanche*, *Phelipanche* (Piednoel et al., *Mol. Biol. Evol.*, 2012), *Solanum* (Torres et al., *G3*, 2011; Zhang et al., *Plant Cell*, 2014) and *Prospero* (Emadzade et al., *Ann.Bot*., 2014) were analyzed in collaboration with the research groups of S. Renner (Univ. Munich, Germany), J. Jiang (Univ. Wisconsin, USA) and H. Schneeweiss (Univ. Vienna, Austria). We also closely collaborated with the lab of A. Leitch (Quenn Mary University of London, UK) on the study of genome changes following interspecific hybridization and polyploidization in *Nicotiana.* In this case the applications of our bioinformatics approaches led to discovery of targeted loss of specific repeats and differential dynamics of high- and low-copy sequences in *Nicotiana* allopolyploids (Renny-Byfield et al., *Mol.Biol.Evol.*, 2011; Renny-Byfield et al., *PLoS ONE*, 2012). Although the data acquired so far do not yet allow making any generalizations, some features of repeat composition of plant genomes start to emerge. One of them is the observation that very large genomes seem to be deficient in repeat removal, leading to their progressive accumulation and continuous degradation, resulting in large genome fractions being constituted by extremely diverse populations of low-copy repeats. This is one of the findings we plan to test in our future analyzes. Another general finding was that genomic repeat composition and quantities, as represented in the results of graph-based clustering analyses, reflect evolutionary distances

of tested species and thus can be used to infer their phylogeny (Dodsworth et al., *Syst.Biol.*, 2015; first published online in September 2014).

2.2.3 Repeat composition of specialized plant chromosomes

We conducted several studies specifically focusing on repeat composition of plant sex and supernumerary B chromosomes. These chromosomes have several unique features such as a lack of meiotic recombination in the case of some sex chromosomes, making them attractive for investigating how such differences affect the accumulation and composition of various types of repetitive elements. Indeed, differential accumulation of satellite DNA and depletion of certain types of retrotransposons was revealed in two model species, *Silene latifolia* (Macas et al., *PLoS ONE*, 2011) and *Rumex acetosa* (Steflova et al., *Genome Biol. Evol.*, 2013) in collaboration with the group of B. Vyskot (Inst. Biophysics, Brno). In both cases, repeats specific for Y chromosomes were identified by comparative clustering analysis of NGS data obtained from male (XY in *S. latifolia*, XY1Y2 in *R. acetosa*) and female plants (XX). A similar approach was used for our contribution to a multi-laboratory study of origin and sequence composition of rye B chromosomes (Martis et al., *PNAS*, 2012). Comparison of NGS data from plants containing versus lacking B chromosomes and of sequenced pools of flow-sorted A and B chromosomes revealed accumulation of specific sequences (e.g. organellar DNA) and evolution of novel repeats on Bs. Some of these sequence types may play a role in the process of B chromosome non-disjunction, ultimately leading to their so-called "selfish" behavior and accumulation in some plant lineages.

2.2.4 Structure and sequence composition of plant centromeres

Plant centromeres are genomic regions known to be predominantly comprised of repetitive DNA; however, the evolutionary implications, molecular mechanisms of repeat accumulation, and basic principles of centromere determination 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. However, our work has significantly contributed to a shift of this view towards one of epigenetic centromere determination, where the primary sequences of the centromeric repeats are of no importance for centromere function. One such case was found in the garden pea (*Pisum sativum*) where our initial studies of repetitive DNA using NGS revealed unusually high diversity of satellite repeats (> 15 different families) compared to the typical plant genome, which contains only a few satellite families. Using fluorescence in situ hybridization, most of these repeats were found to be localized in extended primary constrictions of pea chromosomes. This finding prompted our further research focused on the identification of pea CenH3-coding genes and production of antibodies against CenH3 proteins to visualize centromeric chromatin domains on pea chromosomes. We found that contrary to most plant species possessing only a single CenH3 gene, there are two divergent CenH3 sequences encoded and expressed in pea. Most surprisingly, we discovered that centromeric regions of pea have a unique structure composed of 3-5 separated CenH3 domains, thus resembling a transition state between monocentric and holocentric chromosomes and that these domains are associated with 13 different families of satellite repeats (Neumann et al., *PLoS Genetics*, 2012). A follow-up study investigating centromere structure in a set of species from three closely related genera (*Pisum*, *Lathyrus* and *Vicia*) revealed that two CenH3 genes as well as complex "meta-polycentric" centromere structure occur in *Pisum* and *Lathyrus* species, whereas *Vicia* species possess a single CenH3 gene and simple centromeres (Neumann et al., in press). Yet another interesting model to study centromere evolution and function emerged from our collaboration with the J. Jiang lab (Univ. Wisconsin, USA) and included potato (*Solanum tuberosum*) and several related wild *Solanum* species. Potato centromeres were found to be very heterogeneous with respect to their sequence composition,

some of them being completely devoid of high-copy repeats, whereas the rest contained mostly chromosome-specific tandem repeats originated from parts of retrotransposon sequences. These repeats were also unique in the extreme length of their monomers (up to 5 kb, compared to monomers of ~160-320 bp typical for most satellites) which could be discovered only thanks to the graph-based modeling of repeat populations from NGS reads (Gong et al., *Plant Cell*, 2012). Similar heterogeneity and rapid evolution of centromeric repeats was also revealed in related *Solanum* species (Zhang et al., *Plant Cell*, 2014).

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Molecular genetics

2.1 Summarization about the previous background to the period 2010-2014 including available resources of the department

The research period evaluated for five successive years 2010-2014, includes following topics, which are mutually interconnected and stably integrated in our research. Ongoing work includes 1) molecular genomic analysis of non-model plant *Humulus lupulus,* 2) viroid (small circular pathogenic RNA) research and 3) analysis of plant nucleases I. These research topics were established more than decade before the evaluation period.

Hop genomic research that we started 15 years ago by analysis of general hop genomic markers important for breeding and by analysis of hop 7SLRNA was shifted to analysis of organization of structural genes encoding prenylflavonoid pathway and later to identification of first regulatory elements and transcription factors (TFs) putatively involved in co-regulation of hop lupulin. Recently, TFs network is investigated and new generation of markers important for practical research is derived from the regulatory genes.

Viroid research included mainly *Pospiviroids* infecting *Solanaceous* species, but also hops, fruit trees and ornamental plants. From previously analyzed mainly evolutionary changes connected to so-called thermomutations this topic was shifted to analyses of viroid-caused pathogenesis that is according to recent hypotheses connected to mechanism of RNA silencing (PTGS). Viroid pathogenesis is interconnected to the regulation of secondary metabolites and lupulin gland development in hop.

The traditional topic of plant nucleases discovery on the surface of pollen (Ph.D. work of the head of this department), further continued by the cloning of these enzymes and analyzing their possible function during viroid pollen transmission and viroid pathogenesis. Nuclease cDNA cloning from tomato, hop and brassica enabled their *in vitro* production and opened the way to use them as anticancer agents to complement or replace utilization of nucleolytic enzymes of animal origin that were usually showing strong unwanted side effects. Much lower side effects of plant nucleases on animals was reported during the evaluated period and currently we are engaged in profound analysis of these enzymes.

During the previous period we accumulated several experimental cDNA, bacterial and library clones, plant expression vectors etc. that continue to be used or characterized up to date. During this period we accumulated important equipment and devices from previous projects and the department established important contacts. These characteristics classify our department as long time established and based on continuation predominantly in the frames described above.

2.2 Personnel and collaboration in the period of 2010-2014

Our department has the personal structure corresponding more to groups in universities in Germany than classical departments, where more long-time employed staff of scientists is present. Our staff included mostly internal Ph.D. students (Dr. Z.Fűssy) or they were working transiently in our group as external Ph.D. students from collaborating institutes. It was true for Dr. Podzimek from Institute of Chemical Technology Prague that was partly employed in the Biology Centre v.v.i. Inst. Plant Mol. Biol. and worked under supervision of Dr. Matoušek as so-called supervisor specialist. Other awarded Ph.D. students visited our group on shorter period from Hohenheim University and Düsseldorf University (Germany).The Ph.D. student work within the group facilitated local and international contacts and collaboration. During evaluating period there were some changes in the staff regarding postdocs; two postdocs worked on the aspect of quantification of RNA and viroid (qRTPCR and other methods) for short (about one-two years) period. They left the department due to their own personal stuff (Relocation to new place (Dr.J. Stehlík) and maternity leave (Dr. J.Procházková). By the end of evaluating period we therefore accepted additional person for the postdoc position. Recent staff as to evaluating period concerns includes three employees on the position of research scientist, two for full-time job [Dr. J. Matoušek (heading most of projects and planned experiments, responsibility in cDNA cloning and modification, preparation of expression vectors, biolistic inoculation, sequence analyses, dsRNAse, degradomes, viroid diagnostics etc), Dr. T. Kocábek, (plant transformation, *A. thaliana* stuff, transient expression systems, microscopy methods etc.] and one for 20% of working time (Dr. J. Bříza, Agrobacterial and bioistic transformation and transient expression, genome blots). The group then includes two postdocs, both foreigners from India [Dr. A. Mishra (bioinformatics, NGS, microRNAs); Dr. G.S. Duraisamy (molecular hybridization, library screenings, sequence analyses etc.) for full-time job and two Ph.D. students (Mgr. A. Týcová and Ing. K. Síglová, mainly working on protein analyses, nuclease activity assays and purifications, real-time qPCR quantifications, cloning and other work under leadership of Dr. J. Matoušek).

The Dept. Mol. Genet. involves three engineers and one person graduated from high school on position of technicians. All these technical assistants are very experienced and besides standard laboratory work, they are involved in plant transformation, transient expression analyses, DNA cloning, RNA isolations and analyses etc.

The work of Dept. of Mol. Genet. involved long-term contacts with several groups abroad and also with institutes of basic and applied research in the Czech Republic. During the evaluation period our department was collaborating on the topic of hop research with the group of Prof. D. De Keukeleire, Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmaceutical Sciences, Ghent University, Belgium on analysis of hop metabolome and with the group of Prof. G. Weber, Dept. of Plant Breeding and Biotechnology, Institute for Plant Breeding, Seed Science and Population Genetics, University of Hohenheim, Stuttgart, Germany, on hop transformation with selected transcription factors. Within the Modbiolin project we collaborate on the field of hop research with Dr. C. Koncz, Max-Planck Institut für Züchtungsforschung, Köln, Germany. The major purpose of the last collaboration is to transform hop plants with transcription factor complexes. The major and long-time collaboration of our group on the hop research involves Hop Research Institute, Žatec, Czech Republic as a major partner of many projects in the past including the evaluation

period, especially Dr. J. Patzak and other collaborators working also on more practical and applied research within the programme of development of markers for hop breeding.

Our group has long-time collaboration on the field of viroid research with former department of Prof. D. Riesner and with the bioinformatic group of Prof. G. Steger, Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Germany covered by several projects within the evaluation period. Viroid collaboration involves within Modbiolin EC project colleagues from Slovenian Institute for Hop Research and Brewing Žalec and from Centre for Plant Biotechnology and Breeding, Agronomy Department, Biotechnical Faculty, Ljubljana University, Slovenia. With these colleagues we collaborate on the research topic of "Slovenian viroid syndrome", the disease that devastated hop fields in Slovenia. During the evaluation period our collaboration on viroids involved group of Dr. J. Schubert, Institute for Biosafety of Genetically Modified Plants, Julius Kűhn-Institute, Quedlinburg, Germany. Quite recently, the viroid collaboration met mutual interests in analysis of multiple viroid infections of hop and apples within bilateral project KONTAKT II of Ministry of education (2014) with our partners from Faculty of Agriculture and Life Science, Hirosaki University, Japan, group of Prof. T. Sano. From CZ partners the collaboration on viroid analysis involves Potato Research Institute, Havlíčkův Brod; Silva Tarouca Research Institute for Landscape and Ornamental Gardening, Publ. Res. Inst., Průhonice and Hop Research Institute, Žatec, CZ.

Nuclease research, that included especially the anti-cancerogenic properties of nuclease as our original finding and fine structural properties that were analyzed during the evaluation period, involved exclusively partners in the Czech Republic. On this topic we collaborated with Institute of Macromolecular Chemistry AS CR, v.v.i., Prague and with Department of Biochemistry and Microbiology, Institute of Chemical Technology Prague for analysis of nuclease structures and biochemical properties. This collaboration involved Institute of Animal Physiology and Genetics, AS CR v.v.i., Liběchov and Institute of Biophysics and Informatics, 1st Medical Faculty of the Charles University, Prague, Czech Republic on experiments using animal systems.

The numerous collaborations facilitate solving crucial issue of the research by the use of complementary materials and devices. This includes metabolome analyses, structural and analytical protein and enzyme analyses, biolistic and transformation methods, facilities for animal systems, bioinformatics methods for small RNAs and NGS etc.

2.3. Grants and laboratory facility

In accordance to main topics our department received some major grant by different funding agencies. For hop molecular biology based investigation research grant was funded by GAČR under GA521/08/0740 entitled "Molecular analysis of transcription factors of hop (*H. lupulus* L.) in relation to lupulin biosynthesis" for the year 2008-2011 and more recently GAČR, GA13-03037S project entitled "Combinatorial regulation and network of transcription factors involved in the biosynthesis of medicinal prenylflavonoids in hop (*Humulus lupulus* L.)" for the year of 2013-2017. For development of hop molecular markers we were co-granted by MZE QH81052 entitled "Development of molecular-genetic markers for modern breeding and gene engineering of hop (*Humulus lupulus*) based on the system of genomic and expression libraries" (2008-2012).

Viroid research was funded by Czech-German bilateral project GAČR P501/10/J018, entitled “Pospiviroid pathogenesis as “regulatory disorder mediated by viroid-specific small RNAs″ for year 2010-2012, MŠMT LH14255 project KONTAKT II entitled “Mechanisms of propagation and crossprotection among viroids infecting hop and apple and their influence on regulation of metabolite pathways of medicinal prenylflavonoids and lupulin″ for the year 2014-2016 and Alexander von Humboldt Stiftung (FRG), Research Group Linkage project entitled “Pospiviroid-induced degradome: the background for the complex and expansive plant disease" for year 2013-2016.

Research based on nucleases was granted by GAČR, GA521/09/1214 entitled “Modification and in planta production of bifunctional nucleases and analysis of their anticancerogenic and biological activities” for the year 2009-2011, where Dr. Matoušek served as principal investigator with other four partners. During the more restrictive period due to economical crisis this collaboration was not further funded.

Besides standard and bilateral projects, the department was also involved in two EC projects: FP7-REGPOT-2008-1 MOBITAG No.229518 entitled “Building up modern biotechnologies for agriculture" (2009-2012) and EP7- REGPOT-2012-2013-1 MODBIOLIN No. 316304 entitled “Use of model organisms to resolve crucial biological problems on the path to innovations" (2012-2015). Both these projects helped to keep international contacts with our partners, owing these project we were able to get new equipments and facilities during the period of strong reduction of investments on AS CR and grant agencies. By the means of standard and bilateral projects we got roughly 475 thousands Euro that covered fully our requirements for materials, traveling, overheads and partly salaries of the department.

From the list of more expensive devices, within the evaluating period the department gained qPCR cycler iQ5 Multicolor Real-Time PCR Detection System and BioLogic DuoFlow Pathfinder 20 System for nuclease chromatography and purification from the MOBITAG EC project. From this project the replacement of light sources in culture unit carried out providing optimal and reliable light conditions for the *in vitro* plant cultures and, at the same time, considerable energy savings were achieved. From MODBIOLIN EC project we were able to get finance for renovation of cultivation room for *A. thaliana* and build biosafety RNA laboratories. From Alexander von Humboldt Stiftung Foundation we financed SuperMicro Server 8 Hot Dual Intel® Xeon, suitable for NGS analyses and bioinformatics.

The department is equipped for standard molecular biology work including DNA cloning, library screenings (e.g. isotope laboratories), protein expression, separation and analysis (cold laboratory), biosafety boxes and laboratories for plant transformation (both biolistic and via *A. tumefaciens*), *A. thaliana* handling unit for tissue culture work, transient expression systems, real-time PCR, devices for thermodynamic analyses of DNA and RNA, ultrasound device for metabolome extraction, specific programmed phytotrone etc.

2.4. Main research directions and achievements

2.4.1 Hop research

During the evaluated period we continued our analysis on genetic co-determination of biosysthesis of anticancerogenic prenylflavonoids and bitter acids in lupulin of *H. lupulus.* The major task included characterization of lupulin-specific transcription factors (TFs) and by middle of the evaluating period we initiated analysis of elements

of molecular network influencing this regulation. The genome of hop as non-model plant was not sequenced during that time. Therefore we preselected important TFs showing expression in hop cones using a cDNA-AFLP approach, using intensive screenings of genomic and expression libraries to extract sequences. Later we analyzed EST trichome-specific libraries available to find homologues of TFs. This was predominantly based on comparison with *A. thaliana* master TFs or factors involved in genetic determination of trichome morphogenesis. The important methodical achievement at this time was the method of extraction and purification of lupulin glands that we adopted from the literature. We prepared our own lupulin-specific expression library which greatly facilitated to identify lupulin- specific TFs by quantitative analyses. Besides TFs from Myb families, on the beginning of the evaluating period we focused on TFs of bZIP oligofamily (Matoušek, J. *et al.,* J. Agric. Food Chem 58:902, 2010). TFs bZIP 1 and 2 having specific expression in lupulin glands were analyzed in detail. It was found that these factors have ability to co-induce anthocyanin biosynthetic pathway in heterologous systems and are able to activate promoters of chalconsynthase chs_H1 and O-metyltrasferase 1 genes. Both these structural genes are responsible for biosynthesis of medicinal compounds of hop mainly anticancerogenic prenylated chalcones as xanthohumol. Further we characterized lupulin-specific WD-40 (WDR) and bHLH sequences. By analogy to *A. thaliana* trichome determination we analyzed a possibility of lupulin-specific MBW complexes. The finding and identification of these complexes was the main achievement published at this period (Matoušek, J. *et al.*, BMC Plant Biol 12:1471, 2012). According to this work at least two complexes M2B2W1 and M3B2W1 and specific inhibitor of their Myb components (Myb7) were found. Unlike to homologues in *A. thaliana*, lupulin specific complexes were not able to induce antocyanins neither in leaves nor in lupulin. It seems likely that this specific regulation contributes to supression of anthocyanins pathway and production of prenylated chalcones as Xanthohumol. This specific regulation in lupulin still has to be resolved, but the complete supression of anthocyanin in lupulin glands is consistent with other results that we performed in the collaboration revealed that ectopic overproduction of *A. thaliana* master TF of anthocyanin pathway PAP1 is able to induce anthocyanins in leaves and in cone bracts, but not in lupulin glands (Gatica-Arias, A. *et al.,* Plant Cell Rep 31:111, 2012). Some of hop lupulin-specific TFs like Myb3 (and also Myb 8-unpublished) have properties similar to master TFs and alternatively they are able to form MBW complexes. We selected the s-Myb3 transcription factor for transformation of hop supporting to the idea of so-called Myb biotechnology, with the aim to change or increase the level of some valuable metabolites. According to our collaborative results obtained transgenic plants indeed showed enhanced activities of some enzymes of hop prenylpropanoid pathway and exhibited modified metabolome (Gatica-Arias, A. *et al.,* Plant cell Tiss. Org. Cult. 113:279, 2013; Gatica-Arias, A. *et al*., Brewing Sci 65:7, 2012; Gatica-Arias, A. *et al*., Acta Hort., p 47-51, 2013). Besides isolation and characterization of TFs in transient expression systems, complementation analyses of TFs in *A. thaliana* were performed (e.g. Kocábek, T. *et al*., Acta Hort., p 77-84, 2013). In the middle of evaluating period within new project we focused more on the network background of lupulin regulation with available TFs and other hop genes (Matoušek, J. *et al*., Brewing Sci 64:151, 2011; Matoušek, J. *et al.*, Acta Hort., p 39-45, 2013). In this respect the most complicated dependency of activation of promoters of structural and regulatory genes of prenylflavonoid pathway is co-determined by WRKY oligofamily of hop genes. Activation by lupulin-specific WRKY1 gene is associated with binary complex predicted from combinatorial transient expression assay with WDR1 TF (e.g. Matoušek, J. *et al*.,

Acta Hort., p 39-45, 2013), as well as with activity of some kinases. In addition, level of WRKY1 mRNA is regulated by PTGS (unpublished). Recently, including years 2013 and 2014 the group is solving these principal unsolved query regarding the molecular regulatory network. The major achievements of the last years also include identification and cloning of proximal promoter elements of important genes of prenylflavonoid pathway and most of cloned TFs. Based of these sequences we are able to predict and assay the basis of the network from combinatorial assays in heterologous systems and in hops using biolistic transient expression systems and hop transformation.

Besides basic molecular genetic analyses, within the hop research we concentrated also on more practical tasks related to development of new generation of molecular markers based on the regulatory sequences extracted from genomic and expression libraries of hop that could be useful for hop breeding programmes. In this research we characterized 30 EST-SSR markers for genotyping hops corresponding to 25 gene loci and 1268 ESTs sequences published by Patzak, J *et al.* (Biol Plantarum 55:761, 2011) and prepared as certified method approved within the research programme of Ministry of Agriculture of CR. Later, based on analysis of hop WRKY oligofamily and WRKY1 regulatory sequences we developed four sets of primers to distinguish among hop genotypes. This procedure was patented in 2013 as Utility model no. 25678 and successfully introduced in practice within the EP7- REGPOT-2012-2013-1 MODBIOLIN „Use of model organisms to resolve crucial biological problems on the path to innovations" granted technology transfer entitled: "Transfer of hop cultivar authenticity control to grower's praxis." As a result the combinations of 4 sets of PCR primers distinguished 181 from 217 hops of world collection of hop genotypes and selection materials.

Because Czech hops are law protected commodity and the hop research is of interests to public, besides some public lectures we publish periodically our results in more popular form. Within the evaluating period we published CZ/English article entitled: „Transgenic" Metabolome of Hop, Some Aspects of its Development and Prospects of Utilization (Matoušek, J., Kvasný prům. 58:13, 2012).

2.4.2.Viroid research

In the work frame of analysis of viroid pathogens during the evaluation period we primarily focused to work on the mechanisms of viroid-mediated pathogenesis, viroid propagation and adaptation to new natural and experimental hosts. Viroids of our interest belong mainly to family Pospiviroidae and include variants of potato spindle tuber viroid (PSTVd) and several hop viroids. According to recent models, RNA silencing of host is the initial phase for the initiation of viroid pathogenesis. Viroid are supposed to induce gene regulation disorders involving vsRNAs originating from Dicer cleavage of structured viroid RNA and from subsequent TASI processes (e.g. Matoušek, J. *et* al., RNA Technologies Springer-Verlag, Berlin pp. 629–644, 2012). During this research period we published the first detailed analysis of so-called small viroid-specific (vs)RNAs of special extraordinary strong variant As1 (PSTVd) (Diermann, N. *et al*., J. Biol.Chem. 391:1379, 2010). It was found that in the spectrum of vsRNAs there are predominant classes of the molecules derived from the viroid pathogenicity domain. Moreover, it was found that during viroid-caused pathogenesis significant changes in the composition of host micro RNAs were induced. As1 PSTVd that we originally described as variant that appeared probably due to thermomutations, is interesting model to solve the complex mechanism of pathogenesis and therefore, we included this viroid variant as comparative model in most experiments to analyze

pathogenesis. In relation to pathogenesis we described a new PSTVd variant C3 that evolved in chamomile (*Matricaria chamomilla*) after biolistic inoculation and produced devastating symptoms (Matoušek, J. *et al*., Biol. chem. 393:605, 2012). By bioinformatics means we characterized high fitness and low variation of lethal viroid compared to mild one. In this work we performed comparative quantifications of selected vsRNAs pool using TaqMan probes. PSTVd-C3 and PSTVdAS1 vsRNAs were compared with mild viroid. Both lethal viroids showed higher stability and lower variation in analyzed vsRNA pools than mild PSTVd strain QFA. In addition, for the first time we showed that although vsRNA homologues from lethal viroids exhibited similar spectra, the distributions in different experimental hosts were quite specific. In this analysis we described impact of viroids on lignin biosynthesis and suppression of several leaf morphogenesis factors. PSTVd spreading was further characterized in the collaborative work, where we described new sequence variants due to adaptation to ornamentals like *Solanum jasminoides, S. muricatum, Datura sp*. and *Brugmansia* sp. and to other ornamental plants after the biolistic transfer. A wide adaptation potential of PSTVd was found (Matoušek, J. *et al*., Eur. J. Plant Pathol. 138:93, 2014). The viroid adaptation and linked pathogenesis is very complex process involving interconnected network on different levels of cellular metabolism. In order to study the interaction on RNA level by more complex way, we projected wide degradome analyses using NGS and bioinformatics tools, this work is currently running.

There was the first observation of viroid devastating effects on hops in Slovenia reported five years ago. This initiated our more intensive collaboration on diagnosis of *Pospiviroids* on hops. During the evaluating period we published first indication of hop stunt viroid on hops in Slovenia (Radišek, S., Majer, A., Jakše, J., Javornik, B., Matoušek, J. First Report of Hop stunt viroid Infecting Hop in Slovenia. Plant Disease 96: 592-593, 2012, the paper did not allow dedications on projects and therefore its not included in the publication list generated for evaluation). According to our first analyses HSVd changes expression of most of transcription factors that we studied within the TFs analyses (see above) (Füssy, Z. *et al*., Acta Hort., p 113-120, 2013). Strong impacts on lupulin-specific TFs was observed in diseased plants suffered from the “Slovenian viroid syndrome” biolistically transferred as RNA to Czech hop (Matoušek, J. *et al*., Acta Hort., p 121-128, 2013). Important finding made during the evaluation period is related to assumed dual action of viroid pathogenesis on lupulin-specific MBW complexes and storng suppression of chalcone synthase chs_H1. Result indicated that viroid pathogenesis cause destabilization of B component targeted activity of the chs_H1-acticvating complex and down-regulation of chs_H1 mRNA which was proposed to be direct target of vsRNAs (Füssy, Z. *et al*., J Plant Physiol. 170:688, 2013). Furthermore, from NGS analyses of small RNAs extracted from diseased hops we characterized the presence of another viroid originated as contaminant from citruses (CVdIV) in the collaboration. Currently we found that there is some cross protection between HSVd and CVdIV viroids (unpublished) therefore, the analysis of multiple viroid infections is more complex issue which is the subject of our recent and future work as a collaborative project KONTAKT II (see above).

Our viroid research included also more practical work on viroid diagnosis in potato and hops, where we prepared some certified methods listed in the list of published results or these data were published in non impact papers (e.g. Ziegler, A. et al., J. Kulturpflanzen 66:7, 2014).

2.4.3. Nuclease research

In 2010 the department of molecular genetic extended comparative analysis of apoptotic plant nucleases with anticancerogenic effects that we discovered earlier. Detailed biochemical properties of originally cloned plant apoptotic nuclease TBN1 were investigated, ability to degrade nucleic acids such as single-stranded and double-stranded RNA and DNA was shown. Antitumor activity and cytotoxicity was analyzed. TBN1 showed antitumor activities on all cancer tumors examined such as human melanoma, prostate carcinoma and neuroblastoma grown *in vivo* on athymic mice even if applied as pegylated form intravenously (Matoušek, Jar. *et al*., Neoplasma 57:339, 2010). In comparison to animal RNases much lower cytotoxic effects of recombinant nucleases such as immunosuppression, depression of blood formation, aspermatogenesis and embryotoxicity were observed, suggesting their potential to be utilized as cytostatics. These properties were discussed in connection to patenting of recombinant nuclease as invention under No 312164, 2010 entitled: "Recombinant plant nuclease as antitumor therapeutics with low side effects". An application of nuclease does not lead to the decrease of activation of lymphocytes as detected in MLC system, the nuclease does not cause the body mass losses and aspermatogenesis. The nuclease is prepared in infiltrated leaves of *Nicotiana benthamiana* transformed by *Agrobacterium tumefaciens* bearing DNA encoding this nuclease (Matoušek, Jar. and Matoušek, Jos. Recent Patents on DNA & Gene Sequences 4: 29, 2010). Later the biochemical and antitumoral activities of three plant recombinant nucleases from tomato (TBN1) brassica (ABN1) and hop (HBN1) were compared (Podzimek, T. *et al*., Plant Sci. 180:343, 2011). Based on these results we assumed that their ability to cleave double-stranded RNA is one of the most essential properties of these enzymes that could degrade small RNAs and influence levels of micro RNAs and regulation of stroma during proliferation of solid tumors.

To accomplish the important part of study, we performed biological, biochemical and structural analyses of plant nucleases by using our staff and internal granting for first year of evaluated period. Biological and structural analyses were successful and enabled us to modify and prepare nucleases in sufficient amounts with high purity for further biomedical assays and crystallography analysis. By site-specific mutagenesis we generated various mutants of TBN1 with modified glycosylation patterns, predicted structural and catalytical properties. Some results performed using these mutated variants will be published in the near future. Glycosylation mutant was successfully crystallized and used for structural analyses. Initially three different crystal forms of TBN1 were identified (orthorhombic, rhombohedral and trigonal) and the structure was solved by a combination of MAD and MR2. Although initially with lower resolution, it was possible to build and describe the TBN1 model that resembled some features of P1 nuclease from *Penicillium citrinum* with differences near the active site and in the glycosylation pattern (Dohnálek, J. *et al.,* J. Synchrotron Radiat. 18:29, 2011). Later crystals were obtained using a combination of salt and polymer and final model was built and refined using data with 2.15 Å resolution and basic structural properties were described. Based on the model it was well predicted that hydrolysis of the phosphodiester bond is caused by a nucleophilic attack of the activated water (hydroxide) molecule followed by formation of pentacoordinated transition state and its breakup into the products. (Kovaľ, T. *et al.,* Acta Crystallogr. F 37:124, 2011). Comparative crystallography analysis including crystals from mutated TBN1 and bioinformatics approaches led to the prediction and draw conclusions regarding more detailed TBN1 catalytic mechanism. The structure solution process required X-ray

diffraction data from two crystal forms. The first form was used for phase determination; the second form was used for model building and refinement. The following characterization of TBN1 as mainly α-helical structure stabilized by four disulphide bridges and by glycosylation with active site localized at the bottom of the positively charged groove containing a zinc cluster essential for the enzymatic activity. In addition, it was found that there is equilibrium of monomers, dimers and higher oligomers of TBN1 in solution. Principles of the reaction mechanism of the phosphodiesterase activity were suggested with the central role of the zinc cluster, nucleobase binding pocket (Phe-site) and Asp70, Arg73 and Asn167. Based on the distribution of surface residues the possible binding sites for dsDNA and other nucleic acids with secondary structure were identified. Phospholipase activity of TBN1, for the first time was reported for a nuclease in our collaborative study (Kovaľ, T. *et al.*, Acta Crystallogr. D 69:213, 2013). This classify anticancerogenic plant nucleases I as enzymes with broad substrate promiscuity and opens new experimental possibilities to explain their anticancerogenic effects in animal models and apoptotic role in plants.

During last years our group analyzed in detail nuclease expression in plants with the aim to increase further yield of recombinant proteins during transient ectopic expression in *N. benthamiana* leaves. Based on analysis of nuclease degradome it was found that nuclease level is suppressed at later stages due microRNA-mediated cleavage followed by some TASI process causing multiple mRNA cleavage (unpublished). Expression is significantly stabilized by several silencing suppressors.

2.4.4 Other topics

Some publications included in the list of results of Dept. Mol. Genet included work of one of our present research staff member, Jindřich Bříza (see above), that was up to January 31, 2011 the head of former Department of Gene Manipulations dealing with plant transgenosis of different plant species within the framework of several research projects. His responsibilities included production of transgenic plantlets using biolistic method, as well as characterization of transgenic plants on molecular biology level. Some papers were published and finished during the evaluating period. This included (i) Pavingerová, D. *et al.*, J. Forest Sci. 57: 277, 2011; Bříza, J., *et al*., Acta Biochim. Pol. 60: 395, 2013 from grant project "Development of transgenic tissue lines of spruce (*Picea abies*) showing high toxicity towards bark beatle (Scolytidae) species" (2007-2011, principal investigator Josef Vlasák); (ii) Vlasák, J. *et al*., Asian J. Plant Sci. Res. 3: 141, 2013 from grant project "Development of a novel recombinogenic technique for chloroplast transformation and its use for production of human papillomavirus E7 protein in plants" (2009-2012, principal investigator Josef Vlasák); (iii) Vlasák, J. *et al*., Afr. J. Biotech. 11: 1133, 2012 from grant project "New biotechnological approaches for nepovirus resistance creation in grapevine rootstock cultivars" (2009-2011, principal investigator Daniela Pavingerová).

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Plant Virology

2. Research Report of the team in 2010–2014

Current personal structure of the team has been established in the middle of 2014. Three major changes included transfer of Assoc. Prof. I. Mráz together with his postdoc Dr. P. Beran to the Faculty of Agriculture (August, 2014), retirement of Daniela Pavingerova (June 2014), and change of the Head of the department (Karel Petrzik -> Igor Koloniuk). There were eight researchers at the department, which were supported by technical staff. Overall, 7 bachelor work, 9 master and three doctoral theses were successfully completed under supervision of team members during the evaluation period.

Our team is mainly interested in plant pathogens of different nature. Most of research activities are tightly connected with plant viruses. Among other pathogens are phytoplasmas and phytopathogenic bacteria. At the end of the evaluation period, few new research directions were taken – exploration of viruses in lichens and fungi (mostly phyto- and entomopathogenic). Main results achieved by our team are outlined below.

Antiviral activities against of several compounds were evaluated using a newly developed methodological approach. It allows time-efficient estimation of in vitro elimination of viruses from vegetatively propagated crops. Turnip yellow mosaic virus and Cauliflower mosaic virus were selected for the studies. The first possess single-stranded RNA genome, while the other is double-stranded DNA viruses that replicates using reverse transcription mechanism. Acyclic nucleoside phosphonates PMEA, (S)-HPMPC, PMEDAP, ribavirin, and tenofovir (R)-PMPA were trialed. The compounds exhibited both various antiviral activity and phytotoxicity. For example, it was shown that tenofovir (R)-PMPA significantly reduced titers of Cauliflower mosaic virus in Brassica pekinensis plants within 6-9 weeks. Antiviral activity was tested by the team members as well as majority of relevant experimental work. Collaborators from the Institute of Organic Chemistry and Biochemistry provided us with the compounds, determined their levels in the culture media, conducted experimental part with catalyzed phosphorylation and HPLC analysis. The results of these studies were published and patented.

A fascinating and quite unexpected result was finding plant viruses in lichens. It should be emphasized that there were no reports about any viruses in lichens at all. A rhabdovirus and Apple mosaic virus (ApMV) were detected in a number of samples of different geographical regions. After obtaining axenic cultures of photobiont, identified as Trebouxia sp., ApMV was repeatedly detected by RT-PCR. For the mycobiont, all attempts to obtain the axenic culture were unsuccessful. Besides experiments design and realization, virological part of work was done in IPMB as well as the cultures maintaining.

During 2010-2014, complete genomic sequences of four plant viruses were determined and characterized.

Broad bean true mosaic virus (Comovirus genus, Secoviridae family) is one of the earliest known comoviruses with genome structure being unknown. Both genomic segments were sequenced with primer walking approach. Comparison of the amino acid sequences of the capsid proteins and the polymerase showed striking differences to other comoviruses and highest similarities to legume infecting comoviruses. The taxonomic position within the genus was established on the basis of molecular data from capsid proteins and RNA polymerase.

For another two comoviruses, Radish mosaic virus and Turnip ringspot viruses, a comprehensive phylogenetic and serological analyses were made. Unlikely other comoviruses, they shared extremely high numbers of genomic identity that were just below the edge of species demarcation line. However, serological differences support their discrimination as two different species. Another study on genetic intraspecies variability of comoviruses suggested inclusion of both capsid proteins to the sequence analysis. It improves species/strains resolution in the genus.

A separate study aimed to explain existence of two large clusters (based on capsid protein sequence) of Apple mosaic virus isolates. One cluster correlated with Maloideae hosts and Trebouxia lichen algae hosts; a second with hop, peer, and other woody hosts. However, correlation was absent between clusters and geographic origin of the isolates. Purifying selection was shown for all populations of Apple mosaic virus isolates. Data analysis including statistical evaluation was done at the department.

An isolate of a novel virus with single-stranded RNA genome from garden lupine was characterized and sequenced. Phylogenetic and sequence analyses suggested that it represents a new species within Potyviridae family. Tentatively it was named as Lupine mosaic virus.

In collaboration with a colleague from Slovenia, a study on Blueberry red ringspot virus (Caulimoviridae family) isolates from Slovenia and Czech Republic was conducted. The Slovenian isolate was sequenced by our collaborator. Both isolates were obtained from old highbush blueberry in the Czech Republic and Slovenia.

During 2013, a double-stranded RNA was isolated from a phytopathogenic fungus, Phomopsis longicolla. It represented an intermediate replicative form of a novel single-stranded RNA virus from Hypoviridae family, provisionally named Phomopsis longicolla hypovirus 1. The fungal isolate, harbouring the virus, was debilitated and showed reduced virulence on leaves of soybean, its primary host. The complete genomic sequence and its phylogenetic position in the family was resolved. It was proposed to include the new species to a recently suggested Betahypovirus genus within Hypoviridae family.

Assoc. Prof. Dr. Vlasák collaborated on the research of worldwide fungal diversity. Exhaustive taxonomic and phylogenetic studies on such fungi as Inonotus sp., Gloeophyllum protractum, Dichomitus albidofuscus, Phellinidium asiaticum, Diplomitoporus sp., Anomoloma myceliosum, Neofomitella polyzonata, Trametes lactinea, Antrodia P.Karst. were performed together with both Czech and abroad collaborators. Besides, a number of species from Polyporales (Basidiomycota) were thoroughly investigated. Molecular analysis, sequencing of difficult templates, and samples preparation was done here. Those pieces of research contributed significantly towards resolving taxonomical positions of the fungi with application of molecular methods.

At the department, a system of recombinant protein expression is exploited to provide collaborators from Institute of Entomology (BC CAS) with modified endotoxins from Bacillus thuringiensis. Many different versions of the toxins are prepared for biological and biochemical testing. One of the versions which exhibited enhanced

toxicity to spruce bark beetle was transferred into Norway spruce (Picea abies) plants. The expression of the recombinant toxin was confirmed with molecular methods.

Another set of experiments was devoted to genetic transformation of plants and algae with different expression constructs. For example, in conifers and other plants with long reproductive cycles, transformed embryogenic tissues can serve as a convenient source of plant material for the testing of insecticidal or fungicidal transgene efficiency. Transormation by Agrobacterium tumefaciens with the gus-intron chimeric gene and other genes was performed and tested. Members of our team participated in planning of the experiments, construction of expression vectors, and transformants characterization.

Phytoplasma research continued in cooperation with breeders of fruit trees and small fruits from Breeding Institute of Pomology (Holovousy), clover breeders (Hladké Životice), researchers from UP (Olomouc) and foreigner researches. Members of our department were actively engaged in the COST Action FA0807 (2010 – 2014): J. Fránová as a leader of working group WG1 (together with B. Duduk, Serbia) and a member of Executive committee; J. Špak as financial rapporteur and the national delegate for the Czech Republic. Training school on phytoplasma DNA extraction from plants and insects was organized and carried out for foreign scientists in July 2013.

In cooperation with Breeding Institute of Pomology Holovousy, survey for 'Candidatus Phytoplasma prunorum' was done in apricot, peach and sour cherry orchards as well as psyllid species were monitored in apple and pear orchards in East Bohemia. 'Ca. P. mali' (AP) and 'Ca. P. pyri' (PD) were sporadically detected in Cacopsylla picta (90 individuals tested/4 positive) and C. pyri (966/11), C. pyrisuga (47/1), C. pyricola (17/1) in apple and pear orchards in East Bohemia, respectively. Occurrence of PD, AP and 'Ca. P. asteris' (16SrI-B and 16SrI-C subgroups) was confirmed in orchards as well as in wild growing fruit trees. Genetic diversity of 'Ca. P. mali' strains based on multilocus gene analyses was demonstrated on 74 apple trees from all over the Czech Republic. Extensive RFLP study of AP revealed prevalence of P-I 16S-23S profile, rpX-A subgroup, subtypes AP-15 and AT-2. Two previously unreported RFLP patterns were observed in the 16S-23S rDNA region in samples from four apple trees; in another one, unique RFLP profile was found after PCR amplification of a region from putative nitroreductase gene. The PCR/RFLP results were confirmed by nucleotide sequence analyses of selected 'Ca. P. mali' strains. This first survey of 'Ca. P. mali' molecular variability using multiple gene analyses in commercial apple orchards and in wild-growing naturally-infected apple trees reported for the first time infection of trees by more than one AP subtype and genetic lineage.

We firstly discovered and characterized in detail 'Ca. P. asteris' (16SrI-B) in Asparagus officinalis and Verbena x hybrida; 'Ca. P. asteris' (16SrI-C) in Rhododendron hybridum, Ribes rubrum, Apium graveolens and phytoplasmas belonging to subgroup 16SrIII-B in Echinacea purpurea. In addition, the complete nucleotide sequences of three extrachromosomal elements from 'Ca. P. asteris' (16SrI-B, two phytoplasma strains originated from the Czech Republic, another one from France) were determined.

The microarray-based system was developed reliably detecting phytoplasmas of 16Sr groups I, II, III, V, VI, VII, IX, X, and XII. Furthermore, the microarray was able to distinguish six out of eight artificial mixed infections, and to reliably distinguish 16Sr groups in five field samples, as well. This represents an advance to two microarrays published previously which allowed only limited determination of 16Sr group in single infection. The microarray can be also used as a suitable alternative to DNA barcoding

or T-RFLP techniques for routine screening of selected 16Sr groups of phytoplasmas, and designed probes can be employed for further molecular tests.

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Photosynthesis

Present team personal structure is determined by the fact that the team is a joint operation together with the Faculty of Science, University of South Bohemia. As a result, all team researchers have fractional time employment varying from 10 to 80 % but overall averaging below 50% per researcher. The second determining factor for the team personal structure is the fact that key sub-team leaders (J. Šantrůček and F. Vácha) as of 10-5 years ago shifted the core of their attention to the University (teaching & management but also research activities). As a result the team composition and functioning has not been static and slowly some changes were implemented as possible (e.g. during contract renewals). Overall, as of December 2014, the team consisted of 10 researchers, supported by 4 technical staff. The contract of one of the researchers (K. Roháček) has not been renewed for 2015.

The major change in team composition during the reporting period was the return of three post-docs from abroad in late 2011, followed by a change in team leadership in 2012. Therefore, as of December 2014, the major sub-team of the department was formed by four young (~35 years old) post-docs with one of them being team leader. The main research interests of this sub-group (D. Bína, Z. Gardian, M. Herbstová, R. Litvín and former team leader F. Vácha) are light harvesting protein structure and function and associated topics. All four young post-docs have spent some time in laboratories abroad (1 - 2 years). Two team members whose main interests are ultrafast dynamics of energy transfer processes in light harvesting systems and carotenoid photophysics (M. Durchan and T. Polívka) work closely with the young post-doc team, benefiting from the capability to isolate pigment-protein complexes and study them by advanced biophysical methods almost under one roof. Nevertheless, the ultrafast laser setup is hosted by the Faculty of Science, which naturally forces all grant funding for the laser lab there.

The sub-group formerly led by J. Šantrůček, interested in plant water regime, stomata and carbon isotope fractionation is undergoing restructuring. M. Hronková and 2 technical staff are currently supposed to work towards the light harvesting sub-group. The most valuable capacity of this sub-group are molecular methods (for example Real-time PCR) which are missing in other parts of the team.

The team is engaged in several key research directions which are summarized below.

a) LHC protein structure and function

Research of light harvesting protein structure, function and evolution has gradually emerged and strengthened in the team over the reporting period. This research direction is grounded by a few key tenets: (i) there is a significant unsampled diversity of light harvesting proteins and pigments among photosynthetic eukaryotes; (ii) the fact that diverse photosynthetic organisms utilize different light harvesting carotenoids means that there has to be a diversity of protein amino acid sequence and structure adaptations to enable these different carotenoids to function effectively; (iii) the team in its current composition is uniquely positioned to study these

systems by biochemical, biophysical and imaging (electron microscopy) methods as there is all the necessary know-how in one place; (iv) upon sufficient sampling of this diversity perhaps a general view of light harvesting strategies and associated peptide structures can be established, facilitating further work in e.g. design of novel materials or sensors. The current running and planned projects of the team key members (Bína, Gardian, Litvín, Polívka) are based on this reasoning. Several ecologically important algae groups have essentially undescribed light harvesting strategies providing this research program with ample opportunities. This is greatly helped by the recent boom of genomic projects which are necessary to provide the background information for the team's work.

Several publications can be mentioned to illustrate the points described above. In 2010, a study of cyanobacterial Pcb antenna proteins was published (Herbstová et al., 2010, BBA), these proteins are related to the plant CP43-CP47 proteins but serve as outer antennas of unique chlorophyll b containing cyanobacteria. Information about the Pcb antenna association with photosystems was collected. In 2011, the team's first foray into Chromalveolate algae was published (Gardian et al., 2011, Photosynth. Res.). The work presented the first available electron microscopy single particle images of light harvesting complexes of any Chromalveolate alga, in this case a relatively obscure Xanthophyte lacking chlorophyll c. Its light harvesting proteins, XLH, assemble into interesting cartwheel-like structures. The team's first joint publication after post-doc stays abroad was published in 2013 (Tichý et al., 2013, BBA). This work presented the results of a comprehensive study of a unique alga *Chromera velia*. Although the alga essentially forms its own phylogenetic group within Chromalveolates, its light harvesting complexes, denoted CLH, are in many aspects very similar to those of Heterokont algae (best example being diatoms). Building on this work, a novel red-shifted chlorophyll *a*-based antenna was described in a two-paper work done together with a group in Třeboň (Kotabová et al., 2014, BBA; Bína et al., 2014, BBA). This red-shifted LHC complex provides a direct link between environmental illumination conditions and light harvesting protein composition and function. Unfortunately this red-shifted antenna complex is too fragile to enable its study by femtosecond spectroscopy.

An electron microscopy study of light harvesting complexes of diatoms (Gardian et al., 2014, Photosynth. Res.) was also recently published, showing that even two different diatom species can in fact have strikingly different spatial arrangement of antenna complexes, providing further impetus for studying more Chromalveolate systems. Presently, there appear to be two supramolecular LHC arrangements in Chromalveolate organisms: i) cartwheel-like structures described from Xanthophytes, *Chromera* and the diatom *Phaeodactylum*; ii) string-like structure found in the diatom *Cyclotella*. The evolutionary and functional consequences of these findings are not clear now. Enhancing the team's electron microscopy capabilities to imaging of membrane patches, a work was also published on membrane complexes' spatial organization in a photosynthetic bacterium *Chloroflexus aurantiacus* (Bína et al., 2014, Photosynth. Res.). Observation of protein complexes in membrane patches offers interesting possibilities to study long range arrangements of membrane complexes and analyze their functions from a different angle. Several other subprojects utilizing this capability are underway.

The contributions of the team on all mentioned papers are significant, easily seen from author lists. All experiments, data processing and interpretation were carried out by the team apart from a few specialized tasks such as tandem mass spectrometry (P. Koník) or genomic information (A. Pain). The exception is one of the *Chromera* red-shifted LHC papers where our contribution to part I. (Kotabová et al., 2014) was minor. J. Tichý, a co-author of several mentioned works, is a PhD student of F. Vácha, carrying out his research in the team.

b) Photophysics of carotenoids and LHC pigment-protein complexes
Essential to light harvesting protein function, carotenoids are a long term interest of one of the team's key members, T. Polívka. The femtosecond laser lab used to study carotenoid photophysics is hosted by the University of South Bohemia, with the team facilities providing some steady state spectroscopy methods and more importantly biochemical support. Several publications resulted from direct collaboration of the facilities in České Budějovice, with significant proportion of work done at the institute. In 2010, an ultrafast study of cyanobacterial Pcb light harvesting complexes was published (Durchan et al., 2010, J. Phys. Chem. B). The Pcb proteins display low efficiency of carotenoid to chlorophyll energy transfer, a characteristic shared with other members of the protein family, IsiA and CP43. On the other hand, the mechanism of energy transfer between carotenoids and chlorophyll is different, using hot S_1 state of carotenoids rather than the S_2 state in the other homologous systems.

As in the publication from 2010, two other systems first studied by the team biochemically were also described in terms of energy transfer pathways. These were XLH antenna from *Xanthonema debile* (Durchan et al., 2012, J. Phys. Chem. B) and CLH antenna of *Chromera velia* (Durchan et al., 2014, BBA). Both of these systems are from Chromalveolate algae and are characterized by high efficiencies of energy transfer from carotenoids to chlorophyll (60 % in XLH and over 90 % in CLH). In XLH the carotenoids use both S_2 and S_1 pathways to transfer energy to chlorophyll *a*. While the S_2 pathway is common, the S_1 was not expected and is probably enabled by protein effecting the carotenoid configuration. CLH, like many other Chromalveolate LHCs (e.g. FCP or PCP), contains a carbonyl carotenoid, in this case an unknown isofucoxanthin-like pigment. Unlike other carbonyl carotenoid systems in CLH the protein suppresses the charge transfer character of the S_1/ICT state. These results demonstrate the important role of protein structure and its influence on the physical properties of bound pigments.

In all mentioned publications the team contributed mostly with sample isolation, steady state experiments, pigment analyses and microsecond transient absorption experiments.

c) Natural and artificial bacteriochlorophyll aggregates
Bacteriochlorophyll aggregates are the basis of light harvesting of several photosynthetic bacteria, building chlorosomes, huge light harvesting structures containing up to 100 000 bacteriochlorophyll molecules with little to no pigment-binding protein. Research on this topic stemmed from a joint project of F. Vácha and a collaborator from Prague J. Pšenčík (Charles University). In 2010, work based on the previous efforts of the team's former PhD student A. Župčanová was published (Pšenčík et al., 2010, Photosynth. Res.). Using transesterified bacteriochlorophylls produced by the team the work studied self assembled aggregates by X-ray scattering, finding direct link between the esterifying alcohol length and lamellar spacing of the aggregates. The team contributed by cell growing, pigment isolation, chemical modification and subsequent purification of bacteriochlorophylls.

Further developing the bacteriochlorophyll aggregate know-how, two papers resulted from collaboration with a group of M. Vácha in Japan (Tokyo Institute of Technology). In 2011 single chlorosomes were studied and their absorption linear dichroism measured (Furumaki et al., 2011, JACS). The work was possible due to the immense absorption cross-section of single chlorosomes. The team contributed with wild type *C. tepidum* cultivation and chlorosome isolation and also with bulk linear dichroism measured on oriented chlorosomes. In another work (Furumaki et al., 2014, ACS Nano) bacteriochlorophyll aggregates were built on gold

nanoparticles resulting in a hybrid self-assembling nanostructure with altered properties. The team contributed by cell cultivation, pigment isolation and preliminary aggregation studies - S. Furumaki visited the team to learn bacteriochlorophyll aggregation and try out a few experimental approaches, some electron microscopy work was also done at the Biology Centre.

Note: it is assumed that a list of all team publications with full citations is included elsewhere in the evaluation materials.

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Fish and Zooplankton Ecology

2.1 Fish and Zooplankton Ecology team introduction:

The Fish and Zooplankton Ecology team represents one of the three departments (Department of Fish and Zooplankton Ecology – DFZE) of the Institute of Hydrobiology (IHB) The research focuses on the highest trophic levels in freshwater ecosystems – zooplankton and fish, and the corresponding needs for different methodological approaches divides the DFZE into two sub-teams with different study approaches.

The Zooplankton Ecology Group (ZEG) conducts traditional zooplankton studies with genetic approaches following the evolutionary adaptations of key *Daphnia* species. A basic principle of their work is the combination of field and laboratory approaches, where working hypotheses for laboratory experiments grow out of data obtained during fieldwork. Their research focus, the genus *Daphnia*, is also a substantial and preferred food resource for planktivorous fish and, as such, forms an important link in the food pyramid and between the two groups of the department. Currently the work of the Zooplankton Ecology Group falls into five research areas: 1) studies of the interactions of trophic state, fish and zooplankton, 2) analyses of long-term changes in the zooplankton of model reservoirs, 3) genetic studies of the populations of the most common European hybrid complex *Daphnia longispina* and of their links to biotic and abiotic factors, 4) “founder effect” of newly colonized ecosystems of lakes in former coal quarries, 5) physio-ecological adaptations of the most common species *Daphnia galeata*, with exceptional plasticity.

The Fish Ecology Unit (FishEcU) functions mostly like a national and international body in charge of quantitative fish stock assessments in lakes and reservoirs with **main research topics being the spatio-temporal distribution of fish abundance and biomass, species- and size-specific behavioural traits, foraging ecology and role in trophic webs, and methods and methodology of quantitative sampling of fish communities**. The research results improve our general knowledge about fish and their role and influence within the whole aquatic ecosystem as well as providing qualified advice and support to practitioners managing fish stocks in lentic water environments. **Great emphasis is placed on research and development of methods for quantitative sampling of fish stocks**. In this particular field FishEcU is a **world leading research group in horizontal acoustic methods**, gauging their limitations, determining the relationships between fish size and the strength of their acoustic echoes, improving the accuracy of acoustic detection of fish larvae, juveniles and aquatic invertebrates, and last but not least the use of acoustic methods in research on fish behaviour. In addition to acoustic methods, **the group draws on its tradition of passive and active fishing gear, developing sampling methods using electrofishing, beach seining, purse seining, trawling and gillnetting**. Results obtained by FishEcU in the field of gillnetting significantly contributed to understanding the ecological value gained by this most common sampling method. During the evaluation period, FishEcU substantially contributed to clarifying the hitherto little-understood behaviour patterns of fish in large inland waterbodies, and their trophic role in these ecosystems. The role of fish was assessed both from a "bottom-up" (food accessibility for fish under different conditions) and a "top-down" perspective (fish as consumers feeding on organisms from lower trophic levels and the implications for the qualitative composition of these lower levels and for water quality). Individual approaches (trophic effectiveness and selectivity) as well as approaches based on evaluating the impact of the whole fish community (food rations, consumption rates, bioenergetic modeling etc.) were used. **An important aspect of the work of the FishEcU is its complex approach: the absolute importance of individual species and size groups is derived by weighing from the total picture of the fish community**. This is made possible by a **unique combination of quantitative and qualitative sampling methods.**

2.2 Team structure and financing

During the past fifteen years substantial effort was put to build a strong team of fish ecology experts competent in obtaining the "true picture" of fish stocks in lakes and reservoirs through more robust and reliable sampling. As a result **the team has grown from four researchers and two technician institutional positions to sixteen researchers and five technician positions (Table 1.), and by eight more doctoral students**. The age structure is well reflecting the expansion period and the actual vital state of the team has a broad base covering young enthusiastic scientist (**70 % of the team researcher staff younger than 40**), but also composed of experienced senior scientists.

However, setting such ambitious research goals was a very demanding task and the group manpower and financial demands went far beyond the possibilities of institutional financing of the CAS via IHB institutional funds (officially three researchers and one technician positions are institutionally supported for fish research in IHB). In order for the task to be achieved, the team had to invest time and effort in securing extensive external financial resources to build its equipment and to develop the required know-how. During the evaluation period, **the institutional expenses granted by the CAS represented on average 44 % of the overall team's budget and hence the majority of the team staff was funded from external sources**. Nevertheless, the team was successful in securing external financing from both, topic-relevant research projects and commercial contracts.

2.2.1 Research projects:

During the evaluation period the team members were principal investigators of 21 research projects - 5 projects provided by the Grant Agency of the Czech Republic, 5 projects provided by Ministry of Education, Youth and Sports, 2 projects provided by the Grant Agency of the Academy of Sciences, 2 projects provided by Ministry of Finance, 1 project provided by the Ministry of Agriculture, 1 project provided by the Ministry of Environment, and 5 projects provided by international institutions (for a complete list of projects see Attachment 3-1), **securing a total of 1,578,734 EUR during 2010-14.**

Selected projects:

2008-2010 – Kubečka J. "Monitoring of the fish stock of Czech reservoirs." (CZ0091) **57,000 EUR**
2008-2012 – Kubečka J. "Optimasation of the biomanipulative effect of predatory fish in ecosystems of water reservoirs." (QH81046) **58,195 EUR**
2009-2011 – Čech M. "Predator avoidance strategies in early life stages of percid fishes ." (GP206/09/P266) **46,498 EUR**
2009-2012 – Macháček J. "Cyclical parthenogenesis in vertically diversified environment: genetic differentiation and reproductive segregation in population of *Daphnia galeata*." (GA206/09/1325) **88,847 EUR**
2009-2012 – Seďa J. "Hybrid zones in pelagic environments: which factors are critical for local dominance of *Daphnia* hybrids within reservoirs?" (IAA600960901) **92,761 EUR**
2010-2012 – Kubečka J. "Fish stock assessment of the Neusiedlersee by acoustic and direct sampling." ("Monitoring-Fischerei"/NP5) **71,000 EUR**
2012-2014 – Prchalová M. "Get out! she signalized: sex segregation of freshwater fish." (GPP505/12/P647) **81,437 EUR**
2012-2015 – Frouzová J. "Hydroacoustical distinguishing between fish and bubbles, and quantification of methane bubble ebullition in freshwater reservoirs of temperate zone." (GAP504/12/1186) **104,658 EUR**
2012-2015 – Matěna J., Kubečka J. "Centre for Ecological Potential of Fish Communities in Reservoirs and Lakes (CEKOPOT)." (CZ.1.07/2.3.00/20.0204) **556,174 EUR**
2014-2017 – Peterka J. "Structuring effect of submerged macrophytes on trophic relationships and distribution of fish in deep lakes (MacFish)." (7F14316) **56,000 EUR**

2.2.2 Commercial contracts

During the evaluation period the team members had 44 commercial contracts dealing mainly with complex fish stock surveys and estimates in large water bodies, manipulative fish stock reductions and rescue samplings and transfers of water bivalves (for a complete list of commercial contracts see Attachment 3-9), **securing a total of 503,118 EUR.**

2.3 International collaboration

Both team subunits are active in international collaboration, which is well reflected in joint projects and co-authorship on research papers. In 2007, Seďa et al. published a crucial study on genetic vertical segregation of *Daphnia* population which is traditionally taken as an exclusively epilimnetic species. One of suspected cause for *Daphnia* sinking down was genetically selective infection by parasites. Since that time the Zooplankton Ecology Group started a very fruitful cooperation with Ludwig Maximilian University

of Munich which yielded 5 original research papers dealing with problems of *Daphnia* parasites in Czech reservoirs **[1, 2, 3, 4, 5, 6]**. FishEcU concentrated both its unique know-how and equipment for quantitative surveys of fish in lakes and reservoirs, thus providing the grounds for extensive international cooperation. Nearly 20 years of complex investigations of the Biesbosh reservoirs, the Netherlands **[7]** have already been conducted and continued in 2014 with dramatic developments when compared to previous surveys. Similar complex surveys of Austrian, German and Latvian lakes were undertaken **[8, 9]**. FishEcU know-how on fry gillnets and trawling was successfully applied during investigations of four Puerto Rican reservoirs **[10, 11, 12]** (this work was led by Mississippi State University). Our combination of hydroacoustic and gillnet techniques facilitated the last study of Lake Turkana (Kenya) before the extensive flow regulation of its main tributary **[13]**. FishEcU advances in sampling methodologies were also reflected in upgrading national sampling standard for lakes and reservoirs **[14]** and in our active participation on upgrades of European fish monitoring standards (mainly acoustic EN 15910:2014 and gillnet FprEN 14757:2014 standards **[15]**, work led by Centre for Ecology & Hydrology, UK). Additionally, FishEcU expertise was used in two manuscripts dealing with marine and diadromous fish species in North America. One study contributed to the anticipated changes in marine fish distributions caused by climate change **[16]** (work led by Bedford Institute of Oceanography, Canada) and the other documented the importance of the post-glacial recolonisation as a major determinant of genetic diversity in American shad **[17]** (work led by Dalhousie University, Canada). The other collaborations were with teams in Austria, Canada, Finland, France, Germany, Norway, Poland, Russia, Sweden, Spain, UK, USA (mainly acoustics, stable isotope, telemetry, trawling, biotic interactions and population dynamics). The high international reputation of both subunits of the team is well reflected in the numbers of study stays of students and researchers from abroad (see report 3-10).

2.4 Awards to the members of the team

I. Matějíčková and L. Vejřík won "Best poster" award in the student poster competition during Ecology of Fish in Lakes and Reservoirs 2014, České Budějovice, Czech Republic; **J. Matěna was awarded with "Thank you letter"** by the president of the CAS **for high quality and devoted work for CAS**; **M. Prchalová was** a**warded with "Otto Wichterle Reward"** of the CAS **for outstanding research outputs; I**. **Vaníčková and M. Říha defended their doctoral theses with "Honoris laudatum".** The thesis of **I. Vaníčková was proposed for the award of the European Federation for Freshwater Sciences**.

2.5 Research outputs

2.5.1 Publication summary

70 IF papers (5 authored by members that were omitted from the evaluation due to their low average full-time equivalent during the evaluation period) **were published by the team during 2010-2014.**

Team members were the first authors in 49 (70%) papers.

Members of other teams or from abroad were the co-authors in 46 (66%) papers.

Out of the 70 papers, 20 (29 %) were published in core journals (i.e. journals of the 1st quartile of JRC categories) **in the fields of ecology, evolutionary biology, parasitology, limnology, multidisciplinary sciences, environmental sciences, veterinary sciences and fisheries.**

Highest impacted outputs (IF>2): Molecular Ecology (1x IF=5.84), International Journal for Parasitology (1x IF=3.64), Limnology and Oceanography (1x IF=3.61), PloS ONE (1x IF=3.53), BMC Evolutionary Biology (2x IF=3.41), Journal of Evolutionary Biology (1x IF=3.28), Ecological Engineering (1x IF=3.04), Parasitology (1x IF=2.36), Journal of Great Lakes Research (1x IF=2.31), Journal of Plankton Research (2x IF=2.26), Hydrobiologia (11x IF=2.21)

Table 1. Team scientometry based on Web of Science Core Collection statistics modified by IHB publication evidence

Name	Researcher ID	Position	IF papers	Sum of times cited	Sum of times cited without autocitations	h index
prof. RNDr. Jan Kubečka CSc.	**A-8230-2011**	**senior scientist**	108	1138	990	21
RNDr. Jaromír Seďa CSc.	**F-9072-2014**	**senior scientist**	59	493	303	14
doc. RNDr. Josef Matěna CSc.	**F-8254-2014**	**senior scientist**	40	339	232	12
RNDr. Jiří Macháček CSc.	**F-9077-2014**	**senior scientist**	20	275	233	11
RNDr. Martin Čech Ph.D.	**C-5797-2014**	**scientist**	52	412	205	12
Mgr. Mojmír Vašek Ph.D.	**C-5839-2014**	**scientist**	39	315	183	12
RNDr. Marie Prchalová Ph.D.	**F-8664-2014**	**scientist**	34	287	178	12
Ing. Jaroslava Frouzová Ph.D.	**F-8518-2014**	**scientist**	32	295	200	11
doc. Dr. Helge Balk	---	**scientist**	13	116	116	6
RNDr. Jiří Peterka Ph.D.	**C-7218-2014**	**associated scientist**	43	317	152	11
RNDr. Vladislav Draštík Ph.D.	**G-2222-2014**	**associated scientist**	30	297	153	11
Mgr. Milan Říha Ph.D.	**G-1065-2014**	**postdoctoral fellow**	29	199	131	9
Mgr. Tomáš Jůza Ph.D.	**G-3159-2014**	**postdoctoral fellow**	30	187	67	8
MRM. Daniel Ricard Ph.D.	**G-1814-2014**	**postdoctoral fellow**	11	1856	1774	8
RNDr. Michal Tušer Ph.D.	**F-8548-2014**	**postdoctoral fellow**	17	133	102	7
RNDr. Milan Muška Ph.D.	**A-7978-2014**	**postdoctoral fellow**	16	41	33	4

Note: Bednarska A., Estlander S., Hohausová E. and Mrkvička T. were omitted from the core evaluated team due to their only partial involvement during the evaluated period and hence low average full-time equivalent (less than 0.2).

2.5.2 Description of research activities

Note: Presented results originated in the team unless specified in the text.

2.5.2.1 Reservoir limnology, reservoir sediment, *Daphnia* eggs and human pathogens

Two papers were using the fish data for explaining spatial patterns of taxa or clones within *Daphnia longispina* species complex in a deep elongated reservoir. The first one is focused on *Daphnia* dormant egg gene bank stored in reservoir sediment showing that observed spatial structuring of the taxa of living *Daphnia* is strengthened by epphipia in the sediment copying the spatial pattern of active stages **[18]**. The second paper shows that if we have just one *Daphnia* taxon from *D. longispina* species complex in the reservoir, there is a nice clonal differentiation along the longitudinal reservoir profile that is detectable during a 10-year period **[19]** (team members contributed by data and main ideas). The spatial patterning of taxa or clones within *Daphnia* population survives even big flood events when clearly not all *Daphnia* are flushed and also the gene bank in the sediment seems to contribute to population recovery **[20]**. Reservoir sediment is not just a place where *Daphnia* dormant eggs are accumulated, it is also a cumulative trap for all particles and dead animal bodies sinking down. Having sensitive molecular methods for detecting human pathogens, we can use reservoir bottom mud as a cumulative biological sample for pooling the occurrence of the target through longer period. The study **[21]** (material from two team members' projects was used in this study) analysed the occurrence of mycobacteria in drinking water reservoirs and also in water treatment plants showing eurytopic occurrence of mycobacteria stressing the importance of sanitary technologies during water treatment technologies. Similar study analyzed occurrence of mycobacteria in fish **[22]** (material from two team members' projects was used in this study). The development of high specific probes for "taxon" identification of bacteria helped us to disentangle the spring succession of plankton components (algae, bacteria, protozoa, rotifera and crustacea) showing that the whole plankton system is working as "fine-tuned symphony" when each player has a defined role and time **[23]** (team members did the sampling and analysis of crustacean and rotifer zooplankton and helped shape the manuscript).

2.5.2.2 *Daphnia* reproduction and genetics

The research on *Daphnia longispina* species complex in Czech reservoirs (most common *Daphnia* in Europe) was accomplished by three additional papers. First, the methodological paper **[24]** (study was done within team members' project), who tested four identification methods of *Daphnia* taxa (visual microscopy, allozyme markers, ITS determination, and Fourier analysis of *Daphnia* outlines) using a large collection of frozen samples from ten Czech reservoirs. The hybridization and effects of past and present introgressions create difficulties in all identifications methods that were tested. The second paper connects sexual reproduction as a potential for hybridization with occurrence of *Daphnia* males mating with sexual females. The production of males during the spring seems to be genetically dependent and male-producing lineages were significantly different from those of female producing lineages **[25]**. The offspring are thus far from randomly mating in the population. Finally, in the third paper **[4]** (the study was done within team members' project), we have applied the most sensitive analysis of microsatelites for clonal microstructure of *Daphnia* populations in two reservoirs. We have

discovered that the concept of clonal erosion at the end of vegetative season need not be valid when we apply a highly sensitive approach. We have also proved the reproductive barrier between maternal taxa as the diversity of hybrids is lower than parents. It seems that the selectivity might often work against the most common genotypes, a model commonly accepted for diseases and parasites.

2.5.2.3 *Daphnia*, their own parasites and ecology of both

The group has done screening of four parasite species that were previously overlooked in eleven Czech reservoirs **[1, 2]** (material from two team members' projects was used in this study). The well-known parasite *Cauleria* (Protozoa), which causes castration of parthenogenetic *Daphnia,* occurred in up to 40 % of inspected animals in some samples. Therefore the effect and consequence to the *Daphnia* population dynamics cannot be overlooked. Among four parasites investigated, the Microsporidia (*Berwaldia*) occurred in all reservoirs. This evokes the old belief that microsporidians have as a secondary host mobile vector (insects). Having the hypothesis that microsporidians have a transmitting secondary host mobile vector (insects) while protozoans are transmitted directly, the genetic structure of these two parasites was compared in three geographically isolated reservoirs. The results matched the hypothesis that the genetic structure of *Cauleria* showed little interconnection between localities, while Microspridia showed less spatial genetic segregation.

The old postulate, known from terrestrial systems, that parasites and diseases are shaping the genetic structure of host was clearly demonstrated on *Daphnia* and parasite data from two Czech reservoirs **[5]** (zooplankton from two Czech reservoirs within team member's project was analysed). Significant differences in the clonal composition between random and infected sub-samples of *Daphnia* were detected on several occasions within both communities, indicating that host genotypes differ in resistance to both parasites.

Using a unique collection of frozen samples from 11 Czech reservoirs, as well as genetic markers for the determination of single species of microsporidia, we did a large survey of microsporidian infections diversity in *Daphnia* especially on the phenomenon of co-infections **[6]** (extensive collection of zooplankton was done within team member's project). There are two conflicting hypotheses on the occurrence of co-infections. First, when the host is infected it makes it more sensitive to secondary infection. Second, when the host is infected because of competition between parasites there is some barrier against the secondary infection. We detected 8 microsporidian species (3 of them completely new species to science) and there was a very interesting pattern that some microsporidian species have occurred as single infection, whereas some others in pairs as co-infections.

Using extensive data set from Czech reservoirs Wolinska et al. **[3]** (the material from two team members' projects was used in this study) have shown that parasite (and host) populations were significantly structured across space, indicating limited dispersal. Moreover, the frequency of parasite genotypes varied significantly over time, suggesting rapid evolutionary change in protozoan parasite (*Caullerya*). However, the distribution of parasite genotypes was similar across different host species, which might in turn have important consequences for parasite epidemiology, in other words the parasite survival strategy.

2.5.2.4 Eco-adaptations of *Daphnia*

There are very few experimental studies mimicking real winter conditions with temperature close to 4°C. The study **[26]** showed that low temperature in the winter leads to small size offspring and low number of primary filtering setae in the filtering apparatus of overwintering *Daphnia*. Our first data on the physiological basis of these results indicate temperature-size relationships that can significantly affect parameters of individual development and population dynamics. However, *Daphnia* are key-stone species and a model organism just under specific conditions, mostly in temperate zones **[27]**. In the tropics, mainly *Ceriodaphnia* take over and should be studied further in the future

2.5.2.5 Spawning site and substrate selection in perch

All fish sizes play significant roles in lakes and reservoirs. Small fish have the highest abundance and fastest production dynamics while large fish represent the parent stock and the bulk of biomass. Therefore, FishEcU studies all important steps of fish ontogeny. A unique series of studies were performed on prenatal history of perch in different waters. The distribution of egg strands of perch and the factors affecting their distribution were studied in Lake Chabařovice and in the Římov reservoir using boat observation and SCUBA divers. In case of Lake Chabařovice, egg strands were found at depths down to 20 m and, in contrast to the previous state of knowledge worldwide, over 80% of perch spawning activity occurred at depths greater than 3 m **[28]**. Using large areas of artificial spawning substrates, the factors influencing the depth distribution of egg strands were identified as waves, temperature, duration of the daylight period and light attenuation in the water column. Factors influencing the selection of spawning sites were identified as wind inducing current, internal seiches and temperature instability of

the water column **[29]**. Surprisingly, for the egg strands deposition, perch strongly preferred dead compared to live submerged vegetation **[30]**. In the case of the Římov Reservoir, factors influencing the depth distribution of egg strands were identified as presence of appropriate spawning substrates and light attenuation in the water column. Factors influencing the selection of spawning sites were identified as presence of littoral vegetation, especially in sheltered bays **[31]**.

2.5.2.6 Spatio-temporal dynamics of ichthyoplankton

As reported earlier by the FishEcU team (Čech et al.), the early life history of percid fishes is extremely interesting due to the variety of behaviours of different groups of fry. FishEcU investigated this topic by evaluating different methods of measuring young fish **[32]** (study was done within project of Čech M.) and by developing the quantitative sampling methods of young fish density and size structure **[33, 34]**. Specially designed square ichthyoplankton trawls were found to be rather quantitative for sampling larvae and early juveniles of both percid and cyprinid fish species. Earlier observations of percid ichthyoplankton from deep stratified lakes were supplemented by the study of shallow well-mixed reservoir, where most juveniles seek day refugium in the bottom benthic habitat **[35]**.

2.5.2.7 Pelagic and late summer fry communities' ecology

The inherent pelagic occurrence of larval and juvenile percid fish is a fascinating story. Sampling the ichthyoplankton of the Římov Reservoir, the most studied model waterbody of the institute, was incorporated into the regular fish monitoring during the evaluation period to elucidate patterns of formation of differently behaving fry groups. The governing factors are obviously not simple so it was not possible to sum-up the story in a publication yet. However, the studies of pelagic fry communities of various waterbodies including the Římov Reservoir led to the discovery of "dwarf" pikeperch subcohorts **[36]**. It appears that the year classes of pikeperch may be supported by late-born or extremely slow growing subcohorts as is common with fish that spawn multiple times per season. This is a surprising discovery as pikeperch is known as a single batch spawner. Another discovery by pelagic fry trawling was the surprising pelagic foraging of tubenose goby (*Proteorhinus semilunaris*) reported by **[37]**. This finding was followed by the discovery of a massive invasion of juveniles of three gobiid species in the open water of Biesbosch resevoirs, the Netherlands in 2014.

The late summer appears to be the best period to estimate the year class strengths of young-of-the-year fish. After establishing a sound methodology for pelagic fry surveys, the team concentrated its attention on fry occurrence in diverse nursery habitats in the littoral zone. The study **[38]** reported the patterns of fry occurrence in different habitats during the first year of life. Diurnal patterns of fry occurrence in the littoral were summarized in **[39]** and the main shifts between littoral and pelagic fry occurrence were described in **[40]**. The reproductive success and the composition of fry community in reservoirs apparently vary significantly from year to year while the patterns of spatial use and the overall older fish composition remains rather constant **[40]**. The above fry distribution papers launched the regular monitoring of the "true picture" of fry composition in all important reservoir habitats in Římov Reservoir in order to achieve a basic understanding of recruitment patterns. Late summer fish fry community is also an important consumer of zooplankton. The study **[41]** summarized capture strategies of perch and roach fry foraging zooplankton. Preference of planktonic food by the fry and its impact on zooplankton was also confirmed by the experiments in **[42, 43]** (Vašek M. contributed to study design) and in **[44]** (Peterka J. contributed significantly to study design and data processing).

2.5.2.8 Ecology of adult fishes and gillnet studies

The status of the older fish community and their biology was the main focus of team's research. Serious attention was dedicated to sampling methodology. Gillnet sampling is at present the most common approach in European lakes and reservoirs **[45]**. A series of significant papers were motivated by the fact that current European standard was not able to properly handle the catches in the localities with dense fish communities where gillnets deployed in a standard way were saturated by the catch **[46]**. The first step was to handle fish diurnal activity which determines the catching power of passive sampling gear such as gillnets **[47]**. This paper revealed very clear rhythms of activity which are also important for general understanding of fish behaviour. A combination of short-term and cumulative catches enabled us to construct a general model that ensures the comparability of short- and long-term gillnet exposure and that corrects for potential saturation of nets by the catch **[48]**. Because of this work, all standard gillnet catches can now be compared. The careful processing of extensive FishEcU gillnet samples from many waterbodies enabled the development of a new methodology that uses gillnet samples to assess the abundance of European eel by using easily identifiable attacked fish as a proof of eel presence **[49]**. Another significant achievement was the introduction of large mesh component of standard gillnets sampling.

2.5.2.9 Improved implementations of hydroacoustic methods

Besides gillnets, the most common sampling tools for lakes and reservoirs are hydroacoustics and active net methods. In 2010, FishEcU organized a worldwide meeting that summarized the state-of-the-art in these active sampling methods **[50, 45]**. The biggest challenge of freshwater hydroacoustics is to achieve an unbiased quantitative recording of fish in shallow layers where most fish are distributed during growing seasons. FishEcU in cooperation with doc. H. Balk (Department of Physics, University of Oslo) has carried a number of experimental tests where unknown signal fluctuations during horizontal sound beam applications under the surface were discovered and prepared for publication (Balk et al. in prep.). A number of experiments were done for fish sizing and determination **[51, 52]**. The last quoted paper is of special importance as it investigated the possibility of employing multi-beam acoustic cameras for quantitative surveys, unfortunately with little positive results. The methods developed by FishEcU were successfully applied for acoustic surveys of Polish and German lakes **[53]** (team members contributed significantly to study design and data processing], **[8]**. Acoustic studies of near-surface fish were also improved by stationary up-looking observations **[54]** and by patent application (pending) for mobile up-looking observation system. With respect to classical down-looking hydroacoustics, two papers contributed to the definition and extent estimation of the near-bottom dead zone and to survey results correction on its effect **[55, 56]**.

2.5.2.10 Active fishing methods – purse seining and trawling

The evaluation period was marked by significant advances in active netting sampling, especially in the open water of lakes and reservoirs. The combination of 48 hours continuous hydroacoustic and netting surveys led to the first overall picture of large fish movement between the littoral and open water **[57]**. The same continuous hydroacoustic data were used in sophisticated calculations of spatial inhomogeneity of fish in the open water **[58, 59]**. Netting support to these studies was done with a specially patented purse seine vessel **[60]**. Furthermore, trawling technology was introduced into Czech reservoir fishery after thorough tests of fish avoidance reactions **[61]** and a comparative study between selectivity of trawl and purse seine showing high inactivity of smaller fish species during the night **[62]**. Trawling was identified as a superior sampling technique for assessment density and distribution of threadfin shad in tropical reservoirs **[10]**. Further development of trawling technology resulted in suggesting a wheeled trawl **[63]**, which may become an ideal sampling tool for the open water of large shallow lakes (like Neusiedlersee in Austria, **[9]**). This approach is now being used in the mainstream section of the River Danube and in lakes in Latvia.

2.5.2.11 Distribution and migrations

Thorough analysis of biological sampling led to an improved understanding of fish distribution and habitat use in reservoirs. The studies **[64]** and **[65]** investigated the patterns of littoral habitat utilization by the fish community. The open water behaviour of fish originating from riverine habitats was even less understood. The study **[54]** followed an interesting phenomenon of open water “sinusoidal swimming” during foraging discovered earlier by FishEcU investigations. This paper together with **[57]** provided an “acoustic” picture of fish use of the open water. The whole effort of understanding the spatial ecology of fish led to the most comprehensive model of fish distribution between day and night. The value of these investigations is that the complete mosaic of fish diurnal and spatial migrations was put together by many different approaches (hydroacoustics, beach and purse seining, trawling, electrofishing) and that several methods gave similar patterns. Besides within lake migrations, FishEcU investigated the migrations between the reservoir and the tributary **[66, 67]** and between river and marsh systems **[68]**. Spatio-temporal distribution of a key prey species of reservoirs in Puerto Rico, threadfin shad, was described in **[11]**, and comparison of its density and biomass in tropical and temperate reservoirs was presented in **[12]**.

2.5.2.12 Water Framework Directive, biomanipulation and fish management

Our unique studies into the role of fish in the trophic webs of lakes and reservoirs coincided with the requirement of the assessment of ecological potential of lakes and reservoirs. FishEcU became an active contributor and national representative of the Czech Republic in EU “Central Baltic Lake Fish Intercalibration Group” and contributed to the officially accepted national methodology for the assessment of ecological potential in lakes and reservoirs **[69, 70]**. The fish part of this national methodology was based on the database of 25 Czech lakes and reservoirs containing more than 70 waterbody surveys (some were surveyed several times). The Czech methodology of detecting the ecological potential of reservoirs using fish was found sufficiently sensitive to anthropogenic stressors. Water Framework Directive issues were also one of the major topics of worldwide conference Ecology of Fish in Lakes and Reservoirs 2014 organized by FishEcU **[71]**. The database was used to assess the biomanipulation potential of predatory fish in the largest reservoirs **[72]**. The role of predatory fish and adaptations of their prey has received increased attention in recent years **[73, 74, 75]**. Also the bird

predators of fish were investigated by **[76]**. In their work, fish losses caused by overwintering great cormorant (*Phalacrocorax carbo*) in the system of the Vltava River were estimated. The study became widely cited in the report to European Parliament "Between fisheries and bird conservation: The cormorant conflict" written by Ian G. Cowx (2013).

2.5.2.13 Fish succession in newly formed lakes, angling and others

The main management priorities of lakes and reservoirs connected to fish investigations are (i) water quality and (ii) satisfaction of recreational fishermen. FishEcU executed over 40 expert studies for fishery managers of reservoirs and lakes (Attachment 3-9). Being done by standardized methods all these results end up in a unique database and will serve to achieve a deeper understanding of fish behaviour and ecological processes. At the same time FishEcU took part in fish stock creation of "post-mining lakes". This included yearly monitoring and stocking of desirable fish species. The fish community of three newly created lakes are undergoing dynamic and extremely interesting successions **[77, 78]** and the main scientific reports will be written in the coming years. With respect to recreation angling, FishEcU participated in a collection and processing of anglers statistics from various reservoirs led by Boukal D. (Faculty of Science, University of South Bohemia). The results on anglers preferences so far were summarized on DINFISH international conference 2010 co-organized by FishEcU and in **[79, 80]**. As part of a participation in projects led by other teams' spatial and temporal changes in benthic macroinvertebrates and rare benthic fauna were researched in streams in the Bohemian Forest **[81]** and Vltava and Labe rivers **[82]**.

2.5.3 Applied outcomes

More than 40 research reports for managers, stakeholders and decision makers (Attachment 3-9).

Kubečka J., Čech M., Peterka J., 2010. **Czech patent** No. 302 159 on Research vessel for fish sampling by the purse seine.

Certified methodology assessing Czech lake-type waterbodies was developed for the Ministry of the Environment of the Czech Republic.

2.6 SWOT analysis

Strengths: Strong and vital team (good recruitment of young researchers) with wide focus, leading position in several fish sampling methods (hydroacoustics, gillnetting, trawling) and adopted methodology of complex surveys of large inland waters. Well established international collaboration and diverse funding (from research grants to commercial contracts). Unique database of reservoir data.

Weaknesses: Delayed dissemination of results to wider ecological perspectives and hence publishing in high impacted, well cited journals. However, recently published first authorship papers in Ecological Engineering (Vašek et al. 2013) and PloS ONE (Šmejkal et al. in press) are demonstrating the turn. Absence of in house molecular techniques.

Opportunities: Implementation of modern state-of-the-art monitoring-sampling-processing methods (3D positioning system, molecular genetics, stable isotopes etc.) and intensification of data obtained during surveying on commercial basis for use in scientific publications. Monitoring and tailoring fish communities of post mining lakes from the very beginning.

Threats: High need for full time position of a statistician with an excellent knowledge of mathematical modelling and ability to process and work with large datasets (this position is currently, but most probably only temporarily, occupied by postdoctoral fellow Daniel Ricard). Low institutional support in project management (scouting, lobbying), which is essential particularly in wide ranging international projects.

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Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Aquatic microbial ecology

2.1 Major characteristics of the team 10, Aquatic Microbial Ecology (head Prof. K. Šimek)

Research of the team typically cover topics in limnoecology, microbial ecology and diversity. Our research approaches frequently combine methods of classical limnology, microbiology, and a broad array of molecular methods, i.e. the combination that is currently rather rare, labor-intensive, and also time-consuming. On the other hand, this seems to be the reason why our publications received considerable attention of scientific society which is well reflected in citation rate, being far above typical values for most of ecological disciplines [**see Table 1 for scientometry**]. For example, two researchers in the age category 35–40 yrs have H-index 16 and 20, two senior researchers 24 and 27, and the department head has H-index 36.

Addressing important questions of contemporary aquatic microbial ecology by the team members has got high international reputation. Especially esteemed are our state of the art experimental designs, with microbial communities manipulated by altering predation pressure and resource levels, combined with molecular or fluorescence microscopy approaches detecting activities and identity of particular microbes *in situ*. Thus it is not surprising that **44% of our papers** are co-authored by foreign scientists [**part 2.3**].

During the last five years, our research scope was extended by implementing a broad array of molecular techniques, by the development of innovative methods for estimating microbial community structure and activity, tools for genome analysis of several groups of Betaproteobacteria and Cyanobacteria, methods for detecting cyanobacterial secondary products and toxins, and the assessment of activities of single bacterial and algal cells that combines fluorescence microcopy and image analysis. Further, new isolation and cultivation approaches have been introduced and combined with molecular taxonomy of both bacteria and Cyanobacteria, addressing issues of their spatial and temporal distributions, toxicity, and biogeography. **Major results of the team are summarized in detail in part 2.4.**

To sum up, **team 10** is an internationally distinguished, effective, and reasonably age-structured research unit of HBI [**Table 2**], with sound scientific performance and well-focused research outcome [**parts 2.3 & 2.4**], ambitious future research lines [**see part 3, "Research plans"**]; with high success rate in project funding [**part 2.2.3**], and a broad network of international collaborations with leading institutions in our research area [**see 2.5.1**]. These statements are also supported by many international awards given namely to the young team members [**2.5.2**] which is very promising for the future team development.

2.2.1 Table 1: Basic scientometric data of the team members *(Core collection, WOS)*

NAME	PhD defense year	Total IF papers	Total WOS citations	H-index	SUM IF	Researcher ID
Komárková Jaroslava	1972	50	707	18	13.3	G-1149-2014
Šimek Karel	1986	100	3531	36	66.4	F-8930-2014
Macek Miroslav	1987	42	644	13	8.6	B-5428-2015
Vrba Jaroslav	1989	71	1613	24	36.8	M-3780-2013
Nedoma Jiří	1991	75	2343	27	33.5	F-9063-2014
Řeháková Klára	2002	22	571	13	23.5	B-2143-2012
Znachor Petr	2003	23	209	10	20.9	F-8901-2014
Horňák Karel	2006	30	856	16	48.5	B-5664-2015
Jezbera Jan	2006	36	798	20	50.2	B-5657-2015
Jezberová Jitka	2006	19	226	10	41.8	G-1024-2014
Salcher Michaela	2008	17	264	11	70.0	A-1141-2013
Kozlíková Eliška	2008	23	283	11	21.3	F-9664-2014
Kasalický Vojtěch	2012	12	168	7	40.3	F-8578-2014
Sirová Dagmar	2012	14	170	6	27.7	F-8239-2014
Shabarova Tanja	2013	7	77	3	23.0	B-5327-2015
Rychtecký Pavel	2014	4	13	2	11.6	B-5621-2008
Mareš Jan	PhD student	18	76	4	37.6	B-2395-2009
Čapková Kateřina	PhD student	5	17	2	16.0	M-1430-2014
Grujčić Vesna	PhD student	0	0	0	0.0	B-5976-2015
Hájek Jan	PhD student	1	0	1	3.5	B-5562-2015
Kust Andreja	PhD student	0	0	0	0.0	B-4816-2015
Matoušů Anna	PhD student	0	0	0	0.0	B-5996-2015

Total IF papers – a sum of papers with impact factor; SUM IF – a sum of impact factors over the 2010–2014 period

2.2.2 Age structure, clearly dominated by young researchers, is reflected in increasing:

(a) Proportions of the young researchers (<32 years, e.g. six PhD students) and those from the category of <35–40 years that become principal investigators of the newly awarded projects (see the complete list the projects in the **attachment 3–1** and a short summary comments in part **2.2.3**);

(b) Number of papers published in high rank journals [see parts **2.3 and** References **in part 6**];

(c) Overall citation impact of the publications of the team members [**Table 1**];

(d) Proportions of the young researchers from abroad, currently two PhD students are from **Croatia (A. Kust & V. Grujčić , 26 years)** and two excellent postdoctoral fellows came from the **University of Zurich, Switzerland (M. Salcher & T. Shabarova, 37 & 35 years, respectively).**

Table 2. Age structure of members of the whole research team including all PhD students not listed in the 1st phase of the evaluation *(the data relate 31 December 2014, including researchers working >3 years in the team over the 2010–2014 period).*

Age category	25–30	30–35	35–40	40–45	45–50	50–55	55–60	60–65	65–70	> 70
# of members	4	6	5 *	2	0	0	4	0	0	1

* **Changes in the age category, 35–40 years:** On 1 January 2013, K. Horňák got a full postdoc position at the University of Zurich. A core young team member, J. Jezbera, unfortunately passed away on 26 November, 2013.

2.2.3 Projects awarded

The team members at the position of principal investigators (PI) got 11 standard projects of the Czech Science Foundation (CSF) and **one international EEF project** over the evaluated period, **giving a sum of ~ 35.00 millions CZK**, equals to **~ 1.294 millions EUR**.

Representative examples of five most significant projects:

2008–2012, **PI: K. Šimek** (Reg. code: CSF 206/08/0015): "Ecophysiological traits and grazing- and virus-induced mortality of bacterial strains representing major bacterioplankton groups in a reservoir"; **4.989 millions CZK**

2010–2013, **CZ-PI: J. Jezbera** (Reg. code EEF/10/E011): "Functional role and ecotype divergence in Actinobacteria of the AcI lineage", a part of international project FREDI, "Functional Role and Ecotype DIvergence in freshwater ultramicrobacteria" (09-EuroEEFG-FP013), coordinated by J. Pernthaler (University of Zurich, Switzerland); **3.159 millions CZK.**

2011–2014, **PI: J. Nedoma** (Reg. code: CSF P504/11/2182): "Phytoplankton release of dissolved organic carbon and its relationship to bacterioplankton composition"; **5.606 millions CZK**

2014–2016, PI: **E. Zapomělová (**Reg. code: CSF 14-18067S): "Toxic potential, evolution of toxin synthesis, and factors driving anatoxin-a production in benthic and soil nostocacean cyanobacteria"; **6.312 millions CZK**

2013–2017, **PI: K. Šimek** (Reg. code CSF 13-00243S): "Unveiling life strategies of important groups of planktonic *Betaproteobacteria* in relationship to carbon flow to higher trophic levels"; **11.405 millions CZK**

Additional funding (a sum of ca 5 millions of CZK = ~ 0.190 millions EUR) was also provided by e.g. (i) the **Postdok_BIOGLOBE project** (CZ.1.07/2.3.00/30.0032), co-financed by the European Social Fund and the state budget of the Czech Republic; (ii) a project financed by the **Ministry of Education, Youth and Sports of CR (**CZ.1.07/2.4.00/17.0130**);** (iii) and a project **of the Ministry of Environment of CR** (ID-code: 05611212); for details see the complete project list in the **attachment 3–1**.

In summary, the project funding accounted for ≥ 50% of all funding of the team 10.

2.3 Comments on publication outcome of the team during the period 2010–2014

The team members authored or co-authored **68 papers** in journals with impact factor:

- **35 papers** (52%) with the first author from our team.
- **33 papers** (48%) with the first author not affiliated to our team.
- **Out of total 68 papers** (for the complete list see the attachment), **30 papers (44%)** were co-authored by colleagues from abroad (see **part. 2.5.1**; "*foreign collaborators"*).
- **Out of the total 68 papers, 31 papers** were published **in the core journals** in the field of microbial ecology and limnoecology, as exemplified below:

- ISME Journal (1×, IF = 9.267); Environmental Microbiology (5×, IF = 6.24); Applied and Environmental Microbiology (3×, IF = 3.952); FEMS Microbiology Ecology (3×, IF = 3.785); Protist (1×, IF = 3.558); International Journal of Systematic and Evolutionary Microbiology (3×, IF = 2.798);
- Limnology and Oceanography (2×, IF = 3.615); Freshwater Biology (1×, IF = 2.905); Ecosystems (1×, IF = 3.531); Biogeosciences (1×, IF = 3.753); Plos ONE (7×, IF =3.534); Journal of Plankton Research (2×, IF = 2.263); Toxicon (1×, IF = 2.581).

2.3.1 Notes regarding the 1st phase of the evaluation

For the 1st phase of the evaluation we selected 16 papers with the first authors exclusively from our team, thus the team members suggested the core ideas and hypotheses of these articles and wrote the papers. The selected articles thus represented an overview of the activities based on original ideas of the team members, which may not necessarily correspond to the publications of the team in journals with the highest impact factor.

Notably the originality of the papers was thus superimposed to simple scientometric parameters such as impact factors of the journals.

2.4 The most important research areas and publication outcome of the team

2.4.1 Factors shaping population dynamics and mortality rates of key bacterioplankton groups and their role in carbon flow to microbial food webs

A broad array of bottom-up and top-down factors shape population dynamics of particular bacterioplankton groups and their role in carbon flow to the grazer food chain. Our studies clearly documented that: (i) bacteria from the genus *Limnohabitans* belong to the key bacterial groups abundant in circum neutral or alkaline lakes (Šimek et al. 2010b); (ii) they display high growth rates and metabolic flexibility, with a tight relationship to algal-derived organic substances and algal exudates (Šimek et al. 2011b); (iii) their high growth potential is counterbalanced by a marked vulnerability to protistan grazing (Šimek et al. 2013, 2014). These ecological traits (see also Kasalický et al. 2013, **part 2.4.3**), make this bacterial group an invaluable model for testing niche separation between closely related strains and their role in carbon flow to higher trophic levels.

For instance, we conducted a comprehensive study focused on niche separation between two coexisting *Limnohabitans* strains. Interactions between these strains, a flagellate predator, and viruses have been examined, suggesting contrasting eco-physiological capabilities and vulnerability to the different sources of mortality between the two closely related ecotypes (Šimek et al. 2010a). The knowledge of major ecological traits in *Limnohabitans* strains also allowed us to conduct a pilot study that convincingly demonstrated strong prey-specific effects of closely related *Limnohabitans* bacteria on the flagellate predator community, as characterized by pyrosequencing. This ecological aspect has been debated for a long time without any direct evidence from natural flagellate communities (Šimek et al. 2013; **Featured Article in ISME Journal, IF = 9.267**). In our experiments, we have designed a novel experimental approach and have contributed significantly to the identification of bacterial strains that are important in the carbon flow to the grazer food chain, as well as predator flagellate groups that are key players in the fluxes.

In a study with high temporal resolution, we analyzed interactions between the major bottom-up and top-down factors modulating the dynamics of particular bacterial lineages from Betaproteobacteria, Flavobacteria, Actinobacteria and their role in carbon flow in food webs of a freshwater reservoir. The results contributed to overcoming an obvious paradox in current microbial ecology: although novel molecular techniques have provided insights to bacterioplankton composition, general knowledge on the growth and loss rates of particular bacterial groups *in situ* is still quite scarce (Šimek et al. 2014) and in need of further study.

Our group was in charge of designing experiments, sampling and processing of samples by microbiological and molecular methods. Sequencing and bioinformatic data related to

flagellate community composition were analyzed in collaboration with the group of J. Boenigk (Essen-Duisburg, Germany). M. Hahn (Mondsee, Austria) assisted with sampling of Austrian lakes and provided several bacterial isolates. M. Weinbauer (Villefranche, France) evaluated virus effect on two closely related *Limnohabitans* strains.

2.4.2 Temporal and spatial distribution of major Betaproteobacterial subgroups and local species adaptations in a broad array of freshwater habitats

We studied the distribution and abundance patterns of 3 genera from Betaproteobacteria – *Limnohabitans* (the R-BT065 lineage), *Polynucleobacter necessarius*, and *Methylophilus* in a large set of limnologically diverse European freshwater systems (i.e. 121–161 habitats; Jezbera et al. 2011, 2012, 2013; Jezberová et al. 2010, Šimek et al. 2010b). Our findings emphasize that at least two genera of Betaproteobacteria, *Polynucleobacter* and *Limnohabitans,* represent ecologically diversified groups with a cosmopolitan distribution and a ubiquitous occurrence in lentic freshwater habitats. They coexisted in majority of the habitats but showed contrasting abundance patterns along the pH gradient of the habitats (pH 3.8–8.5). Environmental factors influencing their distribution and composition appeared to be pH, conductivity, the amount and nature of dissolved organic carbon, oxygen, and altitude. The observed distribution patterns could be explained by different preferences for substrate sources, that is, those prevailing in humic substances-rich acidic brown-colored waters compared to autochthonously-produced algal-derived substances prevailing in circum neutral or alkaline waters. Thus, the ecological diversification turned out to be the main reason for distribution patterns of the *Polynucleobacter* and *Limnohabitans* groups across lentic freshwater habitats. However, we found that the ubiquity of both groups could be only explained by the detailed population analysis of both genera.

The microdiversity of each of the two groups was further studied by the cultivation-independent detection method, Reverse Line Blot Hybridization, with newly designed probes specific for different subgroups within the genus *Limnohabitans* and *Polynucleobacter* (Jezbera et al. 2011, 2013). In some cases differences in environmental preferences resulted in complete niche separation between the probe-defined subgroups. The *Limnohabitans* microdiversity increased significantly along the gradient of rising pH of habitats and these bacteria showed preference for circum neutral habitats. Interestingly, similar distribution patterns were revealed for *Limnohabitans* and *Polynucleobacter acidiphobus*/*difficilis* phylotypes, suggesting similar ecological adaptations of these distantly related taxa (Jezbera et al. 2012). Furthermore, the genotype composition of *Polynucleobacter* genus in a small pond was studied on the genomic basis (Hahn et al. 2012). Surprisingly, only a low degree of genetic diversification and a very passive lifestyle of the populations were revealed. Moreover, such a lifestyle appeared to be successful in a highly dynamic and nutrient-richer shallow pond undergoing complete mixis and pronounced stratification in diurnal cycles.

Our group was in charge of designing experiments, sampling and processing of samples by microbiological and molecular methods. M. Hahn (Mondsee, Austria) assisted with sampling of Austrian lakes, phylogenetic analysis of sequence data and conducted genome analysis of *Polynucleobacter* bacteria.

2.4.3 Isolation, ecophysiology, and genomic characterization of freshwater bacteria

We isolated and characterized bacterial strains from ecologically highly relevant groups of freshwater Betaproteobacteria, which have been mostly investigated by our team using

culture-independent approaches (Šimek et al. 2010b, Jezbera et al. 2012). Newly isolated strains belonged to the previously uncultured R-BT065 cluster (Kasalický et al. 2013). It allowed us to establish a new genus *Limnohabitans* (Comamonadaceae, Betaproteobacteria) with four species, described according to the bacteriological code (Hahn et al. 2010a, b, Kasalický et al. 2010). The low variability of 16S rRNA gene within the genus highly contrasted with their very different metabolic profiling. Therefore, we used a more variable ITS1 sequence to obtain a more robust phylogenetic marker. Based on this new marker, we proposed a completely new system for this genus, with five main lineages and six subgroups (Kasalický et al. 2013), that has been widely accepted in the most recent literature. The metabolic differences of tested strains suggested that each of these subgroups could represent a distinct taxonomic unit – "a species". Thus, the group is highly diversified; however its diversity is usually underestimated by the cultivation-independent tools.

We also determined the genomic and ecophysiologic properties of the *Polynucleobacter* (Burkholderiaceae, Betaproteobacteria) strain isolated from a population (F10 lineage) of a small acidic pond in the Alps (Hahn et al. 2012). We discovered a relatively small number of genes involved in transduction of environmental signals and the lack of motility and quorum sensing. The sequenced strain possessed an evolutionary streamlined genome, interestingly so far only known among free-living bacteria from pelagic marine taxa.

The isolation of new strains and their physiological characterizations enabled us to study the life strategies of ecologically relevant members of freshwater bacterioplankton (**part 2.4.1**). Moreover, it enabled the design of new probes for microdiversity studies of both investigated groups (**part 2.4.2**). The diversity and function of aquatic bacteria is still an understudied topic because only < 5% of the total bacterial genotypes have been isolated and characterized. We conclude that considering only higher taxonomical units would lead to wrong conclusions on the generalist ecology of these bacteria. Certain caution should be exercised when making generalizations based only on the ecology and adaptation of one investigated lineage.

Our team designed experiments, isolated the majority of new strains from the genus *Limnohabitans* and V. Kasalický wrote two papers. M. Hahn in collaboration with our group isolated and described two species of *Limnohabitans,* coordinated the research of the F10 lineage and assisted with sampling campaigns on a large number of Austrian lakes.

2.4.4 Examination of physiological status and activity of single microbial cells by combining fluorescence microscopy and digital image analysis

A typical hallmark of many of our studies is the application of fluorescence dyes and probes in studying ecophysiology and activity of single microbial cells, which is one of the modern trends in microbial ecology. Using unique methods developed in our laboratory, based on fluorescence microscopy coupled with image analysis, we are able to employ these probes for not only tagging labeled cells but also for quantitative estimation of processes and cellular activities by measuring cell-associated fluorescence intensity. We demonstrated very good comparability of our rapid method with the time-consuming and sophisticated 3D-deconvolution microscopy and with flow cytometry (Diaz de Quijano et al. 2014).

We used the fluorochrome PDMPO, a tracer of silica deposition, as a proxy for diatom growth. Diatoms are important components of both freshwater and marine phytoplankton. Znachor et al. (2013) showed close relationship between PDMPO fluorescence (silification rate) and diatom growth rate in several important freshwater diatoms *in-situ*. Using the PDMPO assay, Znachor et al. (2012) also studied environmental factors affecting bacterial colonization of

diatoms at both single cell and population levels, and the importance of organic carbon utilization in diatom nutrition (Znachor et al. 2011, Znachor & Nedoma 2010).

To study cell death in phytoplankton, which represents important but often neglected loss process in phytoplankton, we used a membrane permeability probe SYTOX Green. Rychtecký et al. (2014) clearly showed that the importance of cell death, both within a particular taxon and in whole communities, varies both spatially and temporally, and that coexisting taxa differ in their responses to environmental stressors.

The Fluorescently Labelled Enzyme Activity (FLEA) assay for cell-specific extracellular phosphatase activity belongs to one of the core lines of our research. Novotná et al. (2010) showed substantial differences in the *in-situ* activities of several dinoflagellate species in different lakes, not agreeing with the whole-system phosphorus availability indices. Rychtecký et al. (2015) measured surprisingly low phosphatase activity of dominant taxa in phosphorus-deficient natural freshwater phytoplankton. Both studies suggest the effect of light availability on phosphatase production.

A rare combination of direct analysis of FISH (Fluorescence In Situ Hybridization)-targeted bacteria in food vacuoles of heterotrophic flagellates with measurements of cell volumes of these flagellates and bacteria indicated rapid adaptations of flagellate size-structure to the changing size of prevailing bacterial prey taxa with important implications for carbon flow through freshwater microbial food webs (Šimek et al. 2014).

2.4.5 Reservoir spatial heterogeneity and phytoplankton dynamics

Man-made reservoirs created by damming the original river valleys represent a transition between lotic and lentic systems, with pronounced longitudinal gradients in various physical, chemical, and biological parameters. It might seem that there is only little space for further research in reservoir limnology; however, classical methods in combination with an intensive sampling program allowed us to address reservoir spatial heterogeneity and phytoplankton dynamics.

In Rychtecký et al. (2011), we used functional classification of phytoplankton to estimate their spatial and temporal heterogeneity in the Římov reservoir under contrasting hydrological scenarios. Our study also emphasized the importance of having an intensive phytoplankton monitoring program, which allows for detecting severe consequences of sudden flood events on phytoplankton distribution.

Znachor et al. (2013) conducted a detailed study of phytoplankton (diatoms, in particular) seasonal dynamics at two distinct sites along the longitudinal profile of the Římov reservoir: (a) a nutrient-depleted lacustrine zone near the dam and (b) a nutrient-rich transition zone upstream near the river inflow. We clearly showed that seasonal variation in diatom growth was driven by daily light exposure, pointing out the importance of both seasonal fluctuations of day length and weather conditions. Despite virtually identical diatom biomass along the reservoir, diatom growth was highest at the transition zone, underlining the fact that growth and loss processes in phytoplankton may run at different rates along the longitudinal profile of a canyon-shaped reservoir.

To interpret our findings in a much broader context, we studied the effect of river water quality, inflow rate, and temperature on planktonic food web composition and activities in the eutrophic Sau reservoir in Catalonia, Spain (Šimek et al. 2011a). Based on the distance of a sampling station from the river inflow, we developed the relative distance model generally applicable to any canyon-shaped reservoir and allowing comparison of the biological

longitudinal gradients under seasonally fluctuating water levels and different types of water circulation patterns.

2.4.6 Polyphasic taxonomy and secondary metabolite production in bloom-forming heterocytous Cyanobacteria

The research team lead by E. Kozlíková (Zapomělová) has achieved significant progress in revising the taxonomy of several genera of heterocytous Cyanobacteria, which frequently form harmful cyanobacterial blooms. Taxonomic classification of Cyanobacteria has undergone extensive restructuring with the advent of phylogenetic analyses based on molecular sequence data. The polyphasic approach, establishing monophyletic taxa characterized by a combination of unique phenotypic traits, has proven to be the most plausible and useful way of understanding the cyanobacterial diversity. Despite intensive study in the recent years, evolutionary reconstruction and systematic revision in the important group of bloom-forming nitrogen-fixing (heterocytous) Cyanobacteria is still in progress. In the systematic studies accomplished by our team, we combined phylogenetic reconstruction with extensive morphological examination based on both cultured strains and environmental samples. These analyses were performed completely by the members of our team (E. Kozlíková, K. Řeháková, J. Jezberová, J. Komárková, J. Hájek and J. Mareš). From the taxonomic point of view, our analyses yielded two new genera (*Chrysosporum* – Zapomělová et al. 2012; *Sphaerospermopsis* – Zapomělová et al. 2010), and substantially improved our knowledge on the genera *Dolichospermum* (Zapomělová et al. 2010, 2011) and *Nodularia* (Řeháková et al. 2014).

As another part of polyphasic characterization, we assessed the production of cyanotoxins and other secondary metabolites (fatty acids, oligopeptides, lipopeptides) in several important taxa (Zapomělová et al. 2011, Řeháková et al. 2014, Mareš et al. 2014) in collaboration with the Institute of Microbiology CAS, Třeboň. Present studies have pursued our long-term scope of polyphasic evaluation in cyanobacterial groups important for water management, particularly in freshwater reservoirs.

2.4.7 Probing plant–microbe interactions using rootless aquatic carnivorous plants as model species

The plant–microbe interactions group combines ecological approaches with molecular tools to study complex microbial communities in association with plant hosts and its influence on plant ecophysiology and evolution. A model rootless aquatic carnivorous plant species from the genus *Utricularia* with minimal genome and its associated microbiome are used in both laboratory experiments and field studies. The system represents a unique opportunity to focus on problems that are extremely difficult to study using rooted plants, due to the high complexity of the soil environment.

The multidisciplinary research is conducted in collaboration with University of South Bohemia, Institute of Experimental Botany CAS, Institute of Botany CAS, and University of California, Davis. Our group is in charge of experimental design and execution, plant ecophysiology, and microbial metagenomics and metatranscriptomics. We asses plant photosynthate composition, allocation, and subsequent utilization by microorganisms (Sirová et al. 2010, 2011), and *Utricularia* microbiome structure and function in relation to plant host and its nutrient acquisition (Sirová et al. 2014).

2.5 Supplemental information: Foreign collaborators, awards and SWOT analysis

2.5.1 Ten major foreign collaborators and coauthors of our publications during 2010-2014

- **Doc. M. Hahn,** Research Institute for Limnology, University of Innsbruck, Mondsee, Austria
- **Doc. T. Posch, Prof. J. Pernthaler & Dr. J. Blom,** Limnological Station, Institute of Plant Biology, University of Zurich, Switzerland
- **Drs. M. Weinbauer & J. Dolan,** CNRS, LOV, Observatoire océanologique Villefranche/mer, France
- **Prof. J. Boenigk & Mgr. J. Nuy,** General Botany, University of Duisburg-Essen, Germany
- **Prof. H. P. Grossart,** Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Germany
- **Dr. S. M. Owens**, Institute of Genomics and Systems Biology, Argonne National Laboratory, Argonne, and Computation Institute, University of Chicago, Chicago, IL, USA
- **Dr. R. Schäufele,** Department of Grassland Study, Technical University of Munich, Germany
- **Prof. J. R. Johansen,** John Carrol University, Cleveland, Ohio, USA
- **Prof. E. Rejmánková,** Dept. of Environmental Science and Policy, Univ. California, Davis, USA
- **Dr. I. Bussmann**, AWI Helmholtz Centre for Polar and Marine Research, Germany

2.5.2 International awards of the team members:

- June 2010 – **K. Šimek** received Doctorate Honoris Causa of Limnology from the University of Clermont Ferrand, France, on the basis of his outstanding publication and educational results.

Important awards of young scientists from the team:

- July 2011 – **Sirová D.,** Title: Hunters or gardeners? Plant–microbe interactions in rootless aquatic *Utricularia*. Joint Meeting of Society of Wetland Scientists, WETPOL and Wetland Biogeochemistry Symposium, Praha (Oral presentation, Best Student Contribution award)
- August 2012 – **M. Salcher** received the 'Tom Brock Young Postdoctoral Award' for the most innovative research by an early career scientist awarded by the ISME (International Society for Microbial Ecology), Copenhagen, Denmark
- August 2013 – **V. Kasalický** – Winner of the Poster session at SAME-13, Symposium on Aquatic Microbial Ecology, Warnemünde, Germany
- 2014 – **D. Sirová**, Winner of the L'Oréal – UNESCO Award for Women in Science, Czech Republic
- November 2014 – **T. Shabarova** – Recognition award for the PhD thesis given by the Swiss Foundation of Limnology and Hydrobiology, Friburg, Switzerland

2.5.3 SWOT analysis of the team

Strengths: well-established network of international collaboration, combination of classical limnological approaches with modern fluorescence and molecular techniques, reasonable team age structure with a high proportion of young scientists, currently even dominated by young and very promising female scientists.

Weaknesses: lack of a qualified specialist in e.g. bioinformatics, ineffective recruitment of students, poor knowledge dissemination towards public, missing age classes from 45–55 years.

Opportunities: long-term data analyses, increasing multi-disciplinarity of our research, new analytical infrastructure available, allocated funds for a new bioinformatics position.

Threats: increasing and time-consuming bureaucracy distracting scientists from their primary research goals.

2.6 References related to part 2.4 (the team members **in bold)**

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Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Hydrochemistry and Ecosystem Modelling

2.1 Characteristics of the Team 11, Hydrochemistry and Ecosystem Modelling (head Prof. J. Hejzlar)

Research of our team typically covers topics of biogeochemical cycles and environmental processes that control composition and quality of surface waters. The range of activities during the evaluation period was interdisciplinary but mainly focused on structure, functions, problems and management of aquatic environments like lakes or artificial reservoirs, and their catchments. The staff also provided lecturing on aquatic and environmental sciences at the University of South Bohemia, České Budějovice (USB), and was involved in advisory services on water quality and aquatic ecosystem functioning for water policy and water management.

The scientific team members:

Josef Hejzlar (58), senior scientist, associate professor at USB – lake and reservoir limnology, phosphorus cycling, eutrophication, modelling of nutrient export from catchment, hydrodynamics and water quality in reservoirs. Author or co-author of 230 scientific (64 SCI) papers, ~1,060 citations, h-index: 23.

Jiří Kopáček (58), senior scientist, full professor at USB – nutrient (C, N, P) cycling in mountain catchment-lake ecosystems, recovery of aquatic and terrestrial ecosystems from acidification, long-term trends in air and water pollution, impact of climate change on water and element fluxes. Author or co-author of 160 scientific (103 SCI) papers, ~1,950 citations, h-index: 26.

Petr Porcal (40) scientist, assistant professor at USB – hydrochemistry, fotochemical processes in surface water, characterization of dissolved organic matter. Author or co-author of 31 scientific (22 SCI) papers, ~300 citations, h-index: 10.

Jakub Borovec (41) scientist, assistant professor at USB – P cycling in freshwater ecosystems, chemistry (C, N, and P) of sediments, processes at the sediment/water interface. Author or co-author of 60 scientific (16 SCI) papers, ~240 citations, h-index: 8.

Jiří Kaňa (37) associated scientist, assistant professor at USB – aluminum, iron, and phosphorus cycling in mountain catchment-lake ecosystems, soil nutrient pools, soil-water interactions. Author or co-author of 20 scientific (13 SCI) papers, ~100 citations, h-index: 6.

Martina Čtvrtlíková (35) postdoctoral fellow – ecology of isoetids, reproduction and phenology of *Isoëtes* in acidified lakes, ecotoxicology, mycorrhizal symbiosis in isoetids. Author or co-author of 7 scientific (6 SCI) papers, ~30 citations, h-index: 4.

The HEM team included also seven skilled technical assistants (full-time employment) and two PhD students (part-time employment) – **Monika Krolová** (ecology of macrophytes in reservoirs) and **Jiří Jan** (phosphorus chemistry in sediments).

2.2 Publication outcome of the team during the period 2010-2014

The team members were authors or co-authors of 46 papers in journals with impact factor:

- 23 papers (50%) were with the first author from out team.
- 23 papers (50%) were with the first author not affiliated to our team.
- Of the total of 46 papers, 20 papers (43%) were authored or co-authored by colleagues from abroad.

- Of the total of 46 papers, 27 papers (59%) were published in the core journals (i.e. journals in the 1st quartile of JRC categories) in the fields of environmental sciences, geosciences, limnology, environmental engineering, water resources, soil sciences, plant sciences, ecology, and multidiscipline sciences as exemplified below:

  Environmental Science and Technology (2×, IF = 5.481); Journal of Experimental Botany (1×, IF= 5.364); Water Research (1×, IF = 4.865); Applied and Environmental Microbiology (1×, IF = 3.952); FEMS Microbiology Ecology (1×, IF = 3.785); Biogeosciences (1×, IF = 3.754); Environmental Pollution (1×, IF = 3.746); Soil Biology and Biochemistry (2×, IF= 3.654); PloS One (2×, IF = 3.534); Biogeochemistry (5×, IF=3.531); Atmospheric Environment (1×, IF = 3.465); Limnology and Oceanography (1×, IF= 3.416); Annals of Botany (1×, 3.298); Science of Total Environment (1×, IF = 3.258); Ambio (2×, IF = 2.973); Functional Plant Biology (1×, IF= 2.929); Preslia (2×, IF = 2.778); Aquatic Sciences (1×, IF= 2.602).

2.3 The most important research areas and publications of the team

2.3.1. Revealing key factors of catchment impacts on surface water quality

A new method of balance analysis and modelling of pollutants in the catchment runoff allowed quantifying how natural and anthropogenic sources contributed to concentrations of N [30, 31], S [34], and Cl [38] compounds in the Vltava River during 1900–2010. It appears that water quality is affected not only by direct inputs of pollutants into streams, but also chains of hydrological and biochemical processes in the soil and rivers caused by agriculture, soil drainage, urbanization and construction of reservoirs. The modelling setup included also a predictive model of atmospheric deposition of S and N [17]. This work was done basically by the team members with participation of a modelling specialist from the National Institute for Public Health and the Environment, Netherlands.

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

We studied natural processes of element cycling and terrestrial losses of nutrients (N, P), base cations, and Al from both natural undisturbed forests and forest dying after bark beetle outbreak [5, 8, 13, 24, 25], and their effects on biodiversity of receiving waters [36]. Concentrations and fluxes of ecologically important elements were measured in litter fall [5] and throughfall deposition [13]. Fluxes of litter and associated elements increased by one order of magnitude for several years after the bark beetle infestation [5], but throughfall deposition changed surprisingly slowly during first 5 years after the forest dieback [13].These studies were mostly done by team members. Forest dieback and elevated litter fall affected chemistry of the uppermost soil horizons. Decomposition of litter and reduced nutrient uptake by trees resulted in a steep increase in soil concentrations of soluble N (NH_4-N, organic-bound N), P forms, and elevated soil concentrations of Ca^{2+}, Mg^{2+} and K^+ (which replaced Al^{3+} and H^+ ions from the soil sorption complex) in the disturbed soils. Consequently, soil concentrations of exchangeable Al^{3+} and H^+ decreased 66% and 50%, respectively, and soil base saturation increased from 40% to 70% [24]. The forest defoliation resulted in elevated N availability in soils. Moreover, N liberated by decomposition of fresh litter exceeded N demand of soil microorganisms. Both processes contributed to elevated fluxes of NO_3-N to waters [8]. Laboratory experiments with soils emphasized the role of microbial N immobilization in preventing NO_3-N losses from N-saturated ecosystems as a function of C availability [25]. These studies were done in a close collaboration of team members with USB. The effect of changing water chemistry after forest dieback on water biodiversity was studied at lake Rachelsee (Germany) [36]. This study was done in collaboration with Team 10 (BC-HBI, Aquatic Microbial Ecology).

2.3.3 Nutrient (P, N, and DOC) leaching from soil to surface water

In this work package, we studied processes responsible for P loading of lakes from soil [9,10] and atmospheric [10] sources, modelled NO_3^- [19] and DOC [42] leaching, and proposed a novel hypothesis on N-saturation of catchments, linking N, organic carbon, and sulphur cycling

in the terrestrial ecosystem [42]. We studied how topographical and morphological characteristics of catchment–lake systems affect in-lake nutrient structures in remote mountain areas [10] and effects of soil composition and acidity to retain/release P in alpine catchments [9]. These studies were mostly done by the team members. Our team contributed with long-term chemical data on N fluxes to setting and verification of a new formulation of the acidification model MAGIC [19]. This new model version uses decomposer dynamics to link N cycling to carbon C turnover in soils. Our team also contributed to MAGIC modelling of DOC leaching. This study was done to more precisely reconstruct pre-industrial pH in stream waters [42]. The most important contribution of our team in this work package is a synthesis of processes involved in terrestrial N-cycling and its interactions with other C and S cycles in soils [32]. We showed that the dynamics of N cycling are intimately linked to the associated C and S cycles. The incorporating mechanistic links of N to the C and S cycles has a potential to improve biogeochemical models [32], as has been already shown by new version of MAGIC model, linking N and C cycling in soils [19]. This study was based on cooperation with USB and other institutions, with a leading role of our team.

2.3.4 Recovery from acidification

Study [11] reconstructs anthropogenic nitrogen emissions during the Holocene and estimates their possible effects on remote ecosystems. Our team had a leading role in this study, which was the first to track and estimate Nr emissions since the advent of human civilization. Studies [43], [45], and [47] in this work package are based on review, modelling, and long-term monitoring of soil and water chemistry. Study [47] reviews history of previous acidification research and provides a base to further political and socio-economical negotiations dealing with S and N emission controls. Study [43] shows that surface water recovery from acidification predicted in 2000 for the year 2010 is very close to the actual recovery observed from measured data in many recovering lake districts in the Northern Hemisphere. Although reductions in emissions of S and N compounds have led to dramatic improvements and recovery in water quality in acidified freshwater ecosystems, biological recovery has lagged and the problem will persist in many areas for next decades. Further reductions are required if the goal is to permit recovery of all impacted ecosystems [45]. Our team contributed to these studies with 25- to 30-year long records from the strongly atmospherically acidified Bohemian Forest and Tatra Mountain lake districts and with its experience with these most rapidly recovering ecosystems from acidification in the world.

2.3.5 Photochemistry of DOM

The effect of natural and simulated solar radiation on photochemical release of organically bound aluminum and iron from dissolved organic matter (DOM) was tested in three Maine streams [3]. Results showed significant dependence of DOM sensitivity to solar radiation on stream discharge. This dependence was further studied in a unique seasonal study aiming at changes in photochemical properties of DOM [27]. This study was the first one studying photochemical properties during the whole hydrologic year. Further details on seasonality of photodegradation of DOM were investigated in respect to organic nitrogen – as an important component of DOM. Obtained results were published in the first comprehensive study on seasonal photochemistry of nitrogen in a small forest stream and lake [44]. Major work on these studies was done by P. Porcal and other team members.

Extensive studies on seasonality aspects of DOM photochemistry were completed with laboratory studies aiming at different factors affecting photodegradation of DOM [26, 33, 35]. The study of photochemical production of particulate organic matter [26] was the first published study describing the mechanism of photochemically induced formation of particulate organic matter. P. Porcal did major work in studies [26, 35] and contributed to the study [33].

2.3.6 Phosphorus in sediments and biofilms

One of the key team research lines involves the study of phosphorus interactions with its binding partners in particles in different types of ecosystems or their parts (biofilms, seston, sediments). We developed, implemented, and tested method modifications which allow us to

trace P, Fe, and Al speciation in particulate matter (step by step fractionation). Owing to method miniaturization and improvements in sensitivity, we were the first to describe in detail diurnal changes in P biogeochemical cycling within cyanobacterial mats from the Belize marches (important biodiversity reservoirs) [6]. Further research development was focused on the discrimination between biogeochemically active minerals from the inert mineral background, which is essential for our better understanding of P cycling at the sediment/water interface [22]. Previously available, widely used procedures allowed for only coarse and inaccurate descriptions, while modern trends call for precise and detailed explanations of mechanisms that drive ecosystem processes. Our results, as contributions towards this direction in research, have also found their application in several paleolimnological studies [12, 23]. Several collaborating institutions collaborated with us in this research area, including the University of California, Davis, University of South Bohemia and University of Maine. Our team participated in hypothesis formulation, method selection and optimization, experimental design, results evaluation and interpretation, and manuscript preparation.

2.3.7 Life strategy of aquatic quillworts (Isoëtes) in acidified lakes

We studied life strategy of two quillworts, ***Isoëtes lacustris*** and *I. echinospora,* that represent submerged and highly specialised isoetid flora dominating oligotrophic softwater lakes in Europe. Despite their undisputed importance for ecosystem functioning, quillworts reproduction and ecological requirements have been poorly understood. In their postglacial refuges in Černé and Plešné lakes, both species survived a forty-year period of severe acidification, but failed to reproduce. We found out that temperature dependence of germination length of these species controls their vulnerability to seasonally high aluminium toxicity in acidified lakes [40, 48]. We revealed substantially distinct phenological pattern of these sympatric congeneric species using a unique combination of various approaches including germination experiments *in vitro* and *in situ*, SCUBA diving survey of population dynamics, and mathematic modelling. As ***Isoëtes*** species do not grow clonally, the relatively high minimum temperatures for germination, determined in our research, may set general limits for quillwort distribution along latitudinal and altitudinal gradients, and to particular depths. This study was done by M. Čtvrtlíková, the team member, with contribution of collaborators from Team 10 (BC-HBI, Aquatic Microbial Ecology), who participated in data analysis and writing of papers.

The long-term survival of both quillwort populations over the acidification period relies entirely on the resistance and long life-span of adult plants. Their vitality in nutrient poor environment might be additionally supported by symbiosis with arbuscular mycorrhizal fungi and dark septate endophytes discovered in the quillwort roots [15, 37, 49]. This research was initiated by M. Čtvrtlíková and was done in cooperation with specialist in the Institute of Botany CAS who focused on the incidence and diversity of the unique fungal communities and their role in survival of isoetids in acidified lakes.

2.3.8 The ecophysiology of rootless aquatic plants from the genus Utricularia

We have long-term successful multidisciplinary research collaboration with the Department of Ecosystem Biology, University of South Bohemia, where we focus on the chemical aspects of plant-microbe interactions using aquatic *Utricularia* as model species. Our contribution constituted approximately 50% of total research efforts conducted in collaboration with Institute of Botany (ASCR) and University of South Bohemia and involved optimization and miniaturization of analytical methods for the very specific high-resolution studies. We have assessed the allocation of plant primary production and plant exudation using stable isotope labelling – the results were featured in the Journal of Experimental Botany, one of the top journals in the field [1].

The stable-isotope approach was also employed in the recently published work [39] quantifying the importance of microbial dinitrogen fixation in the nutrition and ecology of aquatic plants. The third research line was based on the assessment of the stoichiometry of plant-microbe interactions, with relation the plant growth and ecology – this included the composition and bioavailability of plant-derived organic carbon [14] and the effects of changes in the

environmental parameters such as light and nutrient availability [14, 20]. Ion chromatography and HPLC protocols have been developed and optimized for this purpose.

2.3.9 Effects of water level fluctuation on macrophytes in reservoirs

Lakes and reservoirs that are used for water storage and flow regulation have usually poorly developed littoral macrophyte communities, which impairs their ecological state and potential, respectively, in terms of the EU Water Framework Directive. The aim of our studies [18, 28] was to reveal controlling factors for the growth of littoral macrophytes in a representative of storage reservoirs with fluctuating water level (Lipno Reservoir, Czech Republic). Macrophytes occur in this reservoir only in the eulittoral zone i.e., the shoreline region between the highest and the lowest seasonal water levels, while submerged species typical for the infralittoral zone are missing. Three eulittoral sub-zones could be distinguished: the upper eulittoral with a stable community of perennial species of high cover, the middle eulittoral with high richness of emergent and amphibious species present at low cover values, and the lower eulittoral without permanent vegetation. Cover and species composition in the sub-zones were primarily influenced by the duration and timing of flooding, less by nutrient limitation and reducing conditions in the flooded organic sediment. Our results indicate ecological importance of eulittoral zone in reservoirs with highly fluctuating water level where submerged macrophytes cannot growth. It was also shown that the growth of eulittoral macrophytes can be supported by targeted management of water level, thus helping reservoir managers in improving the ecological potential of this type of water bodies.

This study was largely done by M. Krolová, the team member, during her PhD studies at the Department of Ecosystem Biology, University of South Bohemia, under supervision of J. Hejzlar, and with partial collaboration with a specialist in plant physiology from the University of South Bohemia.

2.3.10 Hydrochemistry analyses as support for ecological studies of other teams

Our team provided background chemical data and limnological expertise for several types of studies conducted with collaboration with other leading teams (Teams 9 and 10 (BC-HBI), team of the Department of Ecosystem Biology USB, and team from the Charles University in Prague):

Geographical distribution of recently defined aquatic bacterial group of the Betaproteobacterial Genus *Limnohabitans* and characterisation of typical conditions of its occurrence was studied in a wide range of lakes and reservoirs in central Europe [2].

Studies on reproduction biology and diel migration of juvenile perch *Perca fluviatilis* of the Team 9 (BC-HBI) [4, 16] were supported by our team via detailed characterisation of water chemistry and physical condition in the studied water bodies.

Study [29] describes the origin, bedrock geology, geomorphology, hydrological stability and physical and chemical characteristics of a representative set of 29 lakes in the ice-free parts of the Ulu Peninsula, James Ross Island (Antarctic Peninsula). We observed relationships between lake type and water chemistry. Bedrock, lake age and morphometry together with altitude were the most important factors responsible for the observed limnological variability. Our results further suggested possible nitrogen limitation in the lake ecosystems.

Study [7] on extracellular phosphatase activity in the Bohemian Forest lakes showed severe P deficiency of their plankton communities. Bioavailability of P substantially differed among the lakes due to differences in their P loading, as well as in concentrations of Al, and was accompanied by species-specific responses of phytoplankton.

Chemical data were provided for two studies on macrozoobenthos in atmospherically acidified Bohemian Forest streams and lakes [21, 41]. The biological recovery of these waters reflects (besides the levels of S and N deposition) on extent of forest disturbances, as well as on the dispersal ability of benthic organisms.

2.4 Projects

The team members were principal investigators or principal co-investigators of 14 research project grants of the European Commission (EC-FP7) (1×), Grant Agency of the Czech Academy of Sciences (GA-CAS) (1×), Czech Science Foundation (GACR) (6×), Norwegian Funds (MF-CR) (1×), Ministry of Education, Youth and Sports CR (MSMT) (2×), Ministry of Agriculture CR (MZE-CR) (1×), and State Environmental Fund CR (SEF-CR) (2×). The support received via research projects was in total EUR 1.2 million. List of major projects is as follows:

REFRESH - Adaptive Strategies to Mitigate the Impacts of Climate Change on European Freshwater Ecosystems – J. Hejzlar, EC-FP7: No. 244121, 2010–2014, 173,100 EUR.

What are the main mechanisms affecting N flow through soil N pools in N saturated mountain soils? – J. Kaňa, GA-CAS: No. KJB600960907, 2009–2011, EUR 22,000.

Controlling factors of phosphorus sorption in lake and reservoir sediments – J. Hejzlar, GACR: No. GA206/09/1764, 2009–2012, 115,400 EUR.

The integrated impact of climate change, air quality, and forest management on water ecosystem in headwater catchments – J. Kopáček, GACR: No. GA526/09/0567, 2009–2013, 66,700 EUR.

Effects of solar radiation on biogeochemical cycling of nutrients and metals in surface waters – P. Porcal, GACR: No. GAP503/12/0781, 2012–2014, 188,700 EUR.

The effect of natural dieback of mountain spruce forest on microclimate, chemistry, and biodiversity of terrestrial and aquatic ecosystems – J. Kopáček, GACR: No. GAP504/12/1218, 2012–2016, 173,100 EUR.

Functional diversity of soil microorganisms in spruce swamp forest and its effect on soil DOM – J. Borovec, GACR: No. GA13-17398S, 2013–2016, 54,300 EUR.

Disentangling the effects of changing environmental chemistry and climate on biogeochemistry and biodiversity of natural alpine soils and waters – J. Kopáček, GACR: No. GA14-09231S, 2014–2016, 64,700 EUR.

Evalution of the effects of the Gothenburg Protocol and acidified and eutrofied soils and waters in the CZ – J. Kopáček, MF-CR: No. FM EHP a CZ 0051, 2007–2011, 42,800 EUR.

Nutrient sources in catchments with complex land use and impacts on the aqautic ecosystems of reservoirs – J. Hejzlar, MSMT: No. OC08040, 2008–2010, EUR 6,500.

Adaptive Strategies to Mitigate the Impacts of Climate Change on European Freshwater Ecosystems – J. Hejzlar, MSMT: No. 7E11059, 2011–2014, 51,600 EUR.

The use of aeration technologies in the reduction cyanobacterial resting stages and nutrient bioavailability in reservoir sediments – J. Borovec, MZE-CR: No. QH81012, 2008–2011, 18,700 EUR.

Assessment of soil erosion and phosphorus loads causing eutrophication of stagnant water bodies – J. Hejzlar, MZE-CR: No. QI102A265, 2010–2013, 84,500 EUR.

The method for evaluation of the ecological potential of heavily modified and artificial standing waterbodies – J. Borovec, SEF-CR: No. 05611212, 2012–2014, 37,200 EUR.

2.5 Application research and consultancies

The team members were involved in applied collaborative research and gave expertise to several Czech River Basin Authorities (Vltava, Morava, Ohře) and the Czech Ministry of Environment in the field of management of water quality and sources of pollutions in reservoirs. Total income from these activities was EUR 0.45 million.

The balance of phosphorus and nitrogen sources in the catchment of Orlík Reservoir. A study for the Vltava River Authorities, Prague – J. Hejzlar, 2009–2010, 27,700 EUR.

Analysis of sources of phosphorus and nitrogen in the river basin Lomnice (the Otava River basin). A study for the Vltava River Authorities, Prague – J. Hejzlar, 2009–2010, 5,000 EUR.

The implementation of mitigation measures against eutrophication and cyanobacterial blooms at the Brno reservoir, phase I, 2009–2012, Part. C Monitoring. Collaborative research study for IMOS Brno, a.s. – J. Borovec, 2010, 190,900 EUR.

Principles of modelling inputs of nutrients to surface waters in order to identify particular pollution sources and inputs P and N in the catchment. A study for the Czech Ministry of Environment, Water Protection Department, Prague – J. Hejzlar, 2011, 4,900 EUR.

Problems of water quality and ecological potential of Lipno Reservoir in the period 1991-2012. A study for the Vltava River Authorities, Prague – Hejzlar J., 2012–2013, 4,700 EUR.

Methodology of assessment of the ecological potential of heavily modified water bodies and artificial water bodies – lake category. Certified methodology developed for the State Environmental Fund and the Ministry of Environment. – J. Borovec, 2012–2014, 37,200 EUR.

Impacts of intensive cage fish farming on water quality in the Nechranice reservoir. A study for the Ohře River Authorities, Chomutov – J. Hejzlar and J. Borovec, 2014, 7,046 EUR.

Investigation of the sediment washing processes for removal of contamination. Collaborative research study for CREA Hydro & Energy. – J. Borovec, 2013–2014, 178,000 EUR.

2.6 SWOT analysis of the team

The SWOT analysis was conducted by the scientific team members with results as follows:

Strengths

- Interdisciplinary knowledge on biogeochemistry of atmospheric, soil and water processes, enabling application of mathematical models to analyse and predict future development of aquatic ecosystems (principal strength);
- Ability to design and accomplish ecosystem analyses and manipulative experiments, including collaboration with other research teams (principal strength);
- Systematic build-up and evaluation of long-term water chemistry data on the studied aquatic ecosystems (reservoirs up to 55-yr, lakes >30-yr trends) that create a solid base for studies of aquatic ecosystem development (marginal strength);
- Ability to apply for successful projects at research grant agencies and application sphere (water authorities, water management institutions, and companies) (marginal strength);
- Development and operating of sensitive and reliable methods of water, sediment and soil analyses (marginal strength).

Weaknesses

- Fragmentation of research interests of the team members (principal weakness);
- High labour-intensity and high cost of studies based on long-term monitoring (marginal weakness);
- Modelling studies are not supported by specialized researchers (marginal weakness);
- Small use of data from applied research and expertise for scientific outputs (marginal weakness).

Opportunities

- Use and development of advanced analytical methods in ecosystem studies – ICP/MS, IRMS, HPLC, etc;
- Expanding our international and interdisciplinary collaborations with other teams;
- Employment of new perspective researchers;
- Expanding our research to behaviour of micropollutants in aquatic ecosystems.

Threats

- Vulnerability of the team due to a small number of team members and their small mutual substitutability;
- Possible unsuccessfulness in applications for projects maintaining the long-term ecological research;

- We will not find new perspective researchers.

Overall assessment and strategies for the future

The principal strength of the team is combined knowledge on atmospheric, soil and water processes enabling application of mathematical models to analyse and predict trends in aquatic ecosystems, and its abilities to conduct experimental studies. However, a continuation and future progress will be dependent on success in engagement of new perspective researchers, mainly for modelling and studies on biogeochemistry of ecosystems.

We will continue our research focus on long-term ecological research in combination with experimental activities. We will try to reduce fragmentation of our research, in particular by application of future projects on our long-term monitored sites. The next studies also should be focused on exploiting the potential of advanced instrumental techniques.

2.7 List of publications in books and impacted journals related to part 2.3 (the team members are printed in bold)

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Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Soil microbiology and soil chemistry

The Soil microbiology and soil chemistry team (BC-team-12) studies fundamental biological and ecological principles and mechanisms related to the roles and interactions of microbiota in soil environment in both natural and human affected ecosystems. We study how soil microbiota response to environmental changes and stress; what are their characteristic traits; how they interact with each other, with soil animals and plants; what role they play in these interactions and processes; how it relates to soil formation, soil functioning, soil fertility, health of soil and plants; and how these processes relate to human activities and human health; i.e. how the microbiota contribute to ecosystem services provided by the soil. Our research spans from microbial communities through key groups to populations and species, using classical soil microbiological methods including cultivations, molecular methods including recent technologies, laboratory experiments, greenhouse mesocosmos experiments, field surveys and monitoring as well as field manipulative experiments. Typical experimental ecosystems affected by human activity include variously managed grasslands and arable soils to study impact of intensive agricultury on soil microbiota and post mining sites to study succession of soil microbial community in processes of soil formation. The caves, deep subsoils, polar, high-mountain and arid ecosystems represent typical natural experimental environments of our team to study the soil microbiota under extreme conditions.

Team included 11 researchers, their's average capacity ranged from 0.4 to 1.0 during the reporting period 2010-2014. The advantage of the team has been a comprehensive profile of professionals for soil microbiology, whose experience and skills allow a variety of perspectives and deeper penetration in to the studied problems. The age structure of the team in the reported period was represented by a main cohort of 5 senior researchers (56-60 years: F. Novák, M. Šimek, A. Nováková, A. Lukešová and V. Krištůfek), group of 2 experienced researchers (48 and 42 years: D. Elhottová and A. Chroňáková) and group of 4 post-doctoral researchers (32-39 years: M. Kyselková, J. Jirout, J. Hynšt and J. Macková). Fifteen students from University of South Bohemia in České Budějovice and Charles University, Prague participated in our research over the past five years. In the mid of reporting period (summer 2012) the leadership of the team has been changed because the team-head M. Šimek became a director of Biological Centre CAS and D. Elhottová took over the leadership of the team.

Our team has build in last five years important scientific infrastructure consisting of (i) facilities of MOLECULAR LABORATORY LOOP (from extraction DNA, through its isolation, purification, PCR detection/ quantification to identification and verification of detected genes; including technical and methodological platform for DGGE and pyrosequencing analyses); (ii) soil CARD FISH LABORATORY; (iii) upgrading instrumental platform of analytical LABORATORY of MICROBIAL LIPIDIC BIOMARKERS with technical and methodological platform for microbial community analyses based on profile of phospholipid fatty acids methyl esters, ether-lipids and sterols, determination of cellular fatty acids of microbial cultures and their identification with MIS Sherlock System (iv) upgrading instrumental and methodical platform of the GAS METABOLITES LABORATORY; (v) methodological background for study antibiotic resistome and mobilome of soil bacteria. Important part of our research platform are also collections of soil/environmental isolates of microorganisms. Nowadays, collections of our team holds over 6000 strains (Fig. 1). Our team has taken significant steps to modernize existing collections, create new methodologies of preservation, catalogization and connection of the collections into one union with one trademark of the Biology Centre Collections of Organisms (BCCO). Collections serve primarily to our scientific work, but the biotechnological potential has been studied more frequently last years.

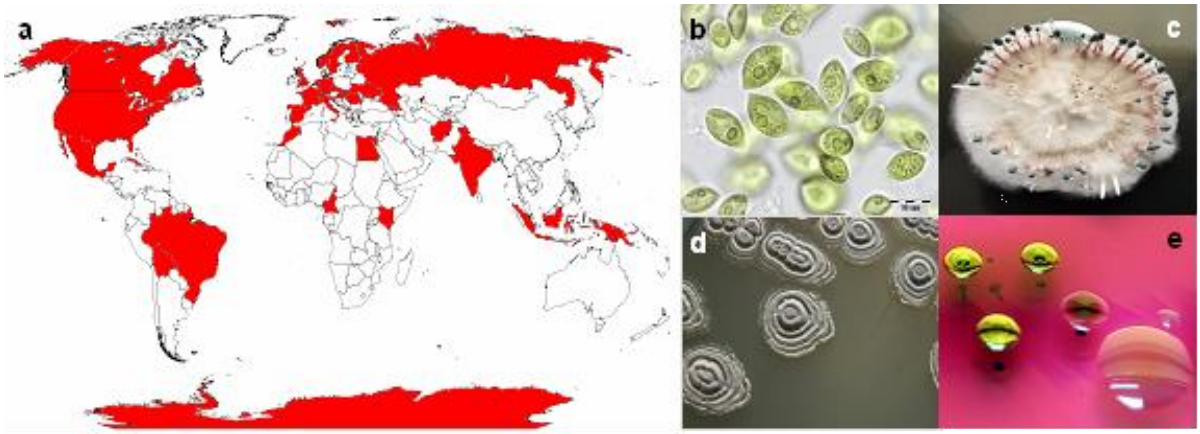

Fig. 1: Biology Centre Collections of Organisms (BCCO) harbours microorganisms collected from around the world. Countries of origin are highlighted in red (a), representing various geographic regions (arctic, sub-arctic, temperate, subtropical, tropical, mountainous, or even underground habitats). Four types of microorganisms are maintained under BCCO: algae and cyanobacteria (b); micromycetes (c); actinomycetes (d); bacteria (e).

The team has solved 25 scientific projects of the Czech Science Foundation, the Czech Ministry of Education, Youngh and Sports (MEYS), COST grants, the National Agency for Agricultural Research, the Ministry of Industry and Trade of the Czech Republic, IAEA Vienna - International Atomic Energy Agency, EU - Programme Interact, including contractual and collaborative projects (e.g. State Nature Conservancy SR, Slovak Caves Administration, Slovak Republic, WARP DRIVE BIO, LLC Cambridge, U.S.A. and R-Biopharm, Darmstadt, Germany, respectively).
Our team is active in domestic and international scientific cooperations. We constituted, together with the teams from Institute of Microbiology CAS and Charles University Prague, the Center of Environmetal Microbiology (2007-2011, MEYS CR). Our scientific cooperation has also led to establishment of an international conference "Ecology of Soil Microorganisms", which represents an interdisciplinary platform conecting different fields and teams of soil microbial ecology. Our foreign partner teams include Department of Microbial Ecology, Center for Evolutionary and Ecological Studies, University of Groningen (J.D. van Elsas); Research Unit of Environmental Genomics, Helmholtz Zentrum Műnchen (M. Schloter); Agroecology Department, INRA, Dijon (L. Philippot); Agroecology Institute for Epidemiology and Pathogen Diagnostics, JKI, Braunschweig (K. Smalla); Institute for Risk Assessment Sciences, Utrecht University, Netherlands (H. Schmitt); Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, University of Firenze (P. Nannipieri); Institute of Microbiology, University of Innsbruck (H. Insam); Faculty of Science, P.J. Šafárík University in Košice, Slovak Republik (L. Kováč); Karst Research Institute Slovenian Acad Sci & Arts, Postojna, Slovenia (J. Mulec); Faculty of Science, Sherbrook University, Quebeck, Canada (R.L. Bradley), John Carroll University, Ohio, USA (J.R. Johansen), University of Helsinki, Finland (M. Yli-Halla).

Our research is closely associated with teaching at the universities (mainly University of South Bohemia in Ceske Budejovice) and scientific training of students of all levels including students of the Erasmus Programme. New research information as well as new methodological trends are transmitted not only to university students, but also to talented and enthusiastic high and secondary school students and teachers.

The main scientific outputs of our team are articles in international peer-reviewed impacted (82) and nonimpacted (13) journals, chapters in books (22), or books (9) and patents (1). The achieved research results fall to Biological sciences including agricultural sciences and biotechnology; the main areas are soil sciences. Majority (more than 75%) of our results are published in journals that are located on the 1st and 2nd quartile for soil sciences, environmental sciences, ecology, microbiology, mycology, biotechnology, applied microbiology or multidisciplinary field. We participated on 58 international conferences with 167 contributions from our research.

Important results of our team were achieved in the following areas (selected results):

Response of microbial community to different forms of environmental attack and stress

Microbial diversity determines the invasion of soil by a bacterial pathogen.
Natural ecosystems show variable resistance to invasion by alien species, and this resistance can relate to the species diversity in the system. Elhottová and Krištůfek partici
ated with collegues from Center for Evolutionary and Ecological Studies, University of Groningen in study which showed that soil microbial diversity is a key factor that controls the extent to which bacterial invaders can establish (van Elsas et al. 2012 *PNAS*, **109,** 1159-1164). The invader's fate (*E. coli* O157:H7, strain T) in soil was determined in the presence of (i) differentially constructed culturable bacterial communities, and (ii) microbial communities established using a dilution-to-extinction approach. Both approaches revealed a negative correlation between the diversity of the soil microbiota and survival of the invader. The relationship was explained by a decrease in the competitive ability of the invader in species-rich vs. species-poor bacterial communities, reflected in the amount of resources used and the rate of their consumption.

Application of manure into the soil contributes to increase bacterial resistance to the antibiotic tetracycline in the environment regardless of the content of tetracycline antibiotics in manure
Emergence of infectious diseases as well as spreading of bacteria resistant to antibiotics is major health concern. Our research was focused on the spread of resistance to tetracycline antibiotics (TET-r), the residues of which represent the strongest loads for agricultural soils. We confirmed the enrichment of soil TET-r-genes through excrement of livestock, regardless of whether the animals were treated with antibiotics or not (Kyselková et al. 2013 *Chemosphere*, **93**, 2413-2418; Fig. 2A). Further we addressed the question whether tetracycline (TC) residues in soils can act as selective pressure enhancing the persistence of TET-r genes in grassland soils receiving cattle feces. We showed that the persistence of genes in manured soils differed according to the type of TET-r-gene. The soil but not the concentration of antibiotics, significantly affected the gene persistence. Certain TET-r genes originating from cattle feces may persist in soil for several months independently from antibiotic selection pressure (Kyselková et al. 2015, *Soil Biology & Biochemistry*, **81,** 259-265; Fig. 2B).

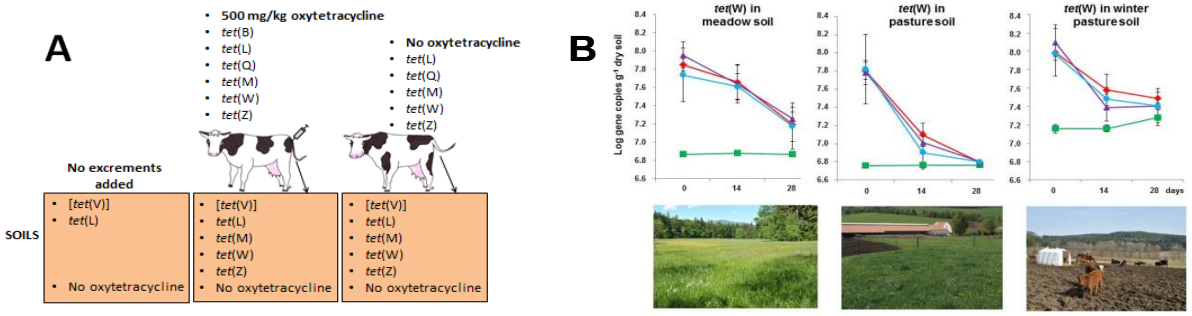

Fig. 2: A) Profile of tet-r genes detected in manure from control and oxytetracycline-treated animal and in soil with/whithout manure application of control/treated-animals; B) Persitance of tet-r gene in grassland soils (with different managment) treated with manure and chlortetracycline antibiotics: Green marks = control soil without manure and antibiotics, red = soil with manure; blue = manured soil with a low concentration of antibiotics (0.2 mg kg^{-1}); violet = manured soil with a high concentration of antibiotics (100 mg kg^{-1}).

Insights into the effect of environment conditions on characters of soil microbial community

Changes in soil microbial communities as affected by intensive cattle husbandry.
Winter pastures represents a model system to study the impact of intensive outdoor husbandry on change of quality and functioning of soil microbial communities. Our results showed that cyclic enrichment of soil by cattle excreta in winter season led to the enrichment by anaerobes (Elhottová et al. 2012 *Applied Soil Ecology*, **58**, *56-65).* We showed that the cattle excreta significantly contributed to both total and extracellular DNA pools in soil which may influence both greenhouse gas production and transmission

of risky genes. (Chroňáková et al. 2013 *Biology and Fertility of Soils*, **49**, 351-361). We confirmed high diversity of methanogenic archaea in a cattle-impacted winter pasture soil (Koubová et al. 2012 *European Journal of Soil Biology*, **48**, 1164-5563). The distinct soil fungal communities in the different sections of the experimental area in dependence on intensity of cattle impact were described. The occurrence of rumen-born anaerobic fungi was documented (Jirout et al. 2011 *Soil Biology and Biochemistry*, **43**, 647-656).

Fungal contribution to nitrous oxide emissions from cattle impacted soils was confirmed in our next detail characterization of soil fungal isolates. A significant effect of cattle impact intensity on the N_2O-production capability of soil fungal consortia was shown (Jirout et al. 2013 *Chemosphere*, **90**, 565-572; Fig. 3).

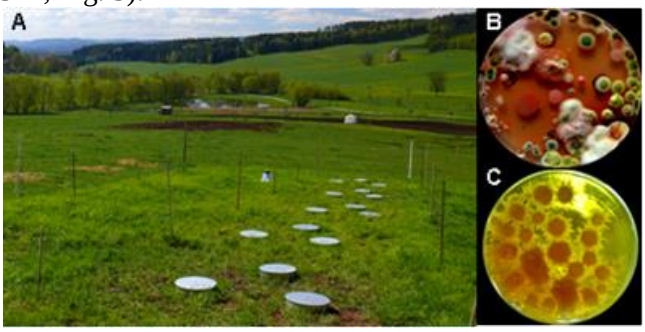

Fig. 3: A) Experimental area of intensive outdoor husbandry used for *in situ* measurements of greenhouse gases emissions; B) Cultivable fungal community isolated from cattle-impacted soils; C) Fungal isolate (*Aspergillus terreus*) tested on production of nitrous oxide.

Anaerobic oxidation of methane in grassland soils used for cattle husbandry.
While the importance of anaerobic CH_4 oxidation has been at first reported for marine ecosystems, the role of this process in soils is still questionable. The specific composition of soil microbiota involved in greenhouse gases production in studied winter pasture soils gave good prospect for detection this specific process. In collaboration with German team (Helmholtz Zentrum Munchen) we arranged a soil experiment under anaerobic conditions using $^{13}CH_4$. High microbial utilization of $^{13}CH_4$ as nutrient source and incorporating of the ^{13}C into microbial PLFA biomass confirmed this process. The relatives of "Candidatus *Methylomirabilis oxyfera*" were suggested as the intermediator of the anaerobic CH_4 oxidation in the winter pasture studied soil (Bannert et al. 2012 *Biogeosciences*, **9**, 3891-3899).

Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. Manipulative experiment at the experimental winter pasture contributed to understanding of the regulation of N fluxes by soil denitrification. Soil pH was shown to be of importance in determining the nature of denitrification end products. The effect on denitrifier community size and structure was shown (Čuhel et al. 2010 *Applied and Environmental Microbiology*, **76**, 1870-1878; Fig. 4). Our team collaborated with colleagues from INRA and UMR, Dijon and Agri-Food and Biosciences Institute, Belfast. Follow-up research studied the proximal and distal control by pH of denitrification rate in a pasture soil. Results indicated that even if the pH-induced changes in the structure of denitrifying microbial community can control the absolute denitrification rate (distal control by pH) the community does not influence the proportion of denitrification products, which is regulated solely by the proximal control by pH (Čuhel & Šimek 2011 *Agriculture, Ecosystems and Environment*, **141**, 230-233).

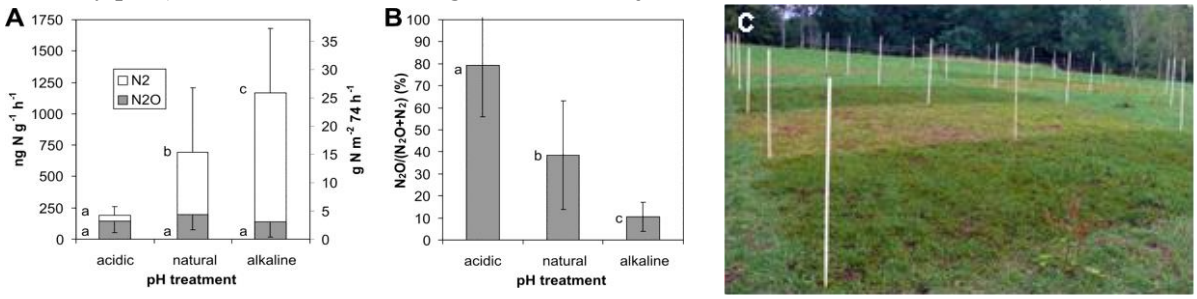

Fig. 4: A) Amount of nitrogen emitted in form of nitrogen gases under various pH levels; B) Ratio of N2O in total emissions of nitrogen gases under various pH levels; C) Manipulative experiment for study of pH-induced changes in soil.

Evidence of rich microbial communities in the subsoil of a boreal acid sulphate soil conducive to greenhouse gas emissions. The largest areas of acid sulphate (AS) soils in Europe are located in Finland, where 67,000-130,000 ha of AS soils are in agricultural use. Our team provided original information on unique microbial and nutritional conditions in C horizons of the AS soil and their high potential as an important source of greenhouse gases (Šimek et al. 2011 *Agriculture, Ecosystems and Environment,* **140**, 113-122). The next comprehensive analyses (Šimek et al. 2014 *Science of the Total Environment,* **466**, 663-672) confirmed the presence of abundant and specific microbial communities and clarified its localization in the deepest Cg2 horizon of the AS soil. This, together with the abundant C and total and mineral N in the deep layers, may result in substantial gas production. Consequently, high GHG emissions could occur, when the generally high water table is lowered because of arable farming. In this research, we have collaborated with colleagues from University of Helsinki.

Interactions between soil microbiota, invertebrates and plant roots

Methane production and methanogenic Archaea in the digestive tracts of millipedes (Diplopoda). Our team and team of soil zoologists BC CAS brought the first detailed survey of methanogens' diversity in the digestive tract of millipedes. We found that methanogens are associated with distinct phylogenetic clades of millipedes and that the CH_4 production reflects differences in the activity and proliferation of the intestinal methanogens rather than an absolute inability of some millipede taxa to host them (Šustr et al. 2014 *PLoS ONE*, **9**, e102659).

Effects of the endemic earthworm *Allolobophora hrabei* (Černosvitov, 1935) on soil microbial communities of steppe grasslands. There is a lack of knowledge about the effects of uncommon earthworms on microbial communities in soil. Results of our team showed that activities of *A. hrabei* - burrowing and deposition of casts in particular - can be considered as an important factor that affects biomass and community structure of soil microorganisms (bacteria, archaea, fungi) in steppe grasslands. Moreover, earthworm casting represents very important process in soil horizon turnover and it should be considered in biogeochemical cycles. Our team collaborated with soil zoologists BC CAS (Jirout & Pižl 2014 *Soil Biology & Biochemistry,* **76**, 249-256).

Evaluation of rhizobacterial indicators of tobacco black root rot suppressiveness in farmers' fields. Disease-suppressive soils are an important natural model for studying interactions between plant roots, plant-beneficial and plant-deleterious microorganisms. Kyselková et al. (2014 *Environmental Microbiology Reports*, **6**, 346-353) show that, despite complex conditions affecting bacterial communities on plant roots in the field, there are specific bacterial taxa correlating with the level of soil suppressiveness towards a disease. These might be helpful for disease-suppressiveness prediction, and represent candidate taxa for biotechnological screening. M. Kyselková from our team collaborated with teams of Université de Lyon, CNRS, Villeurbanne, France and Crop Research Institute, Praha-Ruzyně, Czech Republic.

Role of soil microorganisms in subterranean ecosystem of caves

New species of micromycetes found in smudge on paleolithic art in Lascaux cave *Ochroconis lascauxensis* and *O. anomala*, (Martin-Sanchez et al. 2012 *Fungal Biology,* **116**, 574-589; Fig. 5), were chosen within the 2013 Top 10 new species list by the International Institute for species Exploration (Arisona State University). The next study from the same locality (Bastian et al. 2010 *Microbiology,* **156,** 644-652; Fig. 5) brought important information on ecology of soil fungi in cave, their bio-interactions and impact on the Paleolithic paintings. A. Nováková from our team provided an expert work on the taxonomy and ecology of soil fungi and collaborated with teams of INRA-Université de Bourgogne, Dijon Cedex, France and Instituto de Recursos Naturales y Agrobiologia, CSIC, Sevilla, Spain.

Fig. 5: From left to right: ***Ochroconis lascauxensis*** A. Nováková & P.M. Martin-Sanchez, sp. nov. Mycobank MB561938; Detail of the Black Cow Panel in the Main Gallery; A black stain on sediments with ***Folsomia candida*** specimens, the vectors of the fungi (the collembolae are about 1 mm long).

Microwhip scorpions (Palpigradi) feed on heterotrophic cyanobacteria in Slovak caves - a curiosity among Arachnida. Extraordinary interaction of soil biota in cave food web was documented (Smrž et al. 2013 *PLoS ONE*, **8,** e75989). Unicellular cyanobacteria conspicuously predominated in guts of all studied cave palpigrades - animals with typically predacious feeding habit. Digestibility of consumed cyanobacteria was supported by the presence of guanine crystals, glycogen deposits and haemocytes inside the palpigrade body. Study indicated that cyanobacteria can cope with extreme conditions, even complete darkness when switch to heterotrophy. A. Lukešová from our team provided an expert work on biology and ecology of cyanobacteria. She collaborated with soil zoologists from Charles University, Prague and P.J. Šafarík University in Košice, Slovak Republic.

The Cave Biota of Slovakia
Three years microbiological monitoring of our team and collaboration with soil zoologists from P.J. Šafarík University in Košice and Slovak Caves Administration, Liptovský Mikuláš, Slovak Republic, resulted in monografy (Kováč et al. 2014 *The Cave Biota of Slovakia*, Liptovský Mikuláš: State Nature Conservancy SR, Slovak Caves Administration, 192 p.). It brought original information on a comprehensive image of the state of Biotop 8310 (Inaccessible cave formations, Natura 2000) in Slovakia. Our research laid the groundwork for a long-term monitoring of the biotope conditions trends including the design of monitoring methodology.

Characterisation of new species and important groups of soil microorganisms

New species in *Aspergillus* section Fumigati from reclamation sites in Wyoming (USA) and revision of *A. viridinutans* complex. An original information on new *Aspergillus* species (Figs. 6), with high importance for mycology and soil microbiology and with significant overlap into the clinical and veterinary mycology were obtained by Nováková et al. (2014 *Fungal Diversity*, **64**, 253-274). *A. udagawae*, an opportunistic animal pathogen with increasing incidence in worldwide, has never been reported as a dominant species in soil. The dump soils could be an important source of this pathogen, together with other non-fumigatus opportunistic pathogens such as *A. felis* and *A. lentulus*.
The mycologists from Charles University and Institute of Microbiology CAS in Prague and Medical Mycology Research Center, Chiba University participated on this research.

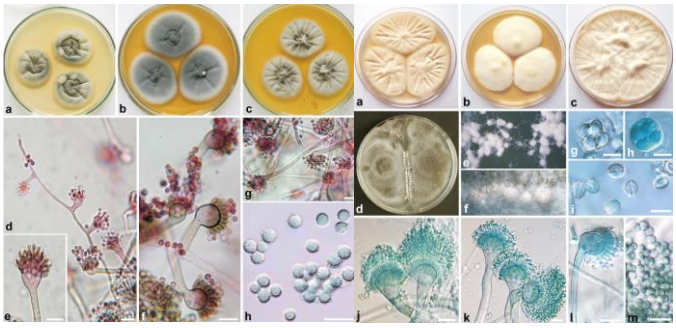

Fig. 6 left: ***Aspergillus brevistipitatus*** **CCF 4149T** *A. Nováková & Hubka, sp. nov. MB803934;* a–c Colonies; d–g conidiophores; h conidia. Fig. 6 right: ***Aspergillus wyomingensis*** **CCF 4417T** *A. Nováková, Dudová and Hubka, sp. nov. MB80393*; a–c Colonies; d fertile cleistothecia as a result of crossing CCF 4416 and CCF 4417T on OA after 6 weeks. e–f cleistothecia. g–h asci; i ascospores; j–l conidiophores; m conidia.

Three soil species, new to science, were isolated by our team (A. Lukešová and PhD students K. Hrčková and A. Bohunická) and described in frame **of morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostocaceae, Cyanobacteria)** (Fig. 7). The study of 26 *Cylindrospermum* strains, mostly isolated from soils, showed that *Cylindrospermum* is unusual

among cyanobacterial genera in that the morphological diversity appears to be more evident than sequence divergence. Study revealed 3 distinct clades. The clade we designate as *Cylindrospermum* sensu stricto contained all 5 of the foundational species, in this clade were described: *C. badium*, *C. moravicum* and *C. pellucidum* (Johansen et al. 2014 *Journal of Phycology*, **50,** 187-202). Teams of John Carroll University (USA), University of South Bohemia (CR) and Institute of Botany CAS (CR) collaborated with our team.

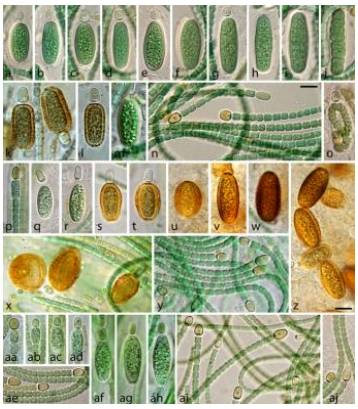

Fig. 7: Light micrographs of *Cylindrospermum:* (a–o) ***C. moravicum*** Johansen et Lukešová **sp. nov.**: (a–g, k–m) variability of akinetes of different age and formed under different growth conditions, (h–j, o) germinating akinetes, (n) vegetative filaments with terminal mature and forming heterocytes; (p–z) ***C. badium*** Johansen et Hrcková **sp. nov**.: (p) formation of proakinete next to a terminal heterocyte, (q and r) maturing akinetes with colorless exospore, (s–x, z) mature akinetes with chestnut colored exospore, (y) vegetative filaments with terminal heterocytes; (aa-aj) ***C. pellucidum*** Johansen et Bohunická **sp. nov.**: (aa-ad) formation of proakinete next to the terminal heterocyte, (ae, ai, aj) vegetative filaments, (af-ah) mature akinetes.

Rapidly growing mycobacteria from agricultural soils and clinical isolates did not differ in tetracycline resistance and presence of tetracycline resistance determinants tet(V) and tap. Rapidly growing mycobacteria (RGM), the common inhabitants of soil and water in past decades, have been increasingly recognized as a cause of human and animal diseases. In frame of our research of antibiotic resistance-reservoirs in soil we characterize tetracycline (TET) resistome of the soil and clinical RGM isolates from Czech hospitals (Fig. 8). Both groups did not difer in TET resistance (>50%) and were characteristic by occurrence of tet(V) and tap genes encoded the TET efflux pumps. The phylogeny of tet(V) correlated with isolates' BOX-PCR profiles, suggesting that this gene evolved along with mycobacterial genomes as a part of the intrinsic resistome. The intrinsic efflux pumps may be more important for TET resistance than horizontally transferred genes in both soil and clinical RGM (Kyselková et al. 2012 *Microbes and Environments*, **27**, 413-422).

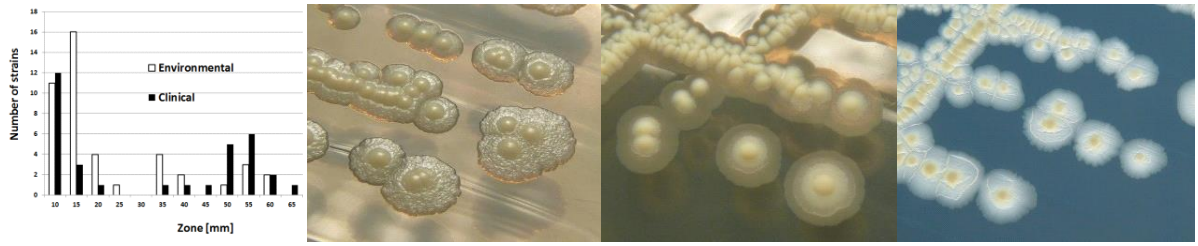

Fig. 8: Tetracycline resistance in the clinical and the environmental isolates of rapidly growing mycobacteria (RGM). Bars represent the number of isolates with the corresponding inhibition zone size around 30 µg. Photos illustrate a typical growth of soil RGM colonies.

Biotechnological transfer from research of soil microbiota

Biosynthesis of Colabomycin E, a new manumycin-family metabolite, involves an unusual chain-length factor. In collaboration with the Institute of Microbiology CAS and the Institute of Clinical and Experimental Medicine (ICEM) in Prague our team participated in discover of a promising agent for the treatment of inflammation (Petříčková et al. 2014 *ChemBiochem*, **15**, 1439-4227). Colabomycin E is a new member of the manumycin-type metabolites. It is produced by the strain *Streptomyces aureus* SOK1/5-04, isolated by our team in frame of primary succession investigation at post-mining sites (Chroňáková et al. 2010 *Microbiological Research*, **165**, 594-608).

Using the newly created card test - RIDA®COUNT *Paenibacillus larvae* - to monitor infectious American foul brood (AFB) pressure from the surroundings. In the last 10-15 years American

foulbrood (AFB), this cosmopolitan and highly infectious disease, occurs increasingly around the world. We propose a new production RIDA®COUNT test with nutrient medium MYPGP and chromogen 2,3,5 - Triphenyltetrazoliumchloride (TTC) suitable for detecting the agent of AFB - *Paenibacillus larvae*. Production of standardized and cheap cards and uniform methodology for their application and evaluation will enable easier assessment of infectious pressure from the surroundings than previously used (Krištůfek et al. 2014 *Patent Number:* **304,336**, January 29, 2014; Ryba et al. 2012 *Open Journal of Veterinary Medicine*, **2**, 233-236). Member of our team V. Krištůfek designed the original solution to the problem of monitoring of foulbrood by a card method, conducted the experimental work and project. He collaborated with colleagues from Faculty of Science, Charles University in Prague, Bee Research Institute at Dol, Libčice nad Vltavou, Czech Republic and Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Science, Prague, Czech Republic.

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Soil zoology and soil microstructure

Team introduction. The Institute of Soil Biology (ISB) is the principal institution in the Czech Republic devoted to complex studies of soil biota and its interactions with the soil environment. It consists of two departments. The Department of Soil Zoology and Soil Microstructure (this team) focuses on the research of soil fauna and its interactions with other members of the soil community and with soil structures, functions and processes. The research on soil fauna includes mainly the studies in the field of community ecology, autecology and taxonomy.

Soil fauna represents a wide variety of organisms differing in size, food resources, ecophysiology and methodological approaches used in their study. As a consequence, specific approaches and specific taxonomic skills are needed to study individual groups of soil fauna. Hence, complex studies including a large array of soil fauna groups are rare. The team possesses a unique capability to study a wide range of soil fauna groups from soil microfauna (nematodes, rotifers and tardigrades) and soil mesofauna (collembolans, oribatid mites, diplurans, proturans and pauropods) to various groups of soil macrofauna (earthworms, millipedes, centipedes, insect larvae and ants). In addition, our team includes an expert in ecophysiology of soil fauna and specialists in field and laboratory manipulation experiments aimed to explore the interactions between soil fauna and soil microflora and the role of these interactions in soil organic matter turnover and nutrient cycling. We also posses unique technical facilities including those necessary for the preparation of thin soil sections and the study of soil microstructure. This unique tool allows us to study the spatial arrangement of individual soil aggregates and other soil particles, gives insight into their origin and the role in soil processes. **Major research topics** can be summarised as follows.

The effect of fauna on soil nutrient turnover. Soil fauna produces large variety of structures such as borrows, faeces, worm casts or ant nests, which alter substantially the environmental conditions and may serve as hot spots of microbial activity. Earthworm activity, namely the creation of casts and burrows, is well known to affect soil conditions and nutrient cycling. Ant nests or earthworm casts are typical examples of such hot spots (Velé et al., 2010, Jílková et al., 2013, Toyota et al., 2013) However, our research shows that the fauna's effect on nutrient cycling can be case-specific and also affected by previous activity of soil fauna i.e. the effects of fauna in soil where no fauna was present differ from effects in soil inhabited by animals for some time. Comparisons of the effects of earthworms on soil nitrogen turnover show that earthworms rather support N leaching from soils under a tillage regime, while the opposite is true for no-tillage soil. Interestingly, experiment tillage soils lack earthworms while no-tillage soils harbour abundant earthworm populations (Toyota et al., 2013). The effect of fauna on N cycling can also alter during the onthogenetic development of animals, as different life stages can rather enhance or slow down nitrogen mineralisation (Toyota and Kaneko, 2012). The effects of soil fauna on nutrient cycling is closely associated with its effects on soil organic matter decomposition and stabilization that will be mentioned below.

Invertebrate - microbial interactions include a large variety of topics; we will mention here two of them in detail. The first one are interactions between invertebrates and the soil microflora during gut passage and consequent effects of these interactions on soil organic matter decomposition, the second one the interactions of soil invertebrates and their pathogens. The effects of soil fauna on the soil microflora and **soil organic matter decomposition** resulting from the passage of soil and litter through the gut of soil invertebrates are very complex. They include the selective ingestion of soil microflora, the selective killing and digestion of soil microbes, as well as the changes in chemistry of ingested material (Frouz et al., 2010). The exact data about such effects should also be based on detailed knowledge of digestive tract morphology (Sosinka et al., 2014, Šustr et al., 2014a). Typically, microbial communities and activity in faeces differ remarkably from those in original material before the gut passage (Jirout and Pižl, 2014). Consequently, the fauna activity can alter metabolic activity of the whole soil profile (Šimek et al., 2010). In most cases, the passage through the intestine of soil animals results in a short-term increase of microbial activity; latter on, however, organic matter in faeces decomposes more slowly than the same

material which remained indigested (Špaldoňová and Frouz, 2014). Introduction of macrofauna feaces in soil also causes a weaker priming effect in comparison with leaf litter; moreover, faeces are also less sensitive to priming effects when supplied by easily available carbon than original litter (Špaldoňová and Frouz, 2014). Similarly to the effects of soil fauna on nutrient cycling described above, these effects of soil fauna were also found to be case-specific and dependent on the duration of fauna effects. When soil fauna (namely earthworms) was introduced into soil previously unaffected by animals, it tended to promote soil organic matter mineralisation; however, after about two years of fauna presence in the same soil, the effect was inverse, and the treatment with fauna showed much smaller loss of organic matter than treatments without soil fauna.

As concerns **invertebrate - pathogen interactions,** an example can be the comparison of innate defence mechanisms of two closely related earthworm species, *Eisenia andrei* and *Eisenia fetida* (Procházková et al., 2013). Currently, *E. andrei* is recorded only from composts and manures, while indigenous populations of *E. fetida* can also be found in the litter layer in forests. Genomic DNA analyses revealed significantly higher levels of fetidin/lysenins (determined using universal primer pairs) in *E. andrei* compared to *E. fetida*. It can be hypothesized that *E. andrei* colonizing compost as a new habitat acquired an evolutionary selection advantage resulting in higher expression of antimicrobial proteins (Dvořák et al., 2013).

Soil microstructure and effects of fauna on soil formation. As already mentioned above, thin soil sections allow us to explore spatial organisation and partly also the origin of soil structures. This we have used in the evaluation of soil genesis in chronosequences of soils after major disturbances. For example, the comparison of soil microstructure in chronosequences of post-mining sites after coal mining located along climatic gradient across USA showed that in wetter sites close to eastern coast of the USA, the bioturbation of soil by soil macrofauna played the principal role in soil formation. By contrast, we found no signs of bioturbation at more dry sites located in the western part of the USA, indicating that mostly physical processes such as soil erosion take part in soil forming process here (Frouz et al., 2013a). In another study, we used thin soil sections and analysis of soil microstructure to show that the proportion of earthworm casts in soil profile (as a measure of earthworm activity) drives many other key soil processes, such as microbial respiration, microbial biomass, composition of the microbial community or the rate of carbon accumulation (Frouz et al., 2013b).

Production of methane and other greenhouse gases. Methane is an important greenhouse gas, and its natural sources are not completely explained. Some invertebrates, including the associated microflora, are known to be important methane producers (Koubová et al., 2012). However, methane production appears to be a complex interplay between methane producing microorganisms, the soil fauna and soil environment. The 16S rRNA gene of methanogens was found in all tested taxa, but only some species emitted methane. The differences in substrate preferences of the main lineages of methanogenic Archaea found in different millipede orders indicate that the composition of methanogen communities may reflect differences in available substrates for methanogenesis or the presence of symbiotic protozoa in the digestive tract. We conclude that differences in methane production in the millipede gut reflect differences in the activity and proliferation of intestinal methanogens rather than an absolute inability of some millipede taxa to host methanogens (Šustr et al., 2014).

Community ecology of soil fauna along various environmental gradients was an important part of research conducted by the unit and included several studies along climatic temperature - moisture or altitudinal gradients (Tajovský et al., 2012, Devetter and Schöll, 2014, Schlaghamerský et al., 2014). An important part of our community ecology research is also represented by studies on soil fauna assemblages in protected areas, which preserve close to natural situations. The results of these studies (Háněl and Čerevková, 2010, Božanič et al., 2013, Schöll et al., 2013, Farská et al., 2014) may often serve as valuable references for other research in such areas.

Particular attention was paid to the **development of soil fauna communities along succession gradients.** Here we want to mention two examples of comprehensive studies, in which we studied a large number of various groups of soil fauna together (Frouz et al., 2013a, 2013b). In both cases we used the chronosequence approach, i.e. we studied the set of sites of different age with presumably very similar history. The first study compared the development of communities of soil biota in several chronosequences of sites after coal mining, which were located along the climatic gradient across the USA. This study shows that at the wetter eastern site overgrown by forest or tall grass prairie, the soil fauna community include a large proportion of saprophagous macrofauna that feed on plant litter. Saprophagous macrofauna also plays an important role in soil bioturbation which can be indicated by thin soil section as described above. At dry western sites, by contrast, most of the soil fauna is represented by root-feeding organisms. As a consequence, communities of soil biota at western sites are less complex with almost no species replacement during succession, which means it takes a shorter time to establish. The more complex communities at wetter eastern sites undergo complex development

over time and their community composition approaches that observed in climax stages much slower. In conclusion, this study shows that simpler, root feeding soil biota communities in short-grass prairies approach the climax faster then more complex, mostly saprophagous communities under tallgrass prairies or forests (Frouz et al., 2013a).

In the second study, we carried out a complex study of soil biota communities along a chronosequence of primary succession of post-mining sites in the Czech Republic. The results revealed that the bacterial channel dominated the food web in early stages of succession. Later on, in shrub-dominated stands, the fungal channel took over. Even later, in the forest stage, the bacterial channel prevailed again. The best predictor of fungal bacterial ratio was the thickness of the fermentation soil layer. We argue that these changes correspond with changes in topsoil microstructure driven by a combination of plant organic matter input and engineering effects of earthworms. In early stages, soil is alkaline, and a discontinuous litter layer on the soil surface promotes bacterial biomass growth, so the bacterial food web channel can dominate. Litter accumulation on the soil surface supports the development of the fungal channel. In older stages, earthworms arrive, mix litter into the mineral soil and form an organo-mineral topsoil, which is beneficial for bacteria and enhances the bacterial food web channel. Here it should be underlined that earthworms themselves contribute rather little to the flow of energy though the food web, so it is not their direct contribution to energy turnover, but an indirect effect via soil modification what changes the food web structure. This example shows that the effect of ecosystem engineers can change the food web composition during succession (Frouz et al., 2013b).

Studies dealing with particular groups of soil animals included studies on the development of nematode assemblages during the secondary succession in old fields (Háněl, 2010) and studies about changes in assemblages of soil rotifers during primary succession at post mining sites (Devetter and Frouz, 2011).

Interaction between the soil fauna and plant communities. The engineering effect of earthworms affects not only the composition of other soil biota, but also plant communities. Previous field observations indicated that earthworms promoted late-succession plant species in a laboratory pot experiment designed to test the effect of litter of various quality on the growth of late-successional plants. Earthworms increased plant biomass, especially that of the large-seeded species, but reduced the number of plant individuals, mainly that of small-seeded ones, most likely due to seed predation. Litter quality did not modify the effect of earthworms on plants; the effect of litter quality and earthworms was only additive. Because the results of these experiments are consistent with field observations, we conclude that earthworms help drive succession in plant communities. However, plant development also affects earthworm establishment during primary succession. Results of experimental introductions of earthworms into mesocosms with soil and plants from various succession stages shows that earthworms can successfully establish at about 25-year-old sites which are already covered by established vegetation cover. Grass is more suitable for earthworm establishment than shrubs. Not only earthworms affect plant succession. Laboratory and field experiments with the larvae of Elateridae show that belowground herbivores can suppress early succession plants, which can promote late-successional plants (Roubíčková et al., 2013).

Effects of agriculture and forestry technologies on soil fauna in relation to ecosystem services provisioning. The study of soil fauna and its interactions with ecosystem functioning and provisioning of ecosystem services in various agriculture or forestry systems is another important filed of our research (Čermák et al., 2011, Koubová et al., 2012). The role of the soil biota in plant - soil interactions clearly emphasizes the important role of the soil biota in provisioning of ecosystem services. This was shown in the frame of the EU project SoilService, in which we participated by maintaining Czech sites and by our expertise in soil biota determination. Here we quantified, across four countries of contrasting climatic and soil conditions in Europe, how differences in soil food web composition resulting from land use systems (intensive wheat rotation, extensive rotation and permanent grassland management) influence the functioning of soils and the ecosystem services that they deliver. Intensive wheat rotation consistently reduced the biomass of all components of the soil food web across all countries. Soil food web properties strongly and consistently predicted processes of C and N cycling across land use systems and geographic locations, and they were a better predictor of these processes than land use. Processes of carbon loss increased with soil food web properties that correlated with soil C content, such as earthworm biomass and fungal/bacterial energy channel ratio, and were greatest in permanent grassland. In contrast, processes of N cycling were explained by soil food web properties independent of land use, such as arbuscular mycorrhizal fungi and bacterial channel biomass. Our quantification of the contribution of soil organisms to processes of C and N cycling across land use systems and geographic locations shows that soil biota need to be included in C and N cycling models and highlights the need to map and conserve soil biodiversity across the world (de Vries et al., 2013). This indicates that introducing biofuel, which may increase the competition about land between food production and other use, and consequently increased pressure on agriculture intensification may have hidden cost in

a decrease of ecosystem services provided. This may even be worsened by using introduced biofuel crops, which affect soil food web more dramatically than native crops (Heděnec et al., 2013).
Another example of the crucial role of the soil biota in the delivery of ecosystem services is offered by plantations of various tree species on post-mining land and also on sites left to natural succession. Soil formation differed markedly among sites afforested with different tree species. At sites with trees producing litter with a low C/N ratio (deciduous species), the organic Oe layer was narrow or absent, and a thick organomineral A layer was evident. At sites with trees producing litter with a high C/N ratio (evergreen species), by contrast, a thick Oe layer and a thin A layer were evident. Besides the C/N ratio, the density and bioturbation activity (measured as the amount of earthworm casts in the topsoil) of earthworms were the strongest predictors of A layer thickness and C accumulation in the mineral topsoil. Sites with higher C accumulation in mineral soil had higher microbial biomass and lower microbial respiration, which may have contributed to the higher C storage. The gradient of bioturbation was correlated with changes in the composition of the bacterial community and other soil biota, but partial correlation showed that the effects of litter quality and bioturbation were largely independent (Frouz et al., 2013c). Overall, the results indicate that the effect of tree species on soil development is substantially mediated by soil fauna activity and especially by earthworm bioturbation, which in turn affects soil chemistry as well as microbial processes (Helingerová et al., 2010, Šnajdr et al., 2013). These results imply a need for diverse management approaches in post-mining areas including the use of spontaneous succession to support the diversity and provisioning of various ecological functions (Hendrychová et al., 2012).
Three species shift leave extensive legacy in forest ecosystems also outside mining sites. For example, the shift from beech to spruce forest increases overall density of soil mesofauna and affects functional traits of soil microarthropods; opportunistic herbifungivorous species increase in the monoculture at the expense of fungivorous species. Similarly, hemiedaphic collembolans increased in a monoculture at the expense of euedaphic species (Farská et al., 2013). Although there was no remarkable influence of management intensity on total densities or diversity indices, significant shifts in functional groups (from fungivory and carnivory to detritivory) indicated that soil functions and processes were affected by forest management (Farská et al., 2014a). Also, the way of forest establishment, i.e. planting vs. natural regrowth, may have a long legacy in soil fauna communities (Farská et al., 2014b).
Taxonomy of soil fauna, methodical approaches and autecology of species. Progress in soil fauna studies would not be imaginable without progress in taxonomy, the development of new methods for soil fauna extraction and studies on species autecology. In this context, extensive taxonomic work has been conducted in key groups of soil fauna (Niedbala and Satrý, 2010, Rusek, 2010a,b, Starý 2010, Kontschán and Starý, 2011, Liu et al., 2011 Niedbala and Satrý, 2011, Ermilov et al., 2012, Kontschán and Starý, 2012abc, Kováč and Rusek, 2012, Rusek 2012, Shrubovych et al., 2012, Kontschán and Starý 2013ab, Kontschán and Starý, 2014ab, Niedbala and Satrý, 2014, Shrubovych et al., 2014abc, Tajovský et al., 2014). Attention was also paid to the description of immature and unknown forms of soil animals (Čermák et al., 2012, Christophoryová et al., 2012). A new method for rotifer extraction from soil was developed (Deveter, 2010), and extensive effort was made to improve our understanding of the autecology and ecophysiology of the keystone species of the soil fauna (Šustr et al., 2010, Hubert et al., 2011).

Applied research and the most important outcomes: As can be seen from the previous text, an important part of the unit research has application potential. This is particularly true for research related to the restoration of degraded and disturbed ecosystems. In this respect, it should be particularly emphasized that research dealing with the evaluation of potential ecosystem services achievable by various ways of reclamation provides new knowledge and tools for restoration planning. Many research reports have been presented to mining companies.
Noteworthy is also our participation in applied research on the recovery of threatened ecosystems in Western Europe, namely heathlands and Nardo-gallion meadows.

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Research Report of the team in the period 2010–2014

Institute	'Biology Centre of the CAS, v. v. i.

Scientific team	Molecular protistology

The main areas of research of our laboratories in the last 5 years are summarized in 9 sections (1-8 – Lukeš lab, 9 – Zíková lab), which are described in detail below.

Our main interest rests with protists of the order Kinetoplastida, but we also did some work on free-living relatives of the obligatory parasitic Apicomplexa. Moreover, we have a general interest in the mitochondrion of protists and some questions dealing with the evolution of protein machineries in protists in particular, and the diversity and evolution of protists in general.

Trypanosomes and related flagellates belong to the most widespread and successful extant parasites, and include serious pathogens responsible for African sleeping sickness, leishmaniasis and Chagas disease. The by far number one model organism is *Trypanosoma brucei*, which is amenable to RNA interference, knock-outs and knock-ins, protein tagging, has its genome sequenced, etc. Hence, absolute majority of functional genomics is being done on this parasite.

Biodiversity of trypanosomatids and other kinetoplastid flagellates is a fast evolving field, which was so far confined to human parasites of the genera *Trypanosoma* and *Leishmania*, but is now aiming to map the presence and diversity of these virtually omnipresent parasites, emergence of their parasitism and co-evolution with hosts of all main kinetoplastid lineages. In part of our research we were also mapping the diversity of Microsporidia and Apicomplexa.

1. Functional genomics of *Trypanosoma brucei*

T. brucei, the causative agents of African sleeping sickness, is very suitable for functional analysis of both proteins that are highly conserved across the eukaryotes (its early-branching position is particularly suitable for this), as well as of proteins that are derived and specific for this group of parasites. During the entire evaluated period we were intensely focused on proteins one way or another associated with the single mitochondrion of *T. brucei*, which during the life cycle undergoes dramatic morphological and biochemical changes. We will subdivide this task into several subtasks:

a/ mitochondrial Fe-S cluster synthesis proteins. We decided to map carefully the function of all proteins that participate in the synthesis of Fe-S clusters located in the mitochondrion. These clusters are probably the most ancient co-factors, associated with the earliest form of life billions years ago and still indispensable for each and every living cell. Their synthesis and metabolism are subjects of intense studies in *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, mouse and humans, but nothing about their synthesis was known not only from *T. brucei* in particular, but from the whole eukaryotic supergroup Excavata in general.

We have devoted to this topic significant efforts, almost completely 3 dedicated PhDs (in the period of last 7 years), and published a series of papers in decent journals. As a consequence, we are now being invited and given talks at Gordon Research Conferences on this subject and were invited by the top expert Prof. Roland Lill to write a review (will come out in 2015). Several papers were published in 2008 and 2009 (e.g. in PNAS), from more recent ones, yet already highly cited, the following was included among our major outputs: ***Long S.***, ***Changmai P.***, *Tsaousis A.D.,* ***Skalický T.***, ***Verner Z.***, *Wen Y.-Z., Roger A.J. &* ***Lukeš J.*** *(2011) Stage-*

specific requirement for Isa1 and Isa2 proteins in the mitochondrion of Trypanosoma brucei *and heterologous rescue by human and* Blastocystis *orthologues.* **Mol. Microbiol**. *81, 1403-1418.*

However, another paper from our lab in this journal (Chagmai et al., 2013) also got a substantial attention, as by rescue studies, we have contributed to our understanding of the function of human ferredoxin. We have also substantially contributed to an important study analysing possible horizontally acquired Fe-S pathways (Tsaousis A.D., Ollagnier de Choudens S., Gentekaki E., **Long S.**, Gaston D., Stechmann A., Vinella D., Py B., Fontecave M., Barras F., **Lukeš J.** & Roger A.J. (2012) The evolution of Fe/S cluster biogenesis in the anaerobic parasite *Blastocystis*. ***Proc. Natl. Acad. Sci. USA*** 109, 10426-10431).

We will publish 2 or 3 papers on Iba57 and three Nfu proteins during 2015.

b/ cytosolic Fe-S cluster synthesis proteins. As an extension of our work on mitochondrial Fe/S pathway, we decided to explore its cytosolic branch, which was discovered only within the last decade and is now subject to a very intense study. This research is done in close collaborations with Prof. Lill (Philipps University, Marburg) and Dr. Antonio Pierik (University of Kaiserslautern), which so far resulted in the following joint paper: **Basu S.**, Netz D.J., Haindrich A.C., Herleth N., Lagny T.J., Pierik A.J., Lill R. & **Lukeš J.** (2014) Cytosolic iron-sulfur protein assembly is functionally conserved and essential in procyclic and bloodstream *Trypanosoma brucei*. ***Mol. Microbiol***. 93, 897-910 (included among our main outputs), and will lead to more publications in the future. Recently initiated collaboration with Prof. Mark Field (University of Dundee) on so-called cryogrinding, which shall be particularly suited to catch transient protein interactions, will be a key for this line of research. In fact, its success or failure will decide on how much attention we will give the cytosolic components of Fe-S synthesis in the years to come.

c/ conserved mitochondrial proteins of unknown function. *T. brucei* has several interesting advantages for mitochondrial studies, such as single organelle per cell, dramatic metabolic changes during the life cycle, DNA concentrated into one compartment of the mitochondrion, etc. Our intention is to use these unique features to our advantage. We are using two approaches: i/ screening of RNA interference (i) libraries, ii/ target RNAi knock-downs of potentially interesting genes. One representative paper attaching new function to a protein responsible for a serious human disease is included among our major outputs: **Hashimi H.**, McDonald L., **Stříbrná E.** & **Lukeš J.** (2013) Trypanosome Letm1 protein is essential for mitochondrial potassium homeostasis. ***J. Biol. Chem.*** 288, 26914-26925. It has been embraced by the community as an unorthodox study with far reaching implications, and is consequently well cited.

We would like to further explore potential of this approach, which is reaching outside of the trypanosome and/or parasitological scientific community, as we believe that screening for missing mitochondrial functions has a good chance for success in *T. brucei*.

d/ mitochondrial proteins involved in translation. Mitochondrial ribosomes of *T. brucei* are the most complex ribosomes known, despite the fact that there are only less than two dozens of transcripts that they are translating into proteins. Their complexity can be caused by complex additional tasks associated with the consequences of RNA editing and other processing of these mRNAs, but may also have other reasons such as regulatory functions. In collaboration with Prof. Dmitri A. Maslov (University of California, Riverside), we have dissected the function of several ribosomal components (Ridlon et al., J. Biol. Chem. 2013) and a study of three highly conserved ribosomal subunits is in process.

e/ proteins involved in maintenance and replication of kinetoplast DNA. Kinetoplast (k) DNA, a hallmark of kinetoplastid flagellates, is a huge amount of mitochondrial DNA composed of thousands of mutually interlocked circles, usually arranged into a single highly organized network that is located in the anterior part of the organelle, adjacent to the basal body

of the single flagellum. We were exploring the functions of heat shock proteins associated with the kDNA network and just published a paper on the subject in prestigious ***mBio*** (**Týč J.**, Klingbeil M.M. & **Lukeš J.** (2015) Mitochondrial heat-shock protein machinery Hsp70/Hsp40 is indispensable for proper mitochondrial DNA maintenance and replication; e02425).
Moreover, we have interesting data on mitochondrial LAP proteins, which are highly conserved throughout eukaryotes, but very little is known about their function. Our unpublished data indicate their active participation in kDNA maintenance, and we assume that such function can also occur in humans and other eukaryotes.

2. <u>RNA editing in *Trypanosoma brucei*</u>

The long-term interest of our lab is RNA editing in trypanosomes and related kinetoplastid flagellates. We are engaged in this field since at least last two decades, with numerous contributions covering both the RNA component of the process as well as its protein players. Within the last 5 years, we have focused on elucidating the function of a quite newly discovered mitochondrial RNA-binding complex 1 (MRB1). Either on our own, or with a very fruitful collaboration with the lab of Prof. Laurie Read (State University of New York, Buffalo), we have described the function of numerous components of this large protein complex, and have also characterized its structure and surprisingly dynamic composition. We have published several papers on this topic in RNA, Nucleic Acids Research and PLoS ONE. For the purpose of this evaluation, we have included well accepted review we wrote on this subject: **Hashimi H.**, Zimmer S.L., Ammerman M.L., Read L.K. & **Lukeš J.** (2013) Dual core processing: MRB1 is an emerging kinetoplast RNA editing complex. **Trends Parasitol**. 29, 91-99.
While we will in 2015 publish functional analysis of the last uncharacterized component of the MRB1 complex, our intention is to move our RNA editing studies to another level. For that purpose we are using the iCLIP method (individual nucleotide cross linking and immunoprecipitation), which allows identification of all RNA molecules bound by the studied protein. This is a brand new method for the field of RNA editing and we are currently putting a lot of efforts into implementation in our lab.
Moreover, we have also done significant work on RNA editing of the early-branching kinetoplastid *Perkinsela*, which lives like an endosymbiont in the cytoplasm of a fish-parasitizing amoeba. We have shown that even this strange organism with extremely reduced nuclear genome retains a huge (probably even more expanded than in other kinetoplastid flagellates) kDNA network, which produces transcripts requiring extensive editing via the insertion and/or deletion of uridines. Moreover, deep sequencing of editing intermediates (to the best of our knowledge first deep sequencing of partially edited mRNAs) revealed the existence of alternative editing patterns. This means that it is highly likely that several proteins are produced from a single transcript which is, however, edited differently. These results will be published in 2015.

3. <u>Whole genome analyses of kinetoplastid flagellates</u>

In the past we have participated in sequencing, assembly and annotation of two nuclear genomes and transcriptomes of *Phytomonas*, an important pathogen of plants (Porcel et al., PLoS Genetics 2013). However, we have also initiated and mostly on our own performed whole genome assembly, annotation and analyses of the i/ monoxenous trypanosomatid *Leptomonas pyrrhocoris*, which will be very important for our understanding of the emergence of dixenous parasitisim; ii/ *Leptomonas seymouri* (in collaboration with Dr. Vyacheslav Yurchenko, University of Ostrava), a monoxenous species that was, unexpectedly, found in the ulcers of *Leishmania* patients in India and other tropical countries; iii/ *Perkinsela*, from the evolutionary perspective perhaps the most interesting kinetoplastid; in collaboration with Prof. John Archibald (Dalhousie University, Halifax), we are now in the process of deep analysis of the

content of its highly reduced nuclear genome; iv/ *Paratrypanosoma* (see below). None of these studies have been published so far, mostly due to the complexity of these projects and our so far limited experience with large-scale genome analyses. However, we are glad to say that it is reasonable to assume that three of them (except *Paratrypanosoma*) will be published during 2015.

4. *Paratrypanosoma* and the diversity of trypanosomatids

We have put a lot of efforts into cataloguing and collecting monoxenous trypanosomatids from all over the world (we have personally collected – outside of Europe – in Ghana, Kenya, Madagascar, The Philippines, Vietnam, South-West China, Ecuador and Papua New Guinea). During these small and low-budget expeditions, we have dissected tens of thousands of insect specimens, inspected them under microscope and, in case of positivity, obtained DNA for sequencing and smears for morphological analysis. Moreover, in several hundred cases we were able to get the found flagellates into culture and transfer them into our lab, so that they are available for future deep analyses, such as whole-genome sequencing.

Within 5 years, we have published more than 10 papers in which we have significantly extended the known diversity of monoxenous trypanosomatids, performed analyses of their geographic and or host-related distribution, mapped the host-parasite relationships, host range, transmission, etc.

However, we have also proceeded with using the strains introduced into culture for biochemical analysis, with the aim to correlate the evolutionary diversity with that of the mitochondrial metabolism. A paper on this quite new subject (**Sverakova** et al., ***Mol. Microbiol***., in press) will be published in the beginning of 2015.

One of the main aims of this line of research was to find a trypanosomatid that would be particularly informative in terms of emergence of parasitism from the free-living life style of bodonids, etc. While we were anticipating such a flagellate (if it existed) in Papua New Guinea or another unexplored exotic place, as a matter of fact such a species was found by us in mosquitoes on the outskirts of Prague. Already initial sequencing of 18S rRNA and glycer-aldehyde dehydrogenase indicated a highly unusual position of the parasite in question. Extensive phylogenetic analysis of a large concatenated alignment confirmed this notion and we have published our finding in rather prestigious journal: **Flegontov P.**, **Votýpka J.**, **Skalický T.**, Logacheva M.D., Penin A.A., Tanifuji G., Onodera N.T., Kondrashov A.S., Volf P., Archibald J.M. & **Lukeš J.** (2013) *Paratrypanosoma* is a novel early-branching trypanosomatid. ***Curr. Biol.*** 23, 1787-1793.

At present, we are studying details of cell biology, interstagial transitions and differences in transcriptomes among morphologically very different stages. Moreover, we are in the process of assembling and annotating the whole genome of *Paratrypanosoma*. We are convinced that this particular flagellate holds information that should provide insight into the switch to parasitism, as well as why and how various stages in complex life cycles of human-pathogenic trypanosomes and leishmanias emerged.

5. Heme metabolism and catalase

Listed among the main outcomes is our finding that the plant pathogen *Phytomonas* is capable of living, under cultivation conditions, in total absence of heme, an unprecedented observation. This story was published (Kořený L., Sobotka R., Kovářová J., **Gnipová A.**, **Flegontov P.**, Horváth A., Oborník M., Ayala F.J. & **Lukeš J.** (2012) Aerobic kinetoplastid flagellate *Phytomonas* does not require heme for viability. ***Proc. Natl. Acad. Sci. USA*** 109, 3808-3813) and received considerable attention (price of the rector of the university; press release, etc.).

Following this publication, we were asked to write a Pearls review on the subject into one of the top journals in the field, which we did (Kořený L., Oborník M. & **Lukeš J.** (2013) Make it,

take it or leave it: heme metabolism of parasites. ***PLoS Pathogens*** 9, e1003088), which got until present over 10,000 downloads. It is our intention to publish in 2015 a much extended version of this paper.
This line of research remains active in our lab, as we now switched to *T. brucei*, both procyclic and bloodstreams stage, with the aim to investigate its heme metabolism. In collaboration with Dr. Benoit Vanhollebeke (University of Brussels, Gosselies), we are analyzing heme import and have preliminary data strongly indicating that, counterintuitively, the bloodstream stage may also go without any heme. This finding and further characterization of two possibly alternative importers of heme will be of general interest, with possible medical implications.
In a separate project, we are trying to determine the function of catalase, a virtually ubiquitous enzyme which was remarkably retained only in monoxenous (insect-only) trypanosomatids, but independently at least three times lost in their dixenous (alternating among insect and vertebrate hosts) kins (our unpubl. data). When a catalase from monoxenous *Leptomonas* is overexpressed in *T. brucei*, it remains active in the procyclic stages, but gets suppressed in the bloodstream (= mammal-infective) stage. Moreover, we have found out that apicomplexan parasites that invade blood of their hosts (malarial *Plasmodium* spp., *Theileria* spp., *Babesia* spp.) have also lost this exceptionally potent enzymes, and therefore conclude that blood parasitism seems to be incompatible with active catalase. We are further exploring this intriguing observation. As a matter of fact, this project already intertwines with the heme projects, as catalase is a heme containing protein, and its activity is therefore fully dependent on the availability of this cofactor.

6. <u>Protist diversity and evolution</u>
Our laboratory is genuinely interested in the diversity of protists, as the current studies on which we have participated (Pawlowski et al., ***PLoS Biology*** 2012) revealed that up to 70% of all extant eukaryotic diversity is hidden in the unicellular groups.
As one of the output is listed a review on microsporidia, one of the most diverse and enormously variable groups (Vávra J. & **Lukeš J.** (2013) Microsporidia and "the art of living together". ***Adv. Parasitol.*** 82, 253-319). This review got substantial attention and has been already cited more than a dozen times.
Even more visible is a joint view of a number of specialists on protists, who teamed up in their revised classification (Adl S.M., Simpson A.G., Lane C.E., **Lukeš J.**, Bass D., Bowser S.S., Brown M., Burki F., Dunthorn M., Hampl V., Heiss A., Hoppenrath M., Lara E., LeGall L., Lynn D.H., McManus H., Mitchell E.A.D., Mozley-Stanridge S.E., Wegener Parfrey L., Pawlowski J., Rueckert S., Shadwick L., Schoch C., Smirnov A. & Spiegel F.W. (2012) The revised classification of eukaryotes. ***J. Euk. Microbiol.*** 59, 429-493.). Head of the team (J. L.) is listed among first authors (not alphabetically) because of his extensive involvement in this work, which has been so far cited more than 170× and serves as a benchmark in the current view of protist diversity.
Diversity of protists goes in hand with their often rather flexible usage of genetic information. We have performed a study, which shed light on the establishment of frequently horizontally transmitted genes EFL and MATX, published in a prestigious journal (Szabová J., **Růžička P.**, **Verner Z.**, Hampl V. & **Lukeš J.** (2011) Experimental examination of EFL and MATX eukaryotic horizontal gene transfers: co-existence of mutually exclusive transcripts predates functional rescue. ***Mol. Biol. Evol***. 28, 2371-2378).

7. <u>Organellar and nuclear genomes of *Chromera velia*</u>
Chromera is a free-living photosynthetic predecessor of the obligatory parasitic lineage of Apicomplexa, which includes some of the most serious pathogens of humans such as *Plasmodium*. It thus represents a unique organism, the study of which shall shed light on the

emergence of the apicomplexan parasitism. With our collaborators (the Oborník lab, Institute of Parasitology; the Keeling lab, University of British Columbia, Vancouver), we have described several unique features of its plastid genome (Janouškovec et al., ***Proc. Natl. Acad. Sci. USA*** 2010), resolving a long-term controversy surrounding the origin of the apicoplast (cited over 100×). We have also participated in the description of the life cycle of *Chromera velia* and currently have demonstrated that its plastid genome departs from the canonical plastid in several unique ways (Janouškovec J., Sobotka R., Lai D.-H., **Flegontov P.**, Koník P., Komenda J., Ali S., Prášil O., Pain A., Oborník M., **Lukeš J.** & Keeling P.J. (2013) Split photosystem protein, linear-mapping topology and growth of structural complexity in the plastid genome of *Chromera velia*. ***Mol. Biol. Evol.*** 30, 2447-2462).
Very recently did we succeed in publishing the mitochondrial genome of *Chromera* and *Vitrella*, again in acclaimed ***Mol. Biol. Evol.***, where it will appear in March 2015. It contains several substantial findings, including the confirmed unprecedented disruption of the respiratory chain. Moreover, in January 2015, in an extensive collaborative effort lead by Dr. Arnab Pain (King Abdullah University, Jedda, Saudi Arabia), but also with a significant contribution from the Institute of Parasitology (we had a joint grant on the subject), a publication on high-quality nuclear genome of *Chromera* and *Vitrella* assembly, annotation and analysis was submitted in a prestigious journal.
Our interest in apicomplexan parasites is reflected by our designation and implementation of an assay capable of diagnosing malaria from human faeces (**Jirků M.**, Pomajbíková K., Petrželková K.J., Hůzová Z., Modrý D. & **Lukeš J.** (2012) Detection of *Plasmodium* in human feces. ***Emerg. Infect. Dis.*** 18, 634-636), published in a respected epidemiology journal. This approach will be useful especially for diagnosis in children, the most vulnerable category of patients.

8. <u>Origins and mechanisms of innovation in the cell</u>

It is beyond doubt that in the course of evolution of the living world, complexity generally increases. However, the ways how this is achieved are hotly debated. We decided to contribute to this discussion by elaborating the constructive neutral evolution theory into what we called "Irremediable complexity". In the end this resulted in 4 papers published in co-authorship with four colleagues from the Canadian Institute for Advanced Research, and especially the Science paper (Gray M.W., **Lukeš J.**, Archibald J.M., Keeling P.J. & Doolittle W.F. (2010) Irremediable complexity? ***Science*** 330, 920-921) stirred interesting discussion in high profile journals.
However, even follow-up paper such as (**Lukeš J.**, Archibald J.M., Keeling P.J., Doolittle W.F. & Gray M.W. (2011) How a neutral evolutionary ratchet can build cellular complexity. ***IUBMB Life*** 63, 528-537) was cited by authorities in the field (including reviews in ***Sci. Amer.*** etc.), as it elaborated the principle of complex cellular machines and processes that we claim might evolve in the absence of selective advantage via constructive neutral evolution (CNE). We suggest that CNE-based evolutionary scenarios are less forced than the selectionist or adaptationist narratives that are generally told.

9. <u>Functional analysis of respiratory complexes in *T. brucei* (Zíková lab)</u>

Characterization of *in vivo* purified respiratory complexes of *T. brucei* showed unexpectedly high divergence in protein composition compared to the well-studied yeast and bovine respiratory complexes. Thus, the unique properties of the *T. brucei* respiratome expand our knowledge about the evolution and function of the mitochondria. Moreover, the differences between parasitic *T. brucei* and mammalian cells might be exploited as potential chemotherapeutic targets (Acestor N.*, **Zíková A.***, Dalley R.A., Anupama A., Panigrahi A.K.,

Stuart K.D. (2011). *Trypanosoma brucei* mitochondrial respiratome: composition and organization in procyclic form. ***Mol Cell Proteomics*** 10 (9): M110.006908).

Another project representing this team involves functional characterization of *T. brucei* mitochondrial (mt) respiratome with i) extreme composition, as respiratory complexes contain many novel and unique subunits; ii) their abundance, activity and function are in *T. brucei* developmentally regulated; iii) the mt F_oF_1 ATPase maintains the mt membrane potential ($\Delta\Psi$) in bloodstream stage and thus possesses unique, essential and irreplaceable function in the infectious stage of the parasite.
A significant progress was achieved in the description of these complexes, for which we have established a routine protocol to purify F_1 ATPase in order to obtain protein crystals. Moreover, we identified two unique subunits of this enzyme, namely subunits p18 and F_1 ATPase subunit alpha, which is cleaved in *vivo* by a putative protease at two different positions. We have also thoroughly characterized function of the inhibitory peptide 1 (IF1), which inhibits futile ATP hydrolysis by F_oF_1 ATPase under ischemic conditions. We studied its function in *T. brucei in vivo* and *in vitro* and showed that when its ectopic copy was over-expressed in the infectious mammalian stage, the mt $\Delta\Psi$ collapsed, leading to the death of the parasite. Importantly, IF1 cannot inhibit bovine F_1 ATPase, strengthening the differences between the parasite and mammals, exploitable for chemotherapeutic interventions.
Using RNAi, we were able to characterize function of several novel subunits of trypanosome F_oF_1 ATPase. We demonstrated that ATPaseTb2 is membrane-bound and localizes with monomeric and multimeric assemblies of the F_oF_1-ATPase, and its loss in flagellates lacking kDNA led quickly triggered growth phenotype (**Šubrtová** et al. & **Zíková A.**, ***PLoS Pathogen***s, in press).
Furthermore, we described functional and structural interactome of *T. brucei* AAC showing that unlike in yeast and mammals, TbAACi is not a stable component of respiratory complexes IV, III or the ATP synthasome, but rather functions as a physically separate entity in this highly diverged eukaryote (**Gnipová**, et al. & **Zíková**, ***Eukaryot. Cell***, in press).

<u>The sum of IFs of papers published by the Lukeš team were as follows:</u>
2010: 99.4
2011: 56.5
2012: 96.3
2013: 111.4
2014: 55.4

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Evolutionary Parasitology

1. Laboratory of Evolutionary Protistology

Head: Miroslav Oborník

The lab is primarily focused on the evolution of single-celled eukaryotes (protists). We are particularly mostly working with members of the SAR group (alveolates, stramenopiles, chlorarachniophyte *Bigelowiella natans*), but also with phototropic excavates (genus *Euglena*) and cryptophyte *Guillardia theta*. We have also participated on phylogenetic studies of other organisms such as phototrophic bacteria, fungi and microsporidia.

Chromerids allow to study evolution of parasitism in Apicomplexa

Chromerid algae *Chromera velia* and *Vitrella brassicaformis* represent the main topic in the lab. We have participated in their discovery (Moore et al., 2008; Oborník et al., 2012), description of ultrastructure, morphology and life cycles (Oborník et al., 2011, 2012), analysis of their plastid genomes (Janouškovec et al., 2010), peculiarities in their heme synthesis (Kořený et al., 2011), as well as their environmental distribution (Janouškovec et al., 2012, 2013). The existence of *C. velia* itself (Moore et al., 2008) in fact confirms the predictive ability of evolutionary theory. It was proposed, based on the discovery of non-photosynthetic relict plastid in Apicomplexa that these dangerous obligate parasites of animals evolved from a phototrophic alga (McFadden et al., 1996). The confirmation came in 2008, when the algae were isolated from Australian stony corals by procedure usually used to isolate intracellular symbionts. It appeared that *C. velia* and also later described *V. brassicaformis* are the closest known phototrophic relatives to apicomplexan parasites. They seem to be actually related to colpodellids (Moore et al., 2008; Oborník et al., 2009; Lille and Slamovits 2014), aquatic alveolate predators possessing a primitive apical complex, and forming a sister group to obligatory parasitic Apicomplexa. The plastids in chromerids are surrounded by four membranes and their pigment compositions show the surprising lack of chlorophyll c, the hallmark of plastids derived from a rhodophyte by secondary endosymbiosis (Moore et al., 2008; Janouškovec et al., 2010; Oborník et al., 2012). The plastid genome of *C. velia* is linear, with inverted repeats of the *orf264*, *psbA* and *atpH2* genes at both ends of the linear molecule. It has also been shown that two generally conserved genes *atpB* and *psaA* are uniquely split into two fragments, which produce separate transcripts and consequently separate proteins, shown to assemble into functional photosystem and ATP synthase (Janouškovec et al., 2013). The genome displays non-canonical gene organization and reduced gene set. The genes encoded on the plastid genome of *C. velia* are highly divergent, unexpectedly long and also pseudogenes are present. In contrast, plastid genome of *V. brassicaformis* forms a compact circle with canonical organization showing high gene density and GC content. Since chromerid plastid genomes overlap with that of dinoflagellates and apicomplexans, reasonable evolutionary comparison was made. It is the structure of plastid super-operon that particularly strongly supports a common ancestry of plastids in apicomplexans, *Chromera*, *Vitrella*, dinoflagellates and heterokont algae (Janouškovec et al., 2010). Chromerid algae, particularly *C. velia*, may became an important model to study evolution of parasitism in Apicomplexa. We are since 2010 participating in sequencing of the genomes of both chromerids; the

genomic paper has just been submitted. The knowledge of the genome sequence is a requisite necessary to become a biological model.

Heme pathway reflects passed endosymbiotic events

We are interested in the evolution of tetrapyrrole biosynthesis in eukaryotes for a long time. During last five years we have studied particularly heme pathway in several algae with secondary plastid and also in kinetoplastid flagellates. First, since the heme pathway is specifically modified in Apicomplexa (Van Dooren et al., 2012), we looked at the pathway in chromerid algae. We showed that *C. velia* synthesizes heme through pathway homologous to that in Apicomplexa: aminolevulinate is synthesized in the mitochondrion by heterotrophic C4 pathway. Thus *C. velia* (and likely also *V. brassicaformis*) becomes to be the only phototroph on Earth synthesizing chlorophyll from glycine instead of glutamate. To investigate intracellular location of enzymes involved in the heme biosynthesis we predicted targeting sequences at N-termini of proteins, suggesting the plastid location of all involved enzymes with a single exception of aminolevulinate synthase (ALAS) (Kořený et al., 2011). The locations of four proteins [ALA synthase (ALAS), ALA dehydratase (ALAD), uroporphyrinogen synthase (UROS), and ferrochelatase (FeCH)] were investigated using xenotransfections in the diatom *Phaeodactylum tricornutum* and the apicomplexan parasite *Toxoplasma gondii*, and specific antibodies. Although performed xenotransfections show ambiguous results, antibodies undoubtedly display location of all four enzymes to the mitochondrion of *Chromera*. Such placement of heme pathway is unprecedented and may even explain the loss of photosynthesis in Apicomplexa and the consequential switch to the parasitic life style (Kručinská et al., in prep). We also mapped the pathway in the excavate alga with green secondary plastid, *Euglena gracilis*. We showed that this alga contains two separated redundant pathways for heme biosynthesis; one is located in mitochondrion and cytosol and represents an original heterotrophic metabolic route, while the second is entirely located in plastid showing origin in the green alga engulfed in the process of secondary endosymbiosis (Kořený and Oborník, 2010). We have found similar arrangement of the pathway also in the chlorarachniophyte *Bigelowiella natans* (Curtis et al., 2012; Cihlář et al, in prep). This may suggest a recent acquisition of secondary green plastids in chlorarachniophytes and euglenozoans. We also propose that such coexistence of redundant heme pathways of an exosymbiont and an endosymbiont origins was present in any plastid endosymbiosis. We have investigated the heme pathway in kinetoplastid flagellates. In a current opinion article we have summarized the knowledge of the heme synthesis and draw the evolutionary history of heme tropism in kinetoplastid flagellates (Kořený et al., 2010). We have also participated in the discovery of the organism, kinetoplastid *Phytomonas serpens*, which does not need heme for viability (Kořený at al., 2012). We summarized the knowledge on the heme pathway in parasites in a short review in PLoS Pathogens (Kořený et al., 2013).

Broken respiratory chain in *Chromera velia*

We have participated on the sequencing the mitochondrial genome of *C. velia* and consequential reconstruction of its respiratory chain. We showed that the chain is broken into two functionally non-related parts. The complexes I and III are missing from respiratory chain of *C. velia*. Function of missing complex III is uniquely substituted by L- and D-lactate cytochrome c oxidoreductases passing electrons on complex IV. Phylogenetic analysis of non-canonical proteins involved in the electrons flow (NDH2, ETFQO, G3PDH, DHODH, SQO, G14LDH) shows eukaryotic origins in most of these proteins; only one of SQO homologs and DHODH shows possible bacterial origins (Flegontov et al., 2015).

Reduced photosystems in chromerids

Composition of photosystems I and II together with ATP synthase complex and b_6f cytochrome complex were investigated in algae with secondary plastids and also in plants, rhodophytes and cyanobacteria. We found substantial reduction mostly of small proteins

stabilizing the complexes throughout complex algae. However, *Chromera* and *Vitrella* appears to possess the most reduced photosystems, 33% of proteins found in the complexes in cyanobacteria are missing from *Chromera*. Phylogenomic analysis of 60 investigated proteins show possible origin of chromerid plastids via tertiary endosymbiosis with a eustigmatophyte (Esson et al., in prep).

Urea pathway in diatoms

We participated on the discovery of urea pathway in diatoms. We contributed with the evolutionary analysis of carbamoylphosphate synthase (CPS), which demonstrates an existence of two CPS in diatoms; one is involved in the pyrimidine biosynthesis while the second is mitochondrially targeted providing substrate for the urea cycle. We identified two duplication events in the CPS evolutionary history and several switches of CPS substrate specificity (Allen et al., 2011).

Gene in the process of endosymbiotic transfer

We have investigated a rare case of gene in the process of endosymbiotic transfer. In the diatom *Thalassiosira pseudonana* we have identified two copies of psb28 gene, one encoded in the nucleus while the second is still encoded in the plastid genome. The nuclear version has changed to such an extent that it was rather difficult to confirm the relations between both psb28 genes. It was finally proved by the use of highly advanced (CAT, CAT-PB) model in frame of PhyloBayes. It is also obvious that location of a gene substantially affects its amino acid composition (Jiroutová et al., 2010).

Other projects

In addition to the mentioned projects we have participated in various studies on the evolution of apicomplexan parasites (Quablan et al., 2012, 2013; Gallusová et al., 2014), ciliates (Pomajbíková et al., 2013), psychotropic fungi (Borovička et al., 2011, 2015), *Chromera velia* (Burki et al., 2013), microsporidia (Valenčáková et al., 2012; Hyliš et al., 2013) and phototrophic bacteria (Koblížek et al., 2011, 2015).

2. Laboratory of Molecular Phylogeny and Evolution of Parasites

Head: Jan Štefka

The laboratory has for several years been focused on two research areas: coevolutionary analyses of host-parasite systems and symbiotic associations between insects and bacterial symbionts. Both of these research lineages utilize mainly the approaches of molecular phylogenetics and genomics. Recently, their methodological and conceptual aspects converged to a degree when complex projects could be established that encompass both research areas into a single framework (support by the GACR grant). The following models and questions were investigated within the last five years:

Turtle blood parasites of the genus *Hemolivia* (Apicomplexa)

The research was supported by four-year (2011–2014) grant P506-11-1738 by GACR: "Population structure and evolutionary relationships of the intracellular parasite *Hemolivia mauritanica*". We established several basic characteristics of this so far neglected organisms: 1) All samples of different morphological forms of *H. mauritanica* form an independent monophyletic lineage within hemogregarines. 2) The genus *Hemolivia* is closely related to the genus *Hepatozoon.* While the *Hemolivia* is clearly monophyletic, its genetic (but not morphological) similarity to *Hepatozoon* is very high and the latter genus may possibly be paraphyletic in respect to *Hemolivia*. 3) The inner genetic structure of *Hemolivia* is sufficient for considering its lineages as independent species.

Eimeria

While eimerians are widespread parasites with broad spectrum of hosts, the degree of their host specificity, its phylogenetic conservativeness and origin are virtually unknown. Our

phylogenetic analyses of extensive sample from different hosts (from 16 small-mammal genera, mostly rodents) indicate that the clustering of eimerian species is partly influenced by the host specificity, but does not arise from a cophylogenetic/cospeciation process; while several clusters are specific to a particular host group, inner topologies within these clusters do not reflect host phylogeny. This observation suggests that the host specificity of *Eimeria* is caused by adaptive rather than cophylogenetic processes.

Host-parasite co-evolution on Galápagos Islands

Parasites' phylogenies often track the evolutionary history of their hosts. Incongruences in the evolutionary history of closely associated lineages can be explained through a variety of possible events including host switching and host independent speciation, but sometimes also methodological inaccuracies. The relatively simple biogeographic arrangement of the Galápagos archipelago, compared with mainland biomes, provides a framework to identify stochastic and evolutionary informative components of genealogic data in recently diverged organisms. Mitochondrial and nuclear sequence data of Galápagos mockingbirds and their parasites were studied on 11 islands (Štefka et al., 2011). Differences in population genetic diversity between all taxa and varying degrees of topological congruence between host and parasite lineages were found. A very low level of genetic variability and lack of congruence was found in one of the louse parasites, which was excluded from subsequent multi-species analysis. Reconciled multi-species tree was congruent with both the nuclear data and the geological history of the islands.

The studied system provided a model for understanding evolutionary forces affecting the genetic character of populations of the hosts and parasites colonizing new habitats. Gene genealogies of Galápagos mockingbirds and two of their ectoparasites showed strong phylogeographic correlations, with instances of incongruence mostly explained by ancestral genetic polymorphism. Genealogy of the third parasite showed low levels of genetic diversity and little evidence of co-phylogeny with their hosts. These differences can mostly be explained by variation in life-history characteristics, primarily host specificity and dispersal capabilities.

Parasites and their hosts: invasion and conservation biology of hosts and parasites

Several studies carried out in the laboratory contain implications for invasion and conservation biology. Study of biogeography of the fish parasite *Ligula intestinalis*, pointed to a cryptic introduction of the parasite to northern Africa with infected fish stock approximately 50 years ago (Bouzid et al., 2013). In a collaborative project the origin of European populations of an invasive parasite of deer, the giant river fluke, was traced back to several areas in North America (Králová-Hromadová et al., 2011). Most recently, the study of allelic diversity of the Major Histocompatibility Complex in Galápagos mockingbirds revealed a relatively high number of alleles in threatened populations of the Floreana mockingbird (MS in preparation). The variability was preserved despite severe bottleneck lasting over multiple generations. Obtained data will provide a baseline for the planned reintroduction efforts.

Phylogenetic origin(s) of insect bacterial symbionts

To overcome well -known problems with phylogenetic reconstructions in bacterial symbionts (due to highly aberrant DNA), we assembled the richest taxon sampling of the Enterobacteriaceae to date (50 taxa, 69 orthologous genes with no missing data) and analyzed both nucleic and amino acid data sets using various modifications of standard phylogenetic methods. Our results strongly suggest independent origins of four symbiotic clusters and confirm the efficiency of several artefact-reducing methods. We also noted and suggested a possible origins of intracellular symbiotic bacteria from gut-associated or pathogenic bacteria. Within this phylogenetic framework, we described and characterized several new lineages of the symbiotic genera *Sodalis* and *Arsenophonus*. As particularly important we see the characterization of new symbiotic system in blood feeding insect *Melophagus ovinus*. This

symbiosis potentially provides a useful comparative model to the symbiosis in tsetse flies symbiont.

This phylogenetic framework has been extended by genomic analyses of the endosymbionts from four species of the Hippoboscidae (*M. ovinus, Lipoptena cervi, Ornithomya biloba,* and *Crataerina pallida*). The results suggest recurrent replacements of obligate symbionts as the main driver of symbiosis in this parasitic group. Facultative *Arsenophonus* symbionts act as source of the obligate symbionts and they frequently replace already established obligate *Arsenophonus* symbionts. This finding sheds light on the long-term discussion about poorly supported and/or ambiguous results of phylogenetic analyses in this group. Perhaps the most illuminating example is *Crataerina pallida*, where we found an obligate *Arsenophonu*s symbiont retaining a large genome highly similar to the genomes of facultative *Arsenophonus* species such as male-killing *A. nasoniae*. PCR-based screening has surprisingly recovered in Olfersini (group within Hippoboscidae) a monophyletic symbiont.

3. Laboratory of Environmental Genomics

Head: Aleš Horák

Our understanding of the basic principles of life is mostly based on the detailed study of few model (and mostly multicellular) organisms, majority of which belongs to metazoans, fungi and plants (including green algae). However, these three lineages represent only a small fraction of eukaryotic diversity. In fact, the vast majority of the world's biota is unicellular, not cultivable and therefore mostly unknown. In light of this, the current biological paradigms are thus inevitably incomplete (if not incorrect).

Laboratory of Environmental Genomics was founded in February 2013 and its main aim is to describe the diversity and the ecological role of selected protistan lineages using the power of the next-generation sequencing and electron microscopy.

Evolution of Apicomplexa

Apicomplexans belong to the major eukaryotic group Alveolata and most representatives are the obligate intracellular parasites of metazoans. However, basal lineages comprise of predators (colpodellids), phototrophs ('chromerids', ARLV clade) and commensals/extracellular parasites (archigregarines). Using the in-house developed bioinformatics protocols, we have discovered the novel, putatively photosynthetic lineage of apicomplexans (clade ARLV) and suggested it is the strong link to coral reefs (Janouškovec et al., 2012; Janouškovec et al., 2013). Unfortunately, our attempts to get the representatives of the ALRV clade into the culture failed.

Most apicomplexan lineages bear remnants of photosynthetic organelle (retained its function in 'chromerids' and probably also ARLV) called apicoplast. Its origin has been subject of debate since its discovery. The detailed phylogenomic analysis of the photosynthetic plastid of 'chromerids' revealed that the organelles of apicomplexans and dinoflagellates share common ancestor and the original donor was red alga (Janouškovec et al., 2010). The most recent results suggest its tertiary origin from heterokont algal lineage Limnista (Ševčíková, Horák et al., submitted).

We also try to bring genomic and transcriptomic data for several minor lineages (archigregarines, agamococcidians), which may be pivotal for our understanding of evolution of parasitism in Apicomplexa.

Analysis of biodiversity of oceanic plankton

We have established an active cooperation with the *Tara* consortium on analysing diversity of excavate protists among the oceanic plankton samples using metabarcoding approach and also general analysis of protistan diversity using scanning electron microscopy and its comparison to molecular data. The preliminary results of the first part revealed unexpected and extreme abundance and diversity of marine diplonemids (ranked as the third most diverse

and seventh most abundant planktonic group). The results were submitted as a part of the global analysis to Science (De Vargas et al., under review). We are also waiting for the full dataset to perform more detailed analysis, focused only on diplonemids. SEM analysis is under way and will continue in 2015.

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Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Tick-borne diseases

Spirochetes from the *Borrelia burgdorferi* sensu lato complex, the causative agents of Lyme borreliosis, and tick-borne encephalitis virus, the causative agent of tick-borne encephalitis, are the main focus areas of research of the Tick-Borne Diseases team. Our research interests include, but are not limited to, molecular biology, clinical microbiology, molecular epidemiology, and pathogenesis of tick-transmitted pathogens of medical importance; molecular and evolutionary genetics of bacterial and viral pathogens; analysis of molecular and cellular factors that are involved in the mechanism of pathogen transmission by tick vectors including protein-carbohydrate interactions; implementation of proteomic and transcriptomic approaches in search of novel candidates for anti-tick vaccines against Lyme borreliosis and babesiosis in animal models; tick immune response to pathogen invasion and analysis of tick antimicrobial and defense proteins for development of new generation antibiotics.

The "Tick-Borne Diseases" team includes two research laboratories: 1) the Laboratory of Molecular Ecology of Vectors and Pathogens (*head L. Grubhoffer*) and 2) the Laboratory of Arbovirology (*head D. Růžek*).

1) Laboratory of Molecular Ecology of Vectors and Pathogens

Molecular epidemiology, pathogenesis and evolutionary genetics of tick-transmitted pathogens of medical importance

•Analysis of genetic diversity of spirochetes from *Borrelia burgdorferi* sensu lato complex worldwide, using protocols developed in our laboratory for multi locus sequence analysis (MLSA) resulted in the description of two novel spirochete species from North America in 2009: rodent originated *B. carolinensis* and bird originated *B. americana*. Validation of the *B. carolinensis* species was completed in 2011 *(**Rudenko N., Golovchenko M., Grubhoffer L.,** Oliver J.H., Jr. 2011:* Borrelia carolinensis *sp. nov., a new species of* Borrelia burgdorferi *sensu lato complex isolated from rodents and a tick from the south-eastern USA. International Journal of Systematic and Evolutionary Microbiology, 61: 381–383).*

•Using multilocus sequence typing (MLST) that involved 8 housekeeping genes (*clpA, clpX, nifS, pepX, pyrG, recG, rplB, and uvrA*), we studied the phylogenetic relationship of spirochaete strains from North America previously assigned to the genospecies *B.bissettii*. Genetic distance analyses confirmed that some strains are more distant to *B. bissettii* than they are to *B. carolinensis*, which suggested that they constitute a new *Borrelia* genospecies, proposed to be named *B.kurtenbachii* sp. nov. in honour of the late Klaus Kurtenbach. We showed that ecological differences between *B. bissettii* and the new *Borrelia* genospecies reflect different transmission cycles *(Margos G., Hojgaard A., Lane R.S., Cornet M., Fingerle V., **Rudenko N.**, Ogden N., Aanensen D.M., Fish D., Piesman J. 2010: Multilocus sequence analysis of* Borrelia bissettii *strains from North America reveals a new* Borrelia *species,* Borrelia kurtenbachii. *Ticks and Tick- borne Diseases, 1: 151–158).*

•The altitudinal shift in the limit of *Ixodes ricinus* occurrence above the previously established altitude of 750 m above sea level has been monitored over the long-term (2002–

2008) in the Krkonose Mts. (Giant Mts.), the highest in the Czech Republic, along two vertical transects in their eastern and central parts (600–1020 and 600–1270 m). TBEV RNA was detected in 26 ticks at up to 1140 m. *B. burgdorferi* s.s. was found at up to 1040–1065 m, *B. garinii* and *B. afzelii* up to 1080–1140 m, and *B. valaisiana* up to 1270 m. Double to quadruple coinfections were detected.The detection of *B. burgdorferi* s.l. and TBEV in host-seeking larvae indicates an autochthonic infection. Upon analysis of the local climate, we consider climate warming to be responsible for the spreading of ticks and tick-transmitted pathogens to higher altitudes *(Danielova V., Daniel M., Schwarzova L., Materna J.,* ***Rudenko N., Golovchenko*** *M., Holubova J.,* ***Grubhoffer L., Kilian P.*** *2010: Integration of a tick-borne encephalitis virus and* Borrelia burgdorferi *sensu lato into mountain ecosystems, following a shift in the altitudinal limit of distribution of their vector,* Ixodes ricinus *(Krkonoše Mts., Czech Republic), Vector-Borne and Zoonotic Diseases, 10: 223–230).*

•The epidemiological data from many European countries and the USA show a dramatic increase in diagnosed cases of LB during the last several decades. Involvement of new or unknown *Borrelia* species into human LB changed drastically the clinical manifestation of the disease. We re-evaluated the impact of known *B. burgdorferi* sensu lato species on public health, proving that the spectrum of species with potential for human infectivity is wider than previously considered, thus LB may now be mimicking a wider spectrum of diseases ***(Rudenko N., Golovchenko M., Grubhoffer L.,*** *Oliver J.H., Jr. 2011: Updates on* Borrelia burgdorferi *sensu lato complex with respect to public health. Ticks and Tick-borne Diseases, 2: 123–128).* This research was an extension of our project dealing with development of novel laboratory techniques for detection and analysis of LB spirochetes in environmental and clinical samples ***(Rudenko N., Golovhenko M.,*** *Oliver Jr. J. H.,* ***Grubhoffer L.*** *2011: Chapter 96th,* Borrelia*. In: D. Liu (Ed.), Molecular Detection of Human Bacterial Pathogens. Taylor and Francis CRC Press Publ., Florida, USA, pp. 1149–1162).*

•The neutrophil extracellular trap (NET) consists of the extrusion of the neutrophil's own DNA, forming traps that can retain and kill bacteria. The production of reactive oxygen species is apparently associated with the onset of NETs (NETosis). We described NET formation at the tick bite site *in vivo* in mice and showed that *B. burgdorferi* sensu stricto spirochetes become trapped and killed by NETs in humans, and that the bacteria do not seem to release significant nucleases to evade this process *(Menten-Dedoyart C, Faccinetto C,* ***Golovchenko M,*** *Dupiereux I, Van Lerberghe PB, Dubois S, Desmet C, Elmoualij B, Baron F,* ***Rudenko N,*** *Oury C, Heinen E, Couvreur B. (2012) Neutrophil extracellular traps entrap and kill* Borrelia burgdorferi *sensu stricto spirochetes and are not affected by* Ixodes ricinus *tick saliva. Journal of Immunology 189: 5393–5401).*

•We determined tick abundance and the prevalence of different *Borrelia* genospecies in three sites in the Siebengebirge, Germany, which were already examined in the years 1987, 1989, 2001 and 2003. We found that, over the last two centuries, tick densities have changed in the Siebengebirge at sites that remained unchanged by human activity since they belong to a nature reserve. Abiotic and biotic conditions most likely favored the host-seeking activity of *I. ricinus* and the increase of multiple *Borrelia* infections in ticks. These changes have led to a potential higher risk of humans and animals to be infected with Lyme borreliosis *(Schwarz A.,* ***Hönig V., Vavrušková Z., Grubhoffer L.,*** *Balczun C., Albring A., Schaub G.A. (2012) Abundance of Ixodes ricinus and prevalence of Borrelia burgdorferi s.l. in the nature reserve Siebengebirge, Germany, in comparison to three ormer studies from 1978 onwards. Parasites & Vectors 5: 268).*

•We conducted the first extensive comparative analysis of *ospC* genes from 127 *B. burgdorferi* s.s. strains collected in European and North American endemic and non-endemic regions. We showed the close relatedness of geographically distinct populations of *B. burgdorferi* s.s. We also demonstrated that *ospC* genotypes commonly associated with human LB in European and North American regions where the disease is endemic were detected in *B. burgdorferi* strains isolated from the non-human-biting ticks and rodent hosts in non-endemic

regions of the United States. We discovered that some *ospC* alleles previously known only from Europe are widely distributed in the southeastern United States, a finding that confirms the hypothesis of transoceanic migration of *Borrelia* species, and questions the 'American dogma' about the Lyme disease in North America *(**Rudenko N., Golovchenko M., Hönig V.,** Mallátová N., Krbková L., Mikulášek P., Fedorova N., Belfiore N.M., **Grubhoffer L.,** Lane R.S., James H. Oliver, Jr. (2013) Detection of* Borrelia burgdorferi *sensu stricto ospC alleles associated with human Lyme borreliosis worldwide in non-human-biting tick* Ixodes affinis *and rodent hosts in southeastern United States. Applied and Enviromental Microbiology 79: 1444–1453).*

•Additional confirmation of the hypothesis of transoceanic spirochete migration was the detection of the globally rare *B. burgdorferi* s.s *ospC* allele L strains, considered to be restricted to Europe, in a coastal plain area of Georgia and South Carolina in the southeastern United States *(**Rudenko N., Golovchenko M., Grubhoffer L.,** Oliver J.H., Jr. 2013. Rare ospC allele L of Lyme disease spirochete* Borrelia burgdorferi *sensu stricto is common in tick* Ixodes affinis *and rodent hosts in southeastern United States. Applied and Environmental Microbiology, 2013, 79: 1403–1406).*

•*OspC* type L was considered to be non-invasive and *B. burgdorferi* strains with this *ospC* type, presumably, were uninfectious to humans. Using direct diagnostics we confirmed the ability of *B. burgdorferi ospC* type L strains to disseminate into host tissues and to cause systemic disease. Invasive potential of *B. burgdorferi* s.s. *ospC* type L strains increases the possible disease risk to humans in the regions of their distribution *(**Golovchenko M.,** Sima R, Hajdusek O., **Grubhoffer L.,** Oliver Jr. J.H. and **Rudenko N.** 2014. Invasive potential of Borrelia burgdorferi sensu stricto ospC type L strains increases the possible disease risk to humans in the regions of their distribution. Parasites & Vectors 2014, 7: 538).*

•Migratory passerine birds have been shown to be responsible for spreading *Borrelia*-infected ticks within and between continents. The dynamics of infection in birds supports the mixing of different species, the horizontal exchange of genetic information and appearance of recombinant genotypes, i.e. presenting a wide opportunity for genetic exchange. We reported the cross-species recombination that led to incorporation of a housekeeping gene from a *B. burgdorferi* s.s. strain into a homologous locus of another bird-associated strain. Our results support the hypothesis that recombination maintains a majority of sequence polymorphism within *Borrelia* populations because of the re-assortment of pre-existing sequence variants. We presented convincing evidence that the diversity and evolution of LB spirochetes is driven mainly by the host *(**Rudenko N., Golovchenko M.,** Belifore N.M., **Grubhoffer L.,** Olver Jr. J.H. (2014) Divergence of* Borrelia burgdorferi *sensu lato spirochetes could be driven by the host: diversity of* Borrelia *strains isolated from ticks feeding on a single bird. Parasites & Vectors, 7:4).*

•Transmission of vector-borne pathogens and infectious diseases between wildlife and domestic animals has become an issue of major interest. Zoos represent a unique environment, where exotic and native vertebrates, arthropods, and humans interact, providing opportunities for pathogen transmission or "sharing" infectious diseases. We collected serum samples from 69 animal species from 5 zoos located in different parts of the Czech Republic to evaluate the prevalence of antibodies against *Borrelia burgdorferi* (Bb) s.l. and tick-borne encephalitis virus (TBEV) in zoo animals. Our results indicate that a high number of animal species in the Czech zoos were exposed to Bb s.l. and that TBEV infection occurred in at least one of the investigated zoos. The risk of tick-borne infections to zoo animals was highlighted after the reported severe case of TBE in a monkey (*Macaca sylvanus*) kept in a monkey park in Germany *(Sirmarová J., **Tichá L., Golovchenko M., Salát J., Grubhoffer L., Rudenko N.,** Nowotny N., **Růžek D.** (2014) Seroprevalence of* Borrelia burgdorferi *sensu lato and tick-borne encephalitis virus in zoo animal species in the Czech Republic. Ticks and Tick-borne Diseases 5: 523–527).*

•*Ehrlichia* species are the etiological agents of emerging and life-threatening tick-borne human zoonoses that inflict serious and fatal infections in companion animals and livestock. We conducted a phylogenetic analysis of *Ehrlichia* isolated from *Rhipicephalus (Boophilus)*

microplus from Minas Gerais, Brazil. Based on molecular and phylogenetic analysis of the *16S rRNA, groEL, dsb* and *gltA* genes, we concluded that this tick-derived microorganism isolated in Brazil is a new species, named *E. mineirensis* (UFMG-EV), with predicted novel antigenic properties in the *gp36* ortholog glycoprotein (***Cabezas Cruz A,*** *Zweygarth E, Ribeiro MF, da Silveira JA, de la Fuente J,* ***Grubhoffer L, Valdés JJ,*** *Passos LM (2012): New species of Ehrlichia isolated from Rhipicephalus (Boophilus) microplus shows an ortholog of the E. canis major immunogenic glycoprotein gp36 with a new sequence of tandem repeats. Parasites & Vectors 5: 291).*

•The results of our analysis of distribution of *Borrelia miyamotoi*, a relapsing fever-related spirochete that has been recently shown to be a human pathogen transmitted by *Ixodes* ticks, confirmed its presence in 16 of the 26 sites surveyed, with infection prevalence as high as 15%. Ticks were collected from California, New York, Connecticut, Pennsylvania, and Indiana in the United States, and from Germany and the Czech Republic in Europe from 2008 through 2012. In addition, an isolate from Japan was characterized. We found 3 distinct genotypes, 1 for North America, 1 for Europe, and 1 for Japan *(Crowder C.D., Carolan H.E., Rounds M.A.,* ***Honig V.,*** *Mothes B., Haag H., Nolte O., Luft B.J.,* ***Grubhoffer L.,*** *Ecker D.J., Schutzer S.E., and Eshoo M.W. (2014). Prevalence of Borrelia miyamotoi in* Ixodes *ticks in Europe and the United States. Emerging Infectious Diseases 20: 1678–*1682*).*

Tick immune response to pathogens and analysis of tick antimicrobial and defense proteins

•Our recent investigation of the genes of *I. ricinus* involved in molecular mechanism of vector-pathogen interaction resulted in isolation of the genes encoding tick immune proteins involved in tick digestion, transmission of *B. burgdorferi*, antioxidant defense (ROS), and pathogen recognition *(****Grubhoffer L., Rego R. O. M.,*** *Hajdusek O., Hypša V.,* ***Kovář V., Rudenko N.,*** *Oliver Jr. J. H. 2010: Tick lectins and fibrinogen-related proteins. In: A. S. Bowman and P. A. Nuttall (Eds.), Ticks: Biology, Disease and Control. Cambridge University Press, Cambridge, UK, pp. 127–142), (****Grubhoffer L, Rudenko N,*** *Vancova M,* ***Golovchenko M, Sterba J*** *.2013: Circulatory system and hemolymph. In: Biology of ticks. Vol. 1. Daniel E. Sonenshine (Editor), R. Michael Roe (Editor). Oxford University Press Inc; 2nd Revised edition edition, pp. 258–286).*

•We found that the Der-p2 allergen-like protein gene was strongly induced by a blood meal in the gut and haemolymph of all developmental tick stages. Showing high similarity to mite allergens (30–35%), Der-p2 allergen-like protein is not an allergen in the sense of triggering an allergic reaction. It possess a different functional activity, or could be involved in the neutralization of IgE antibodies received with blood from people repeatedly bitten by ticks *(****Horackova J., Rudenko N., Golovchenko M., Grubhoffer L.*** *2010: Der-p2(Dermatophagoides pteronyssinus) allergen-like protein from the hard tick* Ixodes ricinus *– a novel member of ML (MD-2-related lipid-recognition) domain protein family. Parasitology, 137: 1139–1149).*

•Another *I. ricinus* gene strongly induced by blood meals is ML-domain containing protein (IrML), which belongs to the ML protein family, commonly occurs in diverse organisms and is involved in lipid binding and transport, pathogen recognition and in immune response. Using *in situ* hybridization, IrML transcripts were detected in type II and III salivary glands acini. Analysis of the predicted structure of *I. ricinus* ML-domain containing protein, and its localization in the tick body, suggest that IrML is a secreted protein and is involved in tick innate immunity *(****Horackova J., Rudenko N., Golovchenko M.,*** *Havlikova S.,* ***Grubhoffer L.*** *2010: IrML – a gene encoding a new member of the ML protein family from hard tick,* Ixodes ricinus*. Journal of Vector Ecology, 35: 1–9).*

•A key element of tick innate immunity is the speed with which immune defense reactions occur. Our earlier research led to identification and characterization of the first two defensins from the whole body cDNA subtracted libraries of *I. ricinus*-def1and def2, with the approximate frequency of appearance having been 4 : 1. Analyzing their genomic structure we

showed, for the first time, that *I. ricinus def1* and *def2* genes involve three exons and two introns, structures characteristic for soft tick defensins but unknown in the hard ticks. More recently, we found that expression of def1 mRNA was induced by blood intake at all life stages – larva, nymph and imago, while def2 mRNA expression was detected only in adult females with a significant increase after a blood meal. The amino acid sequences of def1 and def2 differ only in one residue in the mature peptide, where phenylalanine is present in def1 and arginine in def2. This substitution results in slightly different antimicrobial potential of def1 and def2. Synthetic def1 and def2 had an ability to inhibit Gram-positive bacteria in very low concentrations. *(**Chrudimska T.,** Slaninova J**., Rudenko N., Růžek D., Grubhoffer L.** 2011. Functional characterization of two defensin isoforms of the hard tick* Ixodes ricinus. *Parasites and Vectors, 4: 63; **Chrudimska T.,** Chrudimsky T., **Golovchenko M., Rudenko N., Grubhoffer L.** 2010: New defensins from hard and soft ticks: similarities, differences, and phylogenetic analyses. Veterinary Parasitology, 167: 298-303).*

• Further, we were able to identify six new defensins in the genome of the tick vector for borreliosis in Europe, *I. ricinus*. We detected their expression profiles in various organs and predicted their putative antimicrobial activity based on protein structure and phylogenetic similarities to other defensins *(**Tonk M., Cabezas-Cruz A., Valdes J.J., Rego R.O.M., Rudenko N., Golovchenko M.,** Bell-Sakyi, de la Fuente J., **Grubhoffer L.** (2014) Identification and partial characterisation of new members of the 2* Ixodes ricinus *defensin family. Gene 540: 146–152).*

•We also investigated the antimicrobial activity of two defensins from the U.S. Lyme disease tick vector *I. scapularis*. They showed antifungal activity against two *Fusarium* species, as well as antibacterial activity against the pathogen *Listeria grayii*. This work was in collaboration with Prof. A. Vilcinskas (Giessen, Germany) *(**Tonk M., Cabezas-Cruz A., Valdés J.J., Rego R.O.M., Chrudimská T., Strnad M.,** Šíma R., Bell-Sakyi L., Franta Z.K., Vilcinskas A., **Grubhoffer L.,** Rahnamaeian M. (2014) Defensins from the tick* Ixodes scapularis *are effective against phytopathogenic fungi and the human bacterial pathogen Listeria grayi. Parasites & Vectors 7: 554).*

•Finally, defensin from the tick *Dermacentor marginatus* was investigated and found to have Gram-positive antibacterial properties. Using for the first time a plating method developed for studying various European *Borrelia* genospecies, we were able to evaluate the anti-borrelial activity of this peptide, given that this tick is a non-vector of Lyme borreliosis in Europe. Quantitative analysis showed that the *D. marginatus* defensin is borreliacidal *(**Chrudimska T.,** Cerovsky V., Slaninova J., **Rego R. O. M., Grubhoffer L.** (2014) Defensin from the ornate sheep tick Dermacentor marginatus and its effect on Lyme borreliosis spirochetes. Developmental and Comparative Immunology 46: 165–170).*

• We are part of a new FP7 consortium (HEALTH call) ANTIDotE. ANTIDotE (ANti-tick vaccines to prevent TIck-borne Diseases in Europe) is an international research consortium that aims to identify and develop novel ways to prevent multiple human tick-borne diseases in Europe. Using state of the art proteomic and transcriptomic approaches, we work on identification and characterization of novel tick salivary gland proteins, which will be subsequently assessed as anti-tick vaccines to protect against Lyme borreliosis, babesiosis and tick-borne encephalitis in animal models *(Sprong H., Trentelman J., Seemann I., **Grubhoffer L., Rego R.O.**, Hajdušek O., Kopáček P., Síma R., Nijhof A.M., Anguita J., Winter P., Rotter B., Havlíková S., Klempa B., Schetters T.P., Hovius J.W. (2014) ANTIDotE: anti-tick vaccines to prevent tick-borne diseases in Europe. Parasites & Vectors 7: 77).*

•Being able to follow the population dynamics of any pathogen in vivo is important in understanding the bottlenecks associated with it. We completed an analysis of population dynamics of the Lyme disease pathogen by creating 7 isogenic clones of *B. burgdorferi* and then following them as a mixed population throughout a mouse/tick infectious cycle. We identified several bottlenecks that stochastically limit the complexity of wild type *B. burgdorferi* during the mouse/tick infectious cycle. We now have an experimental system that can be used to model the population dynamics of *B. burgdorferi* during its natural infectious

cycle. We anticipate that mathematical models based on these data can be used to predict key points when strategies to block transmission and reduce the prevalence of *B. burgdorferi* infection would be most effective *(**Rego R.O.,** Bestor A., Stefka J., Rosa P.A. (2014) Population bottlenecks during the infectious cycle of the Lyme disease spirochete* Borrelia burgdorferi. *PLOS ONE 9: e10100).*

Protein-carbohydrate interactions

•Fibrinogen-related proteins (FRePs) with lectin activity were studied in *Dermacentor* and *Rhipicephalus* ticks. All of the proteins were found to be glycosylated, and a cross-reaction of anti-FReP antibodies with the tick storage protein Hemelipoglycoprotein (HLGP) was discovered; however, the cross-reactivity is most probably dependent on the epitope similarity, as sequence similarity was not found between fibrinogen and FRePs on one hand or HLGP on the other. The hemagglutination activity of *Rhipicephalus* ticks was inhibited by sialic acid and sialylated glycoproteins, GalNAc, and GlcNAc, suggesting similarity to another FReP from *Ornithodoros moubata*, the Dorin M protein *(**Sterba J., Dupejova J.,** Fiser M., Vancova M., **Grubhoffer L.** 2011: Fibrinogen-relatedproteins in ixodid ticks. Parasites & Vectors, 4: 127*).

•Mass spectrometric analysis of *D. marginatus* HLGP N-glycans in collaboration with Prof. Novotny laboratory (Indiana University, IN, USA) showed the presence of high-mannose and complex type; paucimannosic type glycans were not observed. Lectin-activity of HLGP was studied in collaboration with Prof. Wimmerová laboratory (CEIT, Masaryk University, Czech Republic). Highest binding activity was found for galactose *(**Dupejova J., Sterba J.,** Vancova M., **Grubhoffer L.** 2011: Hemelipoglycoprotein from theornate sheep tick, Dermacentor marginatus: structural and functional characterization. Parasites & Vectors, 4: 4).*

•Sialylated N-glycans were detected in *Ixodes* and *Dermacentor* ticks. Both N-acetylneuraminic and N-glycosylneuraminic acid (NeuGc) were detected again in collaboration with Prof. Novotny group in Bloomington, IN. Antibodies against NeuGc were utilized for tracking of sialylated glycans in tick tissues, and thus we showed the route of sialylated host glycoproteins from the tick gut through the hemolymph to the salivary glands, where they are in part excreted back into the host *(Vancova M., **Sterba J., Dupejova J.,** Simonova Z., Nebesarova J., Novotny M.V.,**Grubhoffer L**. 2012: Uptake and incorporation of sialic acid by the tick* Ixodes ricinus. *Journal of Insect Physiology, 58: 1277–1287*).

•We used a combination of sialic acid quantitation and detection of metabolically incorporated sialic acid (Click-iT chemistry) to determine expression of sialylated glycoproteins by the ticks themselves. We showed that majority of sialic acid present in fed female *Ixodes ricinus* ticks is coming from the host and not the tick itself. This can be one of the immune system evasion strategies employed by ticks *(**Sterba J., Vancova M., Sterbova J.,** Bell-Sakyi L., **Grubhoffer L.** 2014: The majority of sialylated glycoproteins in adult* Ixodes ricinus *ticks originate in the host, not the tick. Carbohydrate Research 389: 93–99).*

•In collaboration with J. de la Fuente (IREC, Universidad de Castilla-La Mancha, Ciudad Real, Spain) we determined the importance of glycan moieties (NeuAc and NeuGc) for the infection of tick and host cells by the *Anaplasma marginale* MSP1a. While it was previously shown that *Anaplasma* infection of tick cells is dependent on the presence of core-fucosylated N-glycans, we confirmed for the first time the importance of sialic acid for the infection of tick cells. The presence of host sialylated molecules in tick tissues and on the surface of tick cells could explain these findings *(will be published in 2015).*

2) Laboratory of Arbovirology

The Laboratory of Arbovirology was established on January 1, 2014, by transformation of the previous Laboratory of Vector-Host Interactions headed by J. Kopecký. The team is quite small (one postdoc full time, two research scientists part time, two students part time and one technician part time), consisting of mostly junior scientists (the head is 33 years old).

Tick-borne encephalitis virus causes serious, potentially fatal neurological infections that affect humans in endemic regions of Europe and Asia. Our research projects (funded by the Czech Science Foundation and the Russian Academy of Medical Sciences) are focused on investigation of the interactions of tick-borne encephalitis virus with cells in the central nervous system (CNS), the role of host genetic background in the development of clinically diverse forms of tick-borne encephalitis, the development of nucleoside antivirals against tick-borne encephalitis virus, andthe development of novel candidate vaccines against tick-borne encephalitis.

• We investigated tick-borne encephalitis virus infection in the host CNS on several levels. We demonstrated that tick-borne encephalitis causes a serious breakdown of the host blood-brain barrier in mice as well as in human patients. This damage is not caused by the migration of immunocompetent cells into the CNS during the development of inflammation, but represents a bystander effect of the cytokine/chemokine and MMP-9 overproduction in the brain tissue (***Růžek D., Salát J.,*** *Singh S.K.,* ***Kopecký J.*** *(2011) Breakdown of the blood-brain barrier during tick-borne encephalitis in mice is not dependent on CD8+ T-cells. PLOS One 6: e20472.****;*** ***Palus M.,*** *Žampachová E.,* ***Elsterová J., Růžek D.*** *(2014) Serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 levels in patients with tick-borne encephalitis Journal of Infection 68: 165-169****;*** ***Palus*** *et al., J. Med. Virol., in press*). Within the CNS, the virus infects primarily neurons (***Růžek*** *et al., J. Gen. Virol., 2009; Bílý et al., Sci. Rep., under review*), but we were the first who described that astrocytes can be infected as well.

•In human primary neurons, we investigated the virus-cell interactions using several approaches, including electron tomography. We were the first to characterize the 3D topographical organization of membranous whorls and autophagic vacuoles in the virus-infected human neurons. We also demonstrated that autophagy, primarily an innate antiviral reaction, is evaded by tick-borne encephalitis virus, and, even more, this process is necessary for efficient virus replication in the neurons. We revealed that the infected astrocytes are the primary source of pro-inflammatory cytokines in the virus-infected brain, and contribute to the virus-induced neurotoxicity and blood-brain barrier breakdown ***(Palus M.,*** *Bílý T.,* ***Elsterová J., Langhansová H., Salát J.,*** *Vancová M.,* ***Růžek D****. (2014) Infection and injury of human astrocytes by tick-borne encephalitis virus. Journal of General Virology 95: 2411-2426.; highlighted on the cover of the journal)*. These results encouraged novel trends of the immunomodulatory therapy in patients with tick-borne encephalitis ***(Růžek D.,*** *Dobler G., Niller H.H. (2013) May early intervention with high dose intravenous immunoglobulin pose a potentially successful treatment for severe cases of tick-borne encephalitis? BMC infectious diseases 13: 306).*

•To test the role of host genetic background in the clinical course of the encephalitis, we developed an animal model of TBE based on BALB/c-c-STS/A (CcS/Dem) recombinant congenic mouse strains showing different severities of the infection. The host resistance to the infection was associated with lower and delayed viremia, lower virus production in the brain and low cytokine/chemokine mRNA production, but the mice had a strong neutralizing antibody response. The most sensitive mice failed in production of neutralizing antibodies, but exhibited strong cytokine/chemokine production in the brain. These data indicate that the genetic control is an important factor influencing the clinical course of tick-borne encephalitis (***Palus M****., Vojtiskova M,* ***Salat J., Kopecky J., Grubhoffer L.,*** *Lipoldova M., Demant P.,* ***Ruzek D.*** *(2013) Mice with different susceptibility to tick-borne encephalitis virus infection show selective neutralizing antibody response and inflammatory reaction in the central nervous system Jornal of neuroinflammation, 10: 77).* Recently, we identified that these differences are associated with a genetic polymorphism in a gene for Irf3 on mouse chromosome 7.

•There are no specific antiviral compounds against tick-borne encephalitis virus. In collaboration with Radim Nencka (Institute of Organic Chemistry and Biochemistry, CAS, Prague) and Eric De Clercq, a pioneer in antiviral research from the Catholic University of Leuven, we identified that a nucleoside analogue, 7-deaza-2'-C-methyladenosine, has a high

inhibitory effect against tick-borne encephalitis, excellent pharmacological properties and low cytotoxicity, and represent, therefore, a very first promising specific therapeutic agent for treating tick-borne encephalitis *(Eyer et al., J. Antimicrob. Chemoth., under review).*

•Our team also participated in ecological and epidemiological studies focused on tick-borne encephalitis in Germany, Switzerland and Russia, and investigated molecular phylogeography of this disease in Central Europe *(Achazi K., **Růžek D**., Donoso-Mantke O., Schlegel M, Ali H.S., Wenk M., Schmidt-Chanasit J., Ohlmeyer L., Ruhe F., Vor T., Kiffner Ch., Kallies R., Ulrich R.G., Niedrig M. (2011) Rodents as Sentinels for the Prevalence of Tick-Borne Encephalitis Virus. Vector-Borne and Zoonotic Diseases 11: 641–647**;** Gaumann R., **Růžek D.,** Muhlemann K., Strasser M., Beuret C.M. (2011) Phylogenetic and virulence analysis pf tick-borne encephalitis virus field isolates from Switzerland. Journal of Medical Virology 83: 853–863**;** Weidmann M., **Růžek D.,** Křivanec K., Zoeller G., Essbauer S., Pfeffer M., Zanotto P. M. de A., Hufert F.T., Dobler G. (2011) Relation of genetic phylogeny and geographical distance of tick-borne encephalitis virus in central Europe. Journal of General Virology 92: 1906–1916**;** Weidmann M., Frey S., Freire C.C., Essbauer S**., Ruzek D.,** Klempa B., Zubrikova D., Vögerl M., Pfeffer M., Hufert F.T., Zanotto P.M., Dobler G. (2013) Molecular phylogeography of tick-borne encephalitis virus in central Europe Journal of general virology 9: 2129-2139; etc.).* We participated in the description of the first case of TBE in Australia *(Chaudhuri A., **Růžek D.** (2013) First documented case of imported tick-borne encephalitis in Australia Internal medicine journal 43: 93-96).*

•*Simulating the host-tick interface.* The tick *R. appendiculatus* secretes histamine-binding proteins (Ra-HBPs) that sequester histamine during blood feeding. By sequestering histamine, Ra-HBPs prevent host inflammatory responses by competing with the host's native receptor for histamine. Competition for ligand/drug binding between two proteins is extensively studied experimentally, but there are no studies on the all-atom exploration of this competition. In Valdés 2014 *(Valdés, J.J. (2014). Antihistamine response: a dynamically refined function at the host-tick interface. Parasites & Vectors 7: 491),* a novel method was employed to simulate, visualize and analyze this competition at the host-tick interface using the PELE algorithm developed by Victor Guallar at the Barcelona Supercomputing Center (BSC).

•*Protein characterization using bioinformatics.* Understanding the function of proteins lies in their structure (primary, secondary and tertiary). We grouped the Kunitz protein family according to their cysteine motif from the recent *Ixodes ricinus* (a European disease vector) salivary gland transcriptome. By doing so, we were able to hypothesize on the evolution of this highly divergent protein family *(Schwarz, A., **Cabezas-Cruz, A., Kopecký, J., Valdés, J.J.** (2014). Understanding the evolutionary structural variability and target specificity of tick salivary Kunitz peptides using next generation transcriptome data. BMC Evolutionary Biology 14: 4).* In collaboration with Dr. Iain Moal at the BSC, this grouping also allowed us, using a machine-learning algorithm, to accurately (>90%) predict the function of several uncharacterised Kunitz peptides as protease inhibitors and/or ion channel effectors *(**Valdés, J.J.,** Moal, I.H. (2014) Prediction of Kunitz ion channel effectors and protease inhibitors from the* Ixodes ricinus *sialome. Ticks and Tick-Borne Diseases 5: 94).*

•*Molecular dynamics of tick salivary proteins.* A tick salivary protein named trypotogalinin from a North American disease vector, *Ixodes scapularis*, was functionally and structurally characterized. Tryptogalinin is a Kunitz peptide and is 42% identical (homologous) to the salivary tick-derived protease inhibitor (TdPI) from *Rhipicephalus appendiculatus*, but tryptogalinin inhibits twice as many proteases than TdPI. Molecular dynamics analysis of the tryptogalinin predicted model showed that there are high conformational changes (compared with TdPI) of the lysine residue that interacts with the aspartic acid at the active site of serine proteases (i.e., trypsin). The flexibility caused by the high degree of conformational changes of a key residue (lysine) may explain the diversity of tryptogalinin host targets *(**Valdés, J.J.,** Schwarz, A., Cabeza de Vaca, I., Calvo, E., Pedra, J.H.F., Guallar, V., Kotsyfakis, M. (2013). Tryptogalinin is a tick Kunitz serine protease inhibitor with a unique intrinsic disorder. PLoS ONE 8: e62562).*

•*Pathogen evolution. Ehrlichia* sp. are obligate intracellular gram-negative, tick-borne bacteria affecting animals and humans. We characterized the evolution of a new *Ehrlichia* clade using TRP36 sequences. We hypothesized that *Ehrlichia* sp. UFMG-EV evolved from a highly variable *E. canis* clade. To support our hypothesis, we found that *Ehrlichia* sp. UFMG-EV and *Ehrlichia* sp. UFMT-BV TRP36 evolved from a highly divergent and variable clade within *E. canis*. This clade evolved under episodic diversifying selection with a high proportion of sites under positive selection *(**Cabezas-Cruz, A., Valdés, J.J.,** de la Fuente, J. (2014a). The glycoprotein TRP36 of* Ehrlichia *sp. UFMG-EV and related cattle pathogen Ehrlichia sp. UFMT-BV evolved from a highly variable clade of E. canis under adaptive diversifying selection. Parasites & Vectors 7: 584).*

•*Reviews and insights.* Ticks are commonly considered ectoparasites; however, given the arsenal of macromolecules in tick saliva that target host defense mechanisms in order to imbibe a blood meal, we consider ticks as venomous animals *(**Cabezas-Cruz, A., Valdés, J.J.** (2014) Are ticks venomous animals? Frontiers in Zoology. 11:47).* An emerging topic is the anaphylaxis of patients with previous reports of tick bites that is caused by an immune response to a specific carbohydrate, alpha-gal. The mechanism on how alpha-gal is introduced by the tick to cause an allergic reaction is unknown. Due to this tick-induced anaphylaxis, we asked the question: Are humans winning the evolutionary *arms race* with ticks *(**Cabezas-Cruz, A., Valdés, J.J.,** de la Fuente, J. (2014b) Cancer research meets tick vectors for infectious diseases. The Lancet Infectious Diseases 14: 916)*?

•*Effect of tick saliva on the immune response against tick-borne encephalitis virus.* After showing that tick saliva inhibits dendritic cell migration, maturation, and function while promoting development of Th2 responses, we studied its effect on the interaction of TBE virus with mouse dendritic cells. We showed that tick saliva modulates virus-mediated alterations in dendritic cells, being involved in the early infection process in the host *(Fialová A., Cimburek Z., Iezzi G., **Kopecký J.** (2010) Ixodes ricinus tick saliva modulates tick-borne encephalitis virus infection of dendritic cells. Microbes and Infection 12: 580–585).* We found that tick saliva increases replication of TBE virus in dendritic cells, thus facilitating transmission of this important tick-borne pathogen. We confirmed that sialostatin L2 attenuates phosphorylation of STATs in spleen dendritic cells upon addition of recombinant IFN-β. LPS stimulated dendritic cells released IFN-β which in turn led to the induction of IFN-stimulated genes (ISG) through JAK/STAT pathway activation *(**Lieskovská J., Kopecký J.** (2012) Tick saliva suppresses IFN signaling in dendritic cells upon Borrelia afzelii infection. Parasite Immunology 34: 32-39**; Lieskovská J., Páleníková J.,** Sirmarová J., **Elsterová J.,** Kotsyfakis M., Campos-Chagas A., Calvo E., **Růžek D., Kopecký J**. (2015) Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells Parasite immunology 37: 70-78).*

•*Effect of tick saliva on cell signalling pathways activated by Borrelia infection.* To illuminate the molecular mechanism of saliva action, we tested the effect of *Ixodes ricinus* tick saliva on signalling pathways activated by TLR-2 ligand and *Borrelia afzelii* in spleen dendritic cells. We showed that the activation of nuclear factor-κB (NF-κB) p65 and phosphatidylinositol-3 kinase (PI3K)/Akt pathways were decreased by tick saliva upon both TLR-2 and *Borrelia*-stimulation. Among mitogen-activated protein kinases (MAPK), the activation of extracellular matrix-regulated kinase (Erk1/2), but not p38, was suppressed by saliva. The amount of proinflammatory cytokine TNF-α in response to spirochetes decreased in the presence of tick saliva. We showed that the decrease in TNF-α was mediated, in part, by selective suppression of Erk1/2, NF-κB and Akt. The production of antiinflammatory IL-10 in response to spirochetes was strongly induced by saliva and independent of saliva-induced suppression of tested signalling pathways *(**Lieskovská J., Kopecký J.** (2012) Effect of tick saliva on signalling pathways activated by TLR-2 ligand and Borrelia afzelii in dendritic cells. Parasite Immunol. 34: 421–429).*

•*Effect of recombinant salivary proteins on the response of dendritic cells to Borrelia infection.* We determined the effect of cystatins on the production of chemokines in *Borrelia*-

infected bone marrow derived DC. The production of MIP-1α was severely suppressed by both cystatins, while IP-10 was selectively inhibited only by Sialo L2. Sialo L2 strongly attenuated the extracellular matrix-regulated kinase (Erk1/2) and phosphatidylinositol-3 kinase (PI3K)/Akt pathway. The activation of nuclear factor-κB (NF-κB) was affected by Sialo L2 and weakly by Sialo L. In response to *Borrelia*, the activation of Erk1/2 and Akt was moderately impaired by Sialo L2. Production of IFN-β was analysed in plasmacytoid DC (pDC) exposed to *Borrelia*, TLR-7, and TLR-9 ligands. Sialo L, in contrast to Sialo L2, decreased the production of IFN-β in pDC and also impaired the maturation of these cells ***(Lieskovská et al.*** *2015, Parasites & Vectors under review).*

•*Effect of tick salivary serpin on Th17 cells.* Exploiting *I. ricinus* sialome, we showed that IRS-2 selectively inhibits production of IL-6 in dendritic cells stimulated with *Borrelia*, which led to attenuated STAT-3 phosphorylation and finally to impaired Th17 differentiation. Th17 cells constitute a subset of CD4+ T-lymphocytes playing a crucial role in protection against extracellular bacteria and fungi. They are also associated with tissue injury in autoimmune and inflammatory diseases ***(Páleníková et al.*** *2015, Infection and Immunity, in press)*

•*Sialostatin L2 as a saliva activated transmission factor.* We showed that *I. scapularis* Sialo L2, but not Sialo L, facilitates the growth of *Borrelia* in murine skin when administered together with the spirochete. This is the second reported SAT factor in addition to Salp15 *(Kotsyfakis M., **Horká H., Salát J.,** Andersen J.F. (2010) The crystal structures of two salivary cystatins from the tick Ixodes scapularis and the effect of these inhibitors on the establishment of Borrelia burgdorferi infection in a murine model. Molecular Microbiology 77: 456–470)*

•*Immunization with tick salivary protein affects tick feeding success.* We found that cystatin OmC2 from the soft tick *O. moubata* affects the function of antigen-presenting mouse DC by reducing production of the pro-inflammatory cytokines TNF-α and IL-12, and proliferation of antigen-specific CD4+ T-cells, suppressing in this way the host's adaptive immune response. Immunization of mice with OmC2 significantly reduces the survival of *O. moubata* in infestation experiments. OmC2 is a promising target for the development of a anti-tick vaccine to control *O. moubata* populations and combat the spread of associated diseases ***(Salát J.,** Paesen G.C., Řezáčová P., Kotsyfakis M., Kovářová Z., Šanda M., Majtán J., Grunclová L., **Horká H.,** Andersen J.F., Brynda J., Horn M., Nunn M.A., Kopáček P., **Kopecký J.,** Mareš M. (2010) Crystal structure and functional characterization of an immunomodulatory salivary cystatin from the soft tick Ornithodoros moubata. Biochemical Journal 429: 103–112).*

•*Tick salivary molecules as potential drugs for human immunopathological disorders.* We demonstrated that *I. scapularis* Sialo L strongly inhibits the production of IL-9 by Th9 cells. Because it was recently shown that Th9-derived IL-9 is involved in the induction of asthma symptoms, Sialo L was used for the treatment of experimental asthma. Application of Sialo L in a model disease almost completely abrogated airway hyper responsiveness and eosinophilia. We suggest that Sialo L can prevent experimental asthma by inhibiting the IL-9 production of Th9 cells. Thus, alternative to IL-9 neutralization Sialo L provides the basis for the development of innovative therapeutic strategies to treat asthma ***(Horká H.,** Staudt V., Klein M., Taube Ch., Reuter S., Dehzad N., Andersen J.F., **Kopecký J.,** Schild H., Kotsyfakis M., Hoffmann M., Gerlitzki B., Stassen M., Bopp T., Schmitt E. (2012) The tick salivary protein Sialostatin L inhibits the Th9-derived production of the asthma-promoting cytokine IL-9 and is effective in the prevention of experimental asthma. Journal of Immunology 188: 2669–2676).* This research was supported by a collaborative grant GACR with the colleagues from the Institute for Immunology, Johannes Gutenberg-University Mainz, Germany.

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Biology of Disease Vectors

Team 17 – **Biology of Disease Vectors** is formed by three research laboratories:

1. **Laboratory of Vector Immunology** (LVI) headed by **Petr Kopáček**
2. **Laboratory of Tick-Transmitted Diseases** (LTTD) headed by **Ondřej Hajdušek**
3. **Laboratory of Genomics and Proteomics of Disease Vectors** (LGPDV) headed by **Michail Kotsyfakis**

All three laboratories have in common their research focus on ticks as disease vectors and tick molecular factors that facilitate transmission of tick-borne pathogens.

Background:

LVI exists as an independent laboratory since 2003, when the principal re-organization of the Institute of Parasitology occurred and the former large Department of Molecular Ecology of Vectors and Pathogens was divided into several more specialized research units under the leadership of individual PIs.
http://www.paru.cas.cz/en/section/ticks-and-tick-borne-diseases/laboratory-of-vector-immunology/

LTTD is a recently (2013) opened laboratory founded with a support of the EU-FP7 Modbiolin project and headed by O. Hajdušek who returned after his three years' postdoctoral stay at CNRS , Strasbourgh, France. Although formally independent, the LTTD and LVI still substantially share personal staff, laboratory equipment and tightly co-operate on overlapping research projects.
http://www.paru.cas.cz/en/section/ticks-and-tick-borne-diseases/laboratory-of-tick-transmitted-diseases

LGPDV was established in 2009 as a newly opened laboratory created under the leadership of M. Kotsyfakis, who joined the Institute of Parasitology as the first foreign scientist awarded by the J.E. Purkyně fellowship from the Academy of Sciences of the Czech Republic.
http://www.paru.cas.cz/en/section/ticks-and-tick-borne-diseases/laboratory-of-genomics-and-proteomics-of-disease-vectors/

LVI&LTTD

The research activities of LVI & LTTD have been focused on four major directions:

1. The immune system of ticks
2. Blood-meal digestion in ticks as a target for rational anti-tick interventions
3. Iron/heme metabolism in ticks and ferritin 2-based anti-tick vaccine
4. *Borrelia* and *Babesia* laboratory transmission models as tools for the study of tick-pathogen interactions

Ad 1) Tick immune system (P. Kopáček, O. Hajdušek)

The versatile vector competence of ticks has to be intimately linked to the ability of transmitted pathogens to evade tick defense mechanisms encountered on their route through the tick body. Therefore, the research of tick innate immunity is in long-term research focus of LVI. Earlier, we have discovered and characterized a number of molecules playing a role in the immune system of soft ticks (*Ornithodoros moubata*) and hard ticks (*Ixodes* spp.). Our primary research in this field has been focused on tick molecules resembling the components of mammalian complement system, such as fibrinogen related lectins (Ixoderins) or universal protease inhibitors of alpha-2-macroglobulin class. Two milestones achieved before 2010, namely availability of the genome-wide database of *Ixodes scapularis* and feasibility of RNA interference in ticks, made us possible to further focus on the European Lyme disease vector *Ixodes ricinus* exploiting the tools of reverse genetics and functional genomics.

Recognition of our team in the field of tick immunity was reflected by invitation to write a review "Tick innate immunity" **(Kopacek et al., 2010)** in which we, together with Sirlei Daffre (University of São Paolo), provided a comprehensive up-to-date overview of tick cellular reactions and humoral defense mechanisms known to occur in the tick hemocoel, midgut and salivary glands. A more recent review **(Hajdusek et al., 2013)** describes the interaction of tick immune system with the most relevant tick-borne pathogens represented by *Borrelia* spirochetes, intracellular rickettsia of the genus *Anaplasma* and malaria-like protist *Babesia* sp.

Since the discovery of the thioester-containing protein (TEP1) as the determinant of the mosquito competence to transmit malaria (group of E. Levashina, CNRS Strasbourg), we have been intensively focused on tick TEP1 homologues. We found that ticks are unique organisms since they possess all major groups of TEPs known in invertebrates comprising three alpha-2-macroglobulins (A2M-1,2,3), three complement C3-like molecules (C3-1,2,3), two macroglobulin-complement-related (MCR-1,2) and one insect type TEP. We established a robust functional assay based on RNAi silencing of individual TEPs followed by *in vitro*-phagocytosis of microbes by tick hemocytes. Using that we showed that phagocytosis of different Gram-negative bacteria depends on a different set of tick TEPs. By RNAi-mediated gene silencing in the European sheep tick *I. ricinus* we demonstrated the central role of a C3-3 like molecule in the phagocytosis of Gram negative bacteria and revealed non-redundant roles of A2M-1 and A2M2- in the engulfment of the tick pathogen *Chryseobacterium indologenes* and TEP in the phagocytosis of the model bacterium *Escherichia coli* **(Buresova et al., 2011)**. This study written together with E. Levashina with the involvement of J. Hoffmann (Nobel Prize 2011) was published in a newly launched, prestigious Journal of Innate Immunity. Our findings promoting ticks as an exceptional model for further research on the origin and function of the primordial complement system were also described as an invited book chapter **(Kopacek et al., 2012).**

The research on the tick complement system continued after return of V. Urbanová (maiden name Burešová) from maternity leave and after O. Hajdušek return from his postdoctoral stay at CNRS in 2013. In order to assess the function of TEPs in tick immunity, a quantitative real-time PCR (qPCR) expression analysis of tick TEPs was performed at various developmental stages of *I. ricinus* and in tissues dissected from adult females. Expression of TEP genes was mostly tissue-specific; A2M1, C3-1, C3-3 were found to be expressed in cells of tick fat body adjacent to the tracheal trunks, A2M2 in hemocytes, TEP in ovaries, Mcr1 in salivary glands and only A2M3, C3-2 and Mcr2 mRNAs were present in multiple organs. Expression of tick TEPs was further examined in response to injection of model microbes representing Gram-negative, Gram-positive bacteria and yeast. The greatest induction of TEP expression was observed from A2M1 and C3-1 upon challenge with *Candida albicans*. Phagocytosis of the yeast was strongly dependent on an active thioester bond and the subsequent silencing of individual tick TEPs by RNA interference demonstrated the involvement of C3-1 and Mcr2. Results suggesting the existence of a distinct complement-like pathway, different from that leading to phagocytosis of Gram-negative bacteria, have been recently published **(Urbanova et al., 2014b)**.

Furthermore, we focused on the characterization and functional study of two putative C3-complement convertases from *I. ricinus* referred to as Factor C (IrFC) and Factor B/C2 (IrBf/C2). The IrFC was described in a recently published paper **(Urbanova et al., 2014a)**, in which we demonstrate the complex primary structure of IrFC composed of N-terminal cysteine-rich domain, four complement

control protein (sushi) domain, one LCCL and one truncated C-lectin domain and C-terminal trypsin like protease. In adult females, IrFC is expressed mainly in tick haemocytes and is up-regulated upon injury of tick body suggesting its role in tick hemolymph clotting. RNAi silencing of IrFC expression resulted in a significant reduction in phagocytic activity of tick hemocytes against the Gram-negative bacteria *C. indologenes* and *E. coli* indicating its function in activation in the tick complement system. A similar amount of data have been achieved on molecular characteristics and function of IrBf/C2 factor, which is one of rare molecules responsive to the *Borrelia* infection (manuscript in preparation). In the frame of the GACR project (GAP506/10/2136), we have also performed a high-throughput sequencing project of *I. ricinus* hemocyte transcriptome (**hemocytome**). The currently reviewed manuscript by Kotsyfakis, Kopáček et al. "Deep sequencing analysis of *Ixodes ricinus* hemocytome" provides data from deep sequencing a *I. ricinus* hemocyte cDNA library and annotation of immune-related transcripts based on their hemocyte abundance as well as their sequence divergence in alignments with arthropod homologues. More than 15,000 extracted coding sequences (CDS) were generated and are displayed in an annotated hyperlinked spreadsheet format. About 300 transcripts were found significantly over-expressed in hemocytes, including those encoding for scavenger receptors, antimicrobial peptides, pathogen recognition proteins, proteases and protease inhibitors. We additionally annotated ubiquitously distributed transcripts associated with immune function and we provided an insight into the evolution of major protein families involved in tick innate immunity by presenting their phylogenetic trees.

Ad2) Blood digestion in ticks (D. Sojka, P. Kopáček)

Blood feeding and digestion in ticks are key physiological processes providing essential nutrients for development and fecundity of the parasite and, ultimately, allow for successful transmission of tick-borne pathogens. To provide a comprehensive characterization of the tick blood digestive system, we have focused on the European hard tick *Ixodes ricinus* and joined our capabilities with the laboratory od M. Mareš (IOCB, CAS, Prague) and the laboratory of J.H. McKerrow (UCSF, San Francisco). By exploiting a complex of biochemical, reverse genetic and proteomic approaches, we have earlier described the *I. ricinus* multi-enzyme digestive apparatus that is based on a network of acidic cysteine and aspartic peptidases (Horn et al., 2009; Sojka et al., 2008). During the past 5 years, the initial "static" image of blood digestive machinery in ticks at defined lifestage (partially engorged females) has been enriched by a dynamic monitoring of the digestive machinery of *I. ricinus* at various tick lifestages and during the course of on-host feeding. The dynamic profiling of concentrations, activities and mRNA expressions of the major hemoglobinolytic enzymes revealed that the *de novo* synthesis of peptidases triggers the dramatic increase of the hemoglobinolytic activity along the feeding period. These results suggested that the egressing proteolytic system in the early stage of feeding might be efficiently impaired by antibodies present in the blood of vaccinated host **(Franta et al., 2010)**. We have further focused on the characterization and functional analysis of the individual components of the hemoglobinolytic apparatus. We described in details two enzymes that initiate hemoglobin cleavage in the tick gut, namely cathepsin L (**IrCL1**) **(Franta et al., 2011)** (Int. J. Parasitology-Journal cover) and cathepsin D (**IrCD1**) **(Sojka et al., 2012)**. IrCL1 is an acidic (pH optimum 3-4) cysteine peptidase that expression in the tick gut peaks during slow feeding period. The enzyme was immune-localized inside the vesicles of digestives using antibody raised against recombinant IrCL1 expressed in *Pichia pastoris*. Recombinant IrCL1 was enzymatically active and allowed for determination of its sensitivity to inhibitors (in collaboration with M. Mareš, IOCB CAS Prague) and cleavage specificity determined by positional scanning of synthetic peptide library (in collaboration with UCSF – C.R. Caffrey, J.H. Kerrow and C.S.Craig). RNAi silencing of IrCL1 impaired tick feeding suggesting its potential as a candidate antigen (Franta et al., 2011).
The tick genome contains three paralogs coding for aspartic peptidases out of which IrCD1 is mainly expressed in the tick gut during feeding. Modeling the 3D structures of tick cathepsin D revealed that IrCD1 is the most distinct enzyme given to the shortened pro-peptide region and a unique pattern of post-translational modifications. Properties of the recombinant IrCD1 are consistent with the endo-lysosomal environment because the zymogen is auto-activated and remains optimally active at acidic pH. Hemoglobin cleavage pattern of recombinant IrCD1 was identical with native enzyme present in the gut homogenates (cooperation with IOCB, Prague). An implementation of novel synthetic

tetradecapeptidyl substrate library (in collaboration with A.J. O'Donoghue and C.S. Craig, UCSF) was used here for the first time and revealed the significant IrCD1 preferences for tyrosine at P3 and alanine at P2' positions. The role of IrCD1 in hemoglobinolysis was further confirmed by RNAi that decreased cathepsin D activity in the tick gut by 90% (Sojka et al., 2012). The intense studies on tick cathepsin D further continued in collaboration with the group of M. Mareš in frame of our joined project (GAP207/10/2183). We obtained a 1.4 A resolution crystal structure of D_{270} - A_{270} IrCD1 inactivated mutant, the first proteolytic enzyme structure from ticks. This result is now followed by a comprehensive structural study of IrCD1 that was completed in 20132014 and includes 4 crystal structures of IrCD1- (i) mature active enzyme; (ii) mature enzyme in complex with substrate-like inhibitor pepstatin; (iii) inactive mutant proenzyme and (iv) complex of the mature enzyme with the pro-part derived inhibitor. This study reveals a unique mechanism of IrCD1 activation among other cathepsin-D related molecules and identifies the auto-regulatory (inhibitory) IrCD1 pro-part as a novel-class cathepsin D inhibitor with a further usage in biological, medicinal and biochemical studies. The completed study will be submitted for publication in a highly impacted journal within 2015.
During this project we have also established a wider collaboration with one of the leading laboratories in molecular parasitology – D. Soldati-Favre, University of Geneva – on functional characterization of cathepsin-D like peptidases in the secretory system of apicomplexan parasites. This collaboration was enabled by SCIEX-NMS^{ch} fellowship to D. Sojka who spent one year of postdoctoral training at the laboratory of D. Soldati-Favre during 2012–2013. An ongoing collaboration involves biochemical and functional characterization of plasmepsins IX, X from *Plasmodium falciparum* and the conditionally knocked-out TgASP5 from *Toxoplasma gondii* (manuscript in preparation for publication in a high-rank scientific journal).
The recognition of our group in the field of tick blood digestion was appreciated by an invited review **(Sojka et al., 2011)** describing the overview of cysteine peptidases in blood-feeding ectoparasites and especially by an prestigious review article **(Sojka et al., 2013)** published in Trends in Parasitology (Journal Cover). In this article we summarized our current knowledge of the molecular mechanisms of tick hematophagy, compared them to proteolytic digestive systems of other blood feeding parasites and raised aims and questions for future research in the field. Consequently, a comprehensive review on parasitic aspartic peptidases by Sojka and Hartman entitled "*Cathepsin D – an old drug target with novel implications for parasite control*" has just been submitted for publication.

Our contribution to the knowledge of the blood digestive system in ticks was evaluated among the top annotated outputs of the Biology Centre in 2012. In addition, D. Sojka was awarded by the prestigious prize of the Czech Academy of Sciences in 2013 for young research workers for outstanding scientific research in the topic "*Blood digestion by ticks – a complete insight into the intestinal multienzyme hemoglobinolytic machinery*" .

The recent activity of LVI in the research of blood digestion in ticks is based on the implementation of artificial membrane feeding of *I.ricinus*, which presents a true milestone for our current and future research focus (see also the research plan section). In order to better understand the importance of hemoglobin in the tick physiology and development, we can feed ticks on hemoglobin-rich (full blood) and hemoglobin-poor (serum) diets. To this end, we discovered that hemoglobin is essential as a source of heme for ticks which are heme auxotrophs. Surprisingly, hemoglobin is not critically required as a source of amino-acid for vitellogenesis and is not a source of iron for ticks. The experimental setup allowed us to investigate the inter-tissue transport of heme from the tick gut to ovaries and other tissues. Achieved results are summarized in the submitted manuscript by Perner et al: "*Haem acquisition and distribution in ticks*". This work includes also our collaboration project on Western blot quantification and normalization using BioRAD THX technology and V3 protein work-flow with BIO-RAD core facility (Ning Liu, BioRAD laboratories, Hercules, CA, USA).

Ad3) Towards anti-tick vaccines based on ferritin 2 and other molecules involved in tick iron/heme metabolism (P. Kopáček, O. Hajdušek)

Previously, we have demonstrated that targeting tick iron metabolism pathway is a promising approach for an efficient tick control. Beside the intracellular ferritin 1 and iron-regulatory protein (IRP), we have discovered a novel ferritin 2, which is secreted from the tick gut to the hemolymph and functions as transporter of non-heme iron to peripheral tissues (Hajdusek et al., 2009). The potential of ferritin 2 as an anti-tick vaccine candidate was patented in the Czech Republic (Patent No. 301541, 2010) and in the U.S.A. (Patent No. US 8,168,763 B2, May 1, 2012). The proof-of-concept of ferritin2-based vaccine was carried out in co-operation with the Laboratory of José de la Fuente (Ciudad Real, Spain and Oklahoma State University, Stillwater, USA). We have demonstrated that vaccination of rabbits with recombinant ferritin 2 from *I. ricinus* and vaccination of cattle with recombinant ferritin 2 from *Rhipicephalus microplus* significantly impaired the tick feeding ability and further reproduction (**Hajdusek et al., 2010**). These promising achievement initiated and supported a co-operation with the Czech vet-company Bioveta a.s and resulted in an applied research project from the Czech Ministry of Industry and Trade (FR-TI3/156) with the ultimate goal to develop a commercial *I. ricinus* ferritin 2-based anti-tick vaccine for the pet animals. In spring 2014, we have performed a novel pen-trial experiment at the University Queretaro, Mexico (José de la Fuente, Juan Mosqueda) that comprised 20 cattle immunized with *R.microplus* ferritin 2, two more chimeric variants Fer2 and the Bm-86 positive control. Ticks fed on the cattle immunized with two variants of recombinant ferritin 2 exerted significantly reduced oviposition and hatching rate compared to the non-immunized control group.
Altogether 11 molecules potentially involved in tick iron/heme metabolism, acquisition intracellular and inter-tissue transport have been systematically studied within a GACR postdoctoral project to O. Hajdušek (GP13-27630P). The study comprised determination of their full sequences, developmental stage and tissue expression profiling by qPCR, response to artificial infection with model microbes including *Borrelia* and dependence on hemoglobin uptake in tick diet (see above).
The cDNA sets prepared within this work comprising various developmental stages, tissues, whole bodies of ticks infected by injection or capillary-feeding with different pathogens (G+ and G- bacteria, yeasts, *Borrelia*, uninjected, PBS-injected) have been exploited as laboratory "golden standards" in published (Urbanova et al., 2014a; Urbanova et al., 2014b) as well as submitted papers (Perner et al., "*Haem acquisition and distribution in ticks*").
The vaccination potential of selected proteins has been screened by a series of individual RNAi knockdowns followed by monitoring their impact on tick feeding, oviposition and hatching. These candidates have been successively tested for their potential to inhibit transmission of *Borrelia* spirochetes from the tick to the host using the laboratory transmission model described below.

Ad4) *Borrelia*-*Babesia* transmission models (O. Hajdušek, R. Šíma)

The **main goal** achieved by joined effort of both LTTD and LVI during past 5 years was the implementation of **Lyme disease transmission model for *Ixodes ricinus*** within the GACR postdoctoral project to R. Šíma (GP13-12816P). This opens us a gate towards better understanding of the mechanisms of pathogen transmission from infected nymphs to laboratory animal models with a significant impact for prevention of Lyme disease in humans.
An intensive effort has been put towards elucidation the host-pathogen interactions during *Borrelia* infection. The main aim of this endeavour was to find out factors that could be used as targets to prevent *Borrelia* transmission. Till now, the only transmission model testing *Borrelia* vaccine candidates has been established using *I. scapularis* nymphs – *B. burgdorferi* sensu stricto – mice system (Yale University**,** group of Erol Fikrig), whereas a similar model for the European tick species *I. ricinus* and European *Borrelia* strains (*B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto) has been painfully missing. Implementation of transmission model in our laboratory thus presents a substantial competitive advantage for Lyme disease research within the Europen research space.
Out of 33 different *Borrelia* strains tested (13× *B. burgdorferi* sensu stricto, 14× *B. garinii*, 3× *B. afzelii*, 3×x *B. americana*), nine *B. burgdorferi* sensu stricto, one *B. afzelii* and four *B. garinii* strains were proved to be infectious for laboratory mice and ticks. These strains represent a comprehensive set

of infectious *Borrelia* spirochetes that is of great importance for any projects and collaborations in future. Of those, *B. afzelii* CB43 was selected as the most typical representative of European Lyme disease agents and is used as a model in all further experiments. One paper regarding infectivity of different *Borrelia* strains has been recently published **(Golovchenko et al., 2014)**.
We have optimized the method of *Borrelia* detection in various tissues dissected from infected mice by standard PCR and found that urinary bladder is the most reliable tissue during persistent murine infection. A protocol for quantification of borrelia infection has been developed using quantitative real-time PCR amplifying part of the flagellin gene.
Another hurdle overcome in the past 5 years was the successful implementation of RNAi in *I. ricinus* nymphs using capillary nano-injection. This achieved milestone allows us to test the function of *I. ricinus* proteins in *Borrelia* transmission. Even though RNAi silencing of individual molecules followed by the transmission experiments are extremely time- and labour-demanding, we have already commenced a comprehensive search for the molecules that might affect transmission of Lyme disease spirochetes.
In parallel to Lyme disease transmission model, we have been developing laboratory transmission model for the tick-borne malaria-like pathogen of *Babesia* spp. So far, the reliable *Babesia* transmission model does not exist anywhere. If we succeed to get the model working, we will be capable to test the homologous molecules that have been identified in mosquitoes to impair their capacity to vector malaria. This work is mainly performed by our PhD student Marie Jalovecká who is co-operating in frame of her dual Czech-French PhD program with the laboratory of Lawrence Malandrin (ONIRIS, Nantes). Our model seems to be appropriate for *B. microti* that can easily infect mice that is further capable to infect tick larvae which molt to infected nymphs. These nymphs can reversely infect naïve mouse and they could be used for our future RNAi/vaccine experiments.
Recently, we have optimized the detection of infection level in ticks using fluorescent *in situ* hybridization (FISH).

LTTD has been also involved in field studies, detecting *Borrelia* and *Babesia* prevalence in unfed nymphs over the Czech Republic. We are able to measure by qRT-PCR number of pathogens per tick and compare these infections with levels in the laboratory-infected ticks. We have developed a sensitive PCR to detect and genotype *Borrelia* and *Babesia* from ticks and vertebrates.
Recently, we collaborate with the Norwegian Institute of Public Health in Oslo and diagnose human blood samples for the presence of these parasites.

The expertise of LTTD and LVI in Lyme disease transmission model, *Borrelia* detection and RNAi-based functional genomics posed a fundamental input for our involvement in the international research consortium ANTIDotE (ANti-tick vaccines to prevent TIck-borne Diseases in Europe) http://www.antidote-fp7.org/ (FP7- 602272). Seven partners from leading academic, research, public health and industrial institutions will use the state-of-the-art proteomics and transcriptomics approaches to identify and characterize novel tick salivary gland proteins, which will be subsequently assessed as anti-tick vaccines to protect against Lyme borreliosis, babesiosis and tick-borne encephalitis in animal models. In addition, through an integrated and multidisciplinary approach involving Central and Eastern European public health institutes, health organizations and industrial companies, we will examine how to develop anti-tick vaccines and implement these in public health systems **(Sprong et al., 2014)** . To this end, our group have prepared nymphs of *I. ricinus* infected with *Borrelia afzelii* and extracted RNA from salivary glands. The RNA has been sent for transcriptomics analysis. Genes upregulated during infection will be further tested by RNAi for their potential to block *Borrelia* transmission. Similar analysis is supposed to be performed with *Babesia* spp. in the later phase of the project.

LGPDV (M. Kotsyfakis)

We were the first to reveal that tick salivary cysteine protease inhibitors are essential for feeding success of *Ixodes scapularis*, a vector of Lyme disease agents in the USA (Kotsyfakis et al., 2007). One of these inhibitors, **sialostatin L2** (SL2), is also essential for both *Borrelia burgdorferi* **(Kotsyfakis et al., 2010**) and *Anaplasma phagocytophilum* **(Chen et al., 2014)** establishment in the vertebrate host. In the case of *A. phagocytophilum*, our experimental findings suggest that SL2 interferes with the assembly of an atypical inflammasome in vertebrate macrophages that recognizes *Anaplasma* (Chen et al., 2014). As a result, we have recruited funds from the National Institutes of Health, USA (*collaborative R01 grant*) to uncover the mechanism of this action of SL2. Collectively our results show that two different tick-borne pathogens exploit a tick protein − which is essential for tick blood feeding success − to colonize the vertebrate host. To rephrase, the arthropod disease vector and the vector-borne pathogens take advantage of the pharmacological action of the same vector protein (SL2) to obtain a blood meal and to promote the establishment of an infection, respectively. To summarize, tick salivary SL2 has a key role in tick life cycle, since it is essential for both blood meal acquisition by the arthropod vector and for the colonization of the vertebrate host by the vector-borne pathogens (*Borrelia* and *Anaplasma*). Interestingly, SL2 is not recognized by the vertebrate humoral immunity upon natural exposure of the vertebrate host to ticks (although it is a constituent of tick saliva); we introduced the term 'silent antigens' to describe proteins (such as SL2) that, although of exogenous origin − and are in contact with the vertebrate host − they are undetected from the vertebrate humoral immunity (Kotsyfakis et al., 2008). When the vertebrate host is artificially pre-sensitized (vaccinated) for SL2, increased tick rejection rates are observed due to the induced humoral recognition of the protein, further demonstrating the potential of SL2 to serve as an antigen for the development of anti-tick vaccines (patent filed, please see CV). The concept of silent antigens is a novel one and may pioneer broad applications in the field of vaccine development; this is the main reason that the related article was published as Cutting Edge article in the *Journal of Immunology* (Kotsyfakis et al., 2008).

Platelet aggregation and acute inflammation are crucial modules of the vertebrate host defense against a feeding tick and more specifically to the skin injury caused by the intrusion of tick mouthparts. Working with a mouse model of acute inflammation, we revealed that an exogenous salivary protein of the tick *Ixodes ricinus*, the vector of Lyme disease pathogens in Europe, extensively inhibits edema formation and the influx of neutrophils in the inflamed tissue **(Chmelar et al., 2011**). We named this tick salivary protein as *I. ricinus* serpin-2 (**IRS-2**) and we showed that it primarily inhibits human cathepsin G and chymase, while in higher molar excess, it affects thrombin activity, too. All these three human serine proteases have an important role in hemostasis and coagulation due to their involvement in the activation of human Protease-Activated Receptors (PARs). The inhibitory specificity of IRS-2 against the above-mentioned human serine proteases was explained using its resolved crystal structure, determined at a resolution of 1.8 Å. Moreover, we disclosed the ability of IRS-2 to inhibit cathepsin G-induced and thrombin-induced platelet aggregation. To conclude, we showed for the first time that an ectoparasitic protein exhibits these pharmacological effects on the vertebrate host with so stringent target specificity. The stringent specificity in the action of IRS-2 combined with the knowledge of its structure can be the basis for the development of future pharmaceutical applications. Indeed, we demonstrated that the anti-inflammatory action of IRS-2 compares to that of indomethacin, a non-steroidal anti-inflammatory, drug which is available in the drug stores and thus we were invited in the cover of the related issue of Blood (Chmelar et al., 2011). In 2013, we published the functional characterization of a Kunitz peptide from *I. scapularis* salivary glands, which possesses an unusual cysteine motif; we named this peptide as **Tryptogalinin** because of its potent inhibitory activity against human skin b-tryptase **(Valdes et al., 2013)**. Skin b-tryptase has a predominant role in vertebrate mast cell biology and by employing homology-based modeling (and other protein structure analysis/prediction programs), we were able to explain the function of Tryptogalinin as a tick-derived serine protease inhibitor (Valdes et al., 2013). In the past, we have also demonstrated the potent pharmacological action of an *I. scapularis* salivary cysteine protease inhibitor named as sialostatin L, which alleviates the symptoms of acute inflammation, autoimmune encephalitis (Kotsyfakis et al., 2006; Sa-Nunes et al., 2009) and allergic asthma in mice **(Horka et al., 2012)**. Next, we resolved the crystal structure of sialostatin L because this

is an essential step to understand the structural determinants of its pharmacological action (Kotsyfakis et al., 2010).

We know that *I. ricinus* salivary secretion regulates diverse vertebrate proteolytic events at the sites of tick bite (unpublished data, please see figure 1 below); accordingly, we completed in the last two years a series of **high-throughput gene discovery projects** that described the molecular composition of *I. ricinus* salivary secretion. More specifically, we employed Next Generation Sequencing analysis of tick salivary transcriptomes (NGS-RNA seq by employing Illumina and 454 pyrosequencing) coupled with quantitative Proteomics and, not surprisingly, approximately one tenth of the 80,000 discovered contigs/transcript sequences encode for potential protease inhibitors **(Schwarz et al., 2013; Schwarz et al., 2014a; Schwarz et al., 2014b)**. The successful completion of these projects was followed by another two systems biology projects aiming to reveal the molecular mediators of blood meal digestion in the tick midgut as well as the gene expression patterns in tick immune cells (manuscripts under review: Kotsyfakis et al. "Tissue- and time-dependent transcription in *Ixodes ricinus* salivary glands and midguts when blood feeding on the vertebrate host" and Kotsyfakis, Kopáček et al. – "Deep sequencing analysis of *Ixodes ricinus* hemocytome". Collectively, our projects transformed the European tick *I. ricinus* to one of the best-studied organisms among the arachnids − in general − in terms of systems biology and as far as it concerns the description of the gene expression regulation in the different tissues involved in pathogen-transmission. Beyond the importance of the produced data in better understanding the life cycle of a disease vector that imposes important threats in public health in Europe (this tick serves as the vector for Tick Borne Encephalitis virus, *Borrelia* spirochetes, various species of *Rickettsia* and protists of the genus *Babesia*), now we have the necessary knowledge of genetics for this model organism to better understand the evolution of important biological mechanisms such as innate immunity, apoptosis/cell death, gene regulation, protein turnover, metabolism.

Through collaborations worldwide, we also demonstrated that salivary proteins from various arthropod disease vectors (mosquitoes, black flies, sand flies) regulate vertebrate proteolytic events − in the sites of feeding − with an unexpected specificity in their action (patent filed, please see CV). More specifically, we described the molecular mechanism of vertebrate Factor Xa (FXa) inhibition by **Alboserpin**, the major salivary gland anticoagulant from the mosquito and yellow fever vector *Aedes albopictus* **(Calvo et al., 2011)**. Apart from inhibiting Factor Xa, Alboserpin displays high binding affinity to heparin and interacts with phosphatidylcholine and phosphatidylethanolamine, but not with phosphatidylserine. Vertebrate annexin V or heparin outcompetes Alboserpin for binding to phospholipid vesicles, suggesting a common binding site. Consistent with its activity, Alboserpin blocks prothrombinase activity and increases both prothrombin time and activated partial thromboplastin time in vitro or ex vivo. Accordingly, Alboserpin prevents thrombus formation provoked by ferric chloride injury of the carotid artery and increases bleeding in a dose-dependent manner. Another collaborative project revealed that **AgESP**, a serine protease from the malaria mosquito *Anopheles gambiae*, is required for *Plasmodium* parasites to effectively traverse the mosquito midgut and salivary gland epithelial barriers **(Rodrigues et al., 2012)**. *Plasmodium* parasites need to modify the actin cytoskeleton of mosquito epithelial cells to successfully complete their life cycle and mosquito AgESP appears to be a major player in the regulation of this process. In another project, we uncovered that the salivary glands of the same mosquito species secrete ***cE5***, a small peptide that is a highly specific and tight-binding inhibitor of human thrombin, thus facilitating the completion of mosquito blood feeding on the host **(Ronca et al., 2012)-equal first authorship**). During 2014 we co-authored a publication in PLoS Pathogens that for the first revealed with a systems biology approach that, upon malaria infection, the human malaria parasite *Plasmodium falciparum* induces the expression of a mosquito salivary protein named as **Agaphelin (Waisberg et al., 2014)**. Agaphelin, in turn, targets the function of the vertebrate host neutrophils. Apparently, Agaphelin is co-injected with malaria parasites in the host skin after an infectious mosquito bite and, because of its immunosuppressive properties, it gives better chances to the malaria parasites to establish an infectious cycle in the vertebrate host.

Under a collaborative frame, we identified the unique salivary anticoagulant of the sand fly *Lutzomyia longipalpis*, which remained elusive for decades. A novel 32.4 kDa molecule, named **Lufaxin**, was characterized as a slow, tight, non competitive, and reversible inhibitor of Factor Xa

(Collin et al., 2012). Notably, Lufaxin's primary sequence does not share any similarity to any physiological or salivary inhibitor of coagulation reported until our publication. Lufaxin prevents protease-activated receptor 2 (PAR-2) activation by Factor Xa and abrogates edema formation triggered by the injection of FXa in the paw of mice (Collin et al., 2012). Next, we contributed to the functional characterization of another two serine protease inhibitors from the salivary glands of the black fly *Simulium vittatum* **(Tsujimoto et al., 2012)**and the salivary glands of the vampire-bat *Desmodus rotundus* (**Ma et al., 2013)**. The identity of the vampire-bat saliva anticoagulant remained elusive for almost a century and we identified **Desmolaris** as an anticoagulant from this bat and as a novel 21.5-kDa form of tissue factor pathway inhibitor. Recombinant Desmolaris is a slow, tight, and noncompetitive inhibitor of vertebrate Factor XIa; Factor XIa inhibition mechanism is modulated by heparin. Desmolaris also inhibits vertebrate Factor Xa with lower affinity and binds to kallikrein, thus reducing bradykinin generation in plasma activated with kaolin. Moreover, Desmolaris reduces the polyphosphate-induced increase in vascular permeability as well as the collagen- and epinephrine-mediated thromboembolism in mice (Ma et al., 2013). Overall, Desmolaris emerges as a novel anticoagulant that targets vertebrate Factor XIa under conditions in which the coagulation activation, and particularly the contact pathway, plays a major pathological role.

To conclude, our results show that the identification, functional/structural analysis and pharmacological studies of proteolytic regulators from hematophagous organisms open a new pathway in drug discovery and towards developing novel strategies to combat arthropod-borne illnesses.

References (in bold - members of the team including research assistants and students)

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Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Fish parasitology

1. FOREWORD

The team is called "Fish Parasitology" but the scope of its research activities is broader as evidenced by the extent of main research lines (see below). The team includes two research laboratories, Laboratory of Fish Protistology (head *I. Dyková* until February 2011; *A. Holzer* since March 2011) and Laboratory of Helminthology (head *T. Scholz*).

2. MAIN LINES OF RESEARCH

2.1. Laboratory of Fish Protistology

Our main interests are eukaryotic microorganisms infecting fish and amphibians, including all aspects of their structure, biology, life-cycles, host-parasite relationships, and especially their phylogeny and evolution. Most recently, we have also initiated a line of functional research, which is still underexplored in many fish parasites. We focus on two main eukaryotic groups, i.e. the Myxozoa (Cnidaria) and amoeboid organisms. Furthermore, we carry out research into a range of parasitic protists that create economic and health consequences for the aquaculture industry, in collaboration with various academic and commercial partners worldwide. Besides several side projects, our main research lines are:

1. Myxozoan biodiversity, phylogeny and evolution. In order to better understand the factors that drive the evolution of the Myxozoa, we try to use a holistic approach by mapping morphological, life-cycle and host characters onto phylogenetic trees, which are based on commonly used SSU rDNA sequences but also on novel molecular markers such as mitochondrial and nuclear genes (funded by the Czech Science Foundation, 2 postdoc projects, 1 Centre of Excellence project).
2. Functional aspects of myxozoans inside their fish hosts. This line focuses on within-host migration and motility mechanisms as well as on exploring molecules of special interest for developing antiparasitic strategies (vaccines) for the aquaculture sector (funded by the AS CR Program for international collaboration and the EU project MODBIOLIN).
3. Identification, description, pathology and phylogeny of amoebae. This work focuses on our large culture collection of different strains from a variety of aquatic habitats and hosts (funded by the Czech Science Foundation).
4. Diagnostic assays and applied approaches aiming at combating fish diseases in aquaculture. We design and validate a number of diagnostic assays and we conduct *in vitro* trials with a number of potentially amoeboicidal substances to be used as an in-feed treatment against amoebic gill disease in Atlantic salmon (2 contracts funded by Skretting ARC, Norway).

2.2. Laboratory of Helminthology

The principal mission of the laboratory is to build an advanced taxonomic, ecological and phylogenetic framework that will serve as a baseline for future studies of selected groups of the helminth parasites of freshwater and marine fishes and other invertebrate and vertebrate hosts including man (fish-borne diseases). A wide range of helminth parasites of animals

related to aquatic environment is studied using morphological, ultrastructural, phylogenetic and ecological methods, within the following research lines:

1. Morphology, systematics and phylogeny of tapeworms (Cestoda) parasitic in teleosts as part of the global mapping of the cestode diversity (funded by the National Science Foundation – Planetary Biodiversity Inventory project from 2008 to 2014, and two projects supported by the Czech Science Foundation from 2008 to 2012 and from 2012 to 2016, respectively);
2. Life-cycles, ecology and molecular taxonomy of larval stages of trematodes (Digenea) in freshwater molluscs and metacercariae in fish (funded mainly by the Czech Science Foundation from 2010 to 2013 and by Marie-Curie Internal Outgoing Fellowship to *I. Blasco-Costa* from 2010 to 2013);
3. Diversity of helminths parasitising teleost fish (funded by the Ministry of Education, Youth and Sport from 2005 to 2011 as the research centre "Ichthyoparasitology" and by the Czech Science Foundation from 2012 to 2018 as the centre of excellence "European Centre of Ichthyoparasitology").

3. **SUMMARY OF SCIENTIFIC OUTCOMES**

During five years (2010–2014), members of the team co-authored a total of 232 scientific articles published in journals with impact factors (IF). Below, a summary of scientific outcomes, i.e. monographs (books), chapters in books and articles in IF journals, is provided separately for every year. We publish in journals that belong to many other fields than parasitology only, i.e. aquaculture, veterinary science, phylogenetics, evolutionary biology, biology in general, etc. Regarding impact factors of parasitological journals, research on fish parasites is lagging behind when compared with human parasitology or even pet and other lifestock parasitology. In fact, research in fish parasitology is advancing mainly because the aquaculture industry has now become the fastest growing lifestock production industry.

Year	Books	Chapters	IF articles	Sum of IF	Range of IF
2010	-	1	40	72.062	0.426–6.574
2011	-	2	52	101.811	0.333–4.153
2012	-	1	56	122.927	0.696–6.275
2013	2	1	39	81.459	0.965–6.217
2014	-	1	45	85.345	0.304–6.217
TOTAL	**2**	**6**	**232**	**463.604**	**0.304–6.574**

4. **MAIN RESULTS**

Some of the results achieved during the period of 2010–2014 are presented herein, but additional data can be taken from a list of 23 selected outcomes with their brief annotations and actual contribution of team members. Papers mentioned in the text below are numbered [in brackets] as those in the complete list of all 232 papers published in journals with impact factor, which forms another part of materials provided for the first phase of the evaluation.

4.1. **Laboratory of Fish Protistology**

4.1.1. *Myxozoan biodiversity, phylogeny and evolution*

DNA sequencing and phylogenetic analyses of previously molecularly uncharacterised type species and of poorly represented genera as well as myxozoans from extreme habitats or unusual hosts has allowed for unique insights into inter-and intraspecific divergences, the ecology, biogeography, phylogeny and evolution of the Myxozoa [papers Nos. 42, 43, 52, 56, 64, 86, 101, 153, 159, 183; other publications Nos. 11, 15]. These studies are absolutely essential for re-designing the myxozoan taxonomic system, which is based on spore

morphology and contradicts molecular phylogeny. In several comprehensive papers we mapped morphological traits as well as life-cycle-related characteristics such as host group/species (both vertebrate and invertebrate host), pre-sporogonic development and cellular characteristics to the phylogenetic tree and provided insights into the evolution of these clades [papers Nos. 153, 189]. These large-scale studies have had considerable impact and feedback from the myxozoan research community. Molecular markers used for phylogeny included ribosomal DNA sequences and, for the first time, genes with an independent evolution [paper No. 23]. We furthermore traced the history of myxozoan character evolution and succeeded in the disclosure of ancestral morphotypes and their development and extreme morphological plasticity over the course of time [paper No. 23]. Only the use of DNA sequencing allowed us to unveil cryptic species assemblages [papers Nos. 42, 149] or the hidden biodiversity of myxozoans belonging to the class Malacosporea [paper No. 183].

4.1.2. *Functional aspects of myxozoans inside their fish hosts*

The first part of our motility studies focused on pre-sporogonic stages of the bile myxozoan *Ceratomyxa puntazzi* [paper No. 98]. We demonstrated that F-actin-rich cytoskeletal elements polarise at one end of the parasites and in the filopodia that are rapidly created *de novo* and re-absorbed. We discovered that the mechanism of budding is an active polarisation process of cytokinesis, which is independent from a contractile ring and thus differs from the mechanism generally observed in eukaryotic cells. We have also performed a comprehensive functional analysis of the unique twitching/dancing behaviour of sphaerosporid blood stages in carp, which represents a new motility type that reveals important insights into the evolution of metazoan motility. Motility studies were based on a combination of confocal laser microscopy, scanning and transmission electron microscopy and gene expression studies/transcriptomic data.

4.1.3. *Pathology caused by myxozoans*

Pathological changes induced by myxozoans were described in detail in fish important for fisheries, such as the spotted seatrout [paper No. 79 and other publications No. 11] and grey snapper [other publications No. 12], and for aquaculture, e.g. the sharpsnout seabream [paper No. 86], but also in small, terrestrial mammals, i.e. shrews [paper No. 81]. Thereby, the pathological alterations observed reached from epithelial slouthing and pericholangitis over severe cardiac lesions to previously unknown xenoma-like formations induced by the parasites.

One important study was focused on a disease entering naïve amphibian populations, a key threatening process contributing to the precipitous global decline of biodiversity. This particular paper investigated the translocation of *Myxidium* spp. into endemic Australian frog populations by introduction of the cane toad (*Rhinella marina*). rDNA sequence distances confirmed their independent evolutionary trajectory and suggest that the cane toad may have played an important spill-back role in parasite emergence and may have facilitated their dissemination [paper No. 53].

4.1.4. *Identification, description and phylogeny of amoebae:*

Research on amoebae focused on free-living species (*Flabella, Hartmanella, Stenamoeba, Vermistella, Vexillifera,* etc.) and their phylogeography, ultrastructure and biology [papers Nos. 26, 60] as well as on amphizoic species (*Acanthamoeba, Grellamoeba, Naegleria* and *Paramoeba* spp.) and their relation to fish [paper No. 8] and invertebrate [paper No. 61] pathology. Amoebae isolated from cases of nodular gill disease and amoebic gill disease (AGD) in rainbow trout, farmed turbot and Atlantic salmon were characterised [papers Nos. 6, 24]. A large part of the unique culture collection of amoebae of the Laboratory of Fish Protistology was documented in detail with regard to their ultrastructure and molecular phylogeny in a comprehensive monograph “Illustrated Guide to Culture Collection

of Free-living Amoebae" [book No. 2]. The well-documented and curated culture collection together with our expertise in culturing a variety of amoebic organisms offers a great potential for future research, as it is of commercial interest (e.g. contracts with Skretting ARC, Norway, see Section 6) as well as of scientific interest, attracting a number of collaborators [e.g. paper No. 65].

4.1.5. *Diagnostic assays and applied approaches aiming to combat fish diseases in aquaculture*

The design of specific PCR and qPCR assays and *in situ* hybridisation protocols for a number of myxozoans contributed important information on the host specificity and localisation of different species as well as on the quantity of infective stages in the water column [e.g. papers Nos. 149, 152, 159, 205].

A quantitative PCR assay for *Ceratomyxa puntazzi* was designed and used on filtered seawater samples, obtained monthly throughout the year at different depths. This myxosporean infects sharpsnout seabream, an important novel aquaculture species in the Mediterranean. In parallel, sentinel fish were exposed to a *C. puntazzi*-enzooic habitat every month and we were able to demonstrate that fish become infected throughout the year but the parasite is not able to proliferate in the winter months. This represents the first study on the seasonality and transmission of myxozoans in the sea, and provides important implications for adaptive management strategies for the aquaculture of this fish species [paper No. 152].

Over the last 2 years we have been working on developing the first *in vitro* culture of myxozoans, using blood stages of *S. molnari*. We are now able to maintain the parasites in culture for > 6 weeks and aim at further improving the protocol. In the future, we plan on using myxozoan *in vitro* model to try out potential myxozoicidal substances as well as molecular interference approaches.

The agent of AGD, *Paramoeba perurans*, has been the focus of numerous *in vitro* trials aiming at the development of an in-feed treatment for Atlantic salmon in aquaculture facilities in northern Europe and Australia (see Section 6).

A number of other important publications focusing on a variety of organisms and topics cannot be ascribed to the above sections but are a result of our expertise in different fields, e.g. protist barcoding, phylogenetics methods, aquaculture pathogens, parasite transmission studies, etc. [papers Nos. 25, 54, 65, 102, 110, 117, 141, 142, 143, 197, 199, 201].

5.2. **Laboratory of Helminthology**

5.2.1. *Diversity, taxonomy and life-cycles of fish eye- and brain-infecting diplostomids*

Intensive sampling of life-cycle stages of *Diplostomum* spp. from snails, fish and birds carried out in the northern, central and southern regions of Europe resulted in establishment of novel *cox*1 (144 sequences) and ITS1-5.8S-ITS2 (64 sequences) reference libraries for the European species of *Diplostomum*. Two of the named species and 11 of the lineages (arguably species) delineated in the datasets studied originate from Europe, thus indicating a substantial unrecognised genetic diversity inferred from molecular evidence. The molecular vouchers of the adults of two named species from birds and the larval stages of five putative species from fish and snails are described in detail [papers Nos. 161, 215, 224, 232].

The first application of a DNA-based approach to diplostomid diversity in the African continent provided evidence for the existence of three distinct brain-infecting species of *Tylodelphys* (originally misidentified as species of *Diplostomum*) co-occurring in natural populations of the catfish *Clarias gariepinus* in four water bodies in Tanzania. Mitochondrial and ribosomal sequences were also generated for a novel species of *Diplostomum* parasitising another African fish host, *Synodontis nigrita* [paper No. 157].

5.2.2. *Diversity and community ecology of trematodes in molluscs*

The application of morphological and molecular markers (mitochondrial *cox*1 and *nad*1 genes and ITS and LSU of the rRNA gene) helped elucidate the diversity of the larval trematodes in freshwater planorbid (*Petasiger* spp.; life-cycle of one species elucidated using molecular evidence) and lymnaeid (*Plagiorchis* spp.) snails in Central Europe and in marine trochid (two species) and littorinid (6 species) snails in the Mediterranean and off New Zealand, respectively [papers Nos. 110, 122, 207, 208, 231].

A series of studies on trematode communities in populations of *Lymnaea stagnalis* and *Radix auricularia* from eutrophic environments (fishponds in South Bohemia and lakes in the River Ruhr catchment in Germany) demonstrated increased, nutrient-mediated levels of parasitism. The first assessment for a pulmonate snail host, and for highly productive aquatic environments, of the rates of colonisation and extinction at the level of individual snail host patches confirmed the prediction that eutrophic environments provide conditions for speeding up trematode transmission, thus increasing the strength of interspecific interactions. Postulation of a dominance hierarchy for trematodes in *L. stagnalis* and the application of a sequential null-model analysis of the effects of spatial and temporal heterogeneity in conjunction with the competition hypothesis demonstrated, for the first time on a freshwater snail-trematode system, a significant bottom-up structuring effect of competition at the infracommunity level and provided evidence for its additivity at the component community level [papers Nos. 15, 44, 63, 68, 121].

5.2.3. *Global diversity of helminths parasitising teleost fish*

Morphological and molecular studies on trematodes parasitising freshwater and Mediterranean fish resulted in descriptions of six new species and modern redescriptions of three species; of these, seven are molecularly characterised [papers Nos. 7, 69, 97, 123, 158, 206, 211]. A series of studies at the population and community levels elucidated the establishment and population dynamics of important fish pathogens (*Ichthyocotylurus* spp. and *Zeuxapta seriolae*) in their freshwater (*Perca fluviatilis*) and marine (*Seriola dumerilii*) fish hosts, respectively [papers Nos. 62, 176]. These studies pinpointed general macroecological patterns capturing essential fundamentals of the structuring of parasite distributions in marine host-parasite systems. A combined assessment of the taxonomic and community diversity was applied to address the use of parasites as indicators of population connectivity and in assessment of the effects of fishing and pollution of host-parasite systems in coastal and deep-sea marine ecosystems of the Mediterranean [papers Nos. 39, 111, 136].

Extensive taxonomic studies of nematode samples from freshwater and marine fishes in different geographical regions made it possible to discover and describe a large number of previously unknown species and to redescribe others. Special attention was paid to the important group of tissue-dwelling philometrid nematodes, in particular those parasitizing marine fishes. A total of 45 new species of philometrids, including two new genera (*Clavinemoides* and *Dentirumai*), were established based on specimens mainly from marine fishes. These studies showed a high degree of host specificity in gonad-infecting species, where congeneric fish hosts in the same locality were parasitised by morphologically very different philometrid species. An additional 28 new species of fish nematodes, including two new genera (*Ascarophisnema* and *Metabronemoides*), were described mainly from freshwater and marine fishes in Asia, Africa and North America. [For individual papers – see a list of all publications of the team; in total, 82 articles were published with F. Moravec mostly serving as the first and corresponding author].

5.2.4. *Species boundaries and utility of molecular markers in phylogenetic studies*

A multidisciplinary approach to the systematics of basal cestodes (Caryophyllidea) and international collaboration (Slovakia, Switzerland, UK and USA) made it possible to define species boundaries in several groups of fish cestodes and to assess reliably the extent of intraspecific variability and taxonomic importance of morphological characters. On one hand,

the existence of cryptic species was detected in species of the genus *Paracaryophyllaeus*, parasites of loaches (Cobitidae) [paper No. 217]. On the other hand, molecular analysis of an extensive specimen collection of morphologically distinct tapeworms of the genus *Caryophyllaeus*, parasites of cyprinid fishes, brought evidence of host-related plasticity in critical morphological characters widely used for species circumscription and classification of these tapeworms. [Papers Nos. 200, 212 and *Hanzelová et al.* 2015: *Systematic Parasitology* 90: 177–190.]

The utility of two nuclear and two mitochondrial molecular markers (*ssr*DNA and *lsr*DNA, *nad*3 and *cox*1) for use in circumscribing generic boundaries and estimating interrelationships of fish cestodes of the order Caryophyllidea, probably the most early evolved group of 'true' tapeworms (Eucestoda), has been evaluated. It has been demonstrated that these commonly employed markers do not contain sufficient signal to infer well supported phylogenetic estimates due to substitution saturation. Moreover, multiple *trnK + nad3 + trnS + trnW + cox1* haplotypes within individuals were detected, indicating a history of gene exchange between the mitochondrial and nuclear genomes. The presence of such nuclear paralogs (i.e. numts) was described in cestodes for the first time [paper No. 103].

5.2.5. *Taxonomic revisions and interrelationships of parasitic flatworms*

The '*revolutum*' species complex of the trematode genus *Echinostoma* was revised based on material from an extensive sampling programme in Europe. Morphological, molecular and experimental evidence supported the validity of six species, including two new to science; keys to the identification of their cercariae and adults were elaborated. Phylogenetic analyses based on the mitochondrial *nad*1 gene identified 17 species within this complex worldwide and resulted in an updated synonymy [papers Nos. 160, 216, 227].

Several taxonomic papers dealing with fish cestodes and based on the evaluation of extensive, newly collected material using tools of molecular phylogenetics and molecular taxonomy provided the first data on the phylogenetic relationships of the taxa studied. Results often indicate incongruence between the current classification inferred largely from morphological characters and natural grouping as revealed by molecular data [papers Nos. 78, 124, 128, 177, 184, 212, 221]. As an example of complex systematic and phylogenetic studies, a paper on the cestode genus with convoluted taxonomic history is briefly commented on.

Current knowledge of parasite diversity in the Indian subcontinent is disastrous due to shortage of rigorous studies of adequate quality. Within the last decades, an excessive number of taxa have been described, but almost all descriptions violated basic rules of modern taxonomy and systematics. Based on two collecting trips to India and Bangladesh, species of the genus *Gangesia* from the Indomalayan zoogeographical region were revised and only 4 species, instead of 48 nominal taxa (with 36 new synonyms), were recognised as valid. This article represents the first study that applies a multidisciplinary approach (morphology, scanning electron microscopy, molecular taxonomy and phylogeny) and thus may serve as a model to guide researchers who are confronted with similar taxonomic inflation problems [paper No. 126].

5.2.6. *Monographs and book chapters*

F. Moravec, researcher emeritus, summarised extensive data on the nematodes parasitising freshwater fish of Europe accumulated since the first edition of his monograph published by Kluwer and Academia in 1994, i.e. during almost 20 years [book No. 3]. Data on the morphology, host associations, geographical distribution, life-cycles and veterinary importance of members of this important group of fish parasites are presented together with keys to the identification of all species found in Europe. Similarly as the first edition of this book, Moravec's monograph, which is based to a great extent on the author's own new data,

will serve as a key reference on fish nematodes in Europe for many forthcoming years or even decades.

A chapter by A. Kostadinova, A. Pérez-del-Olmo (Spain) and S. Morand (France) [chapter No. 5] on metapopulation dynamics in marine parasites defines the use of metapopulation concepts in addressing marine parasite dynamics. It illustrates a continuum of demographic connectivity among local parasite populations considering the degree of host specificity and parasite dispersal abilities. Moreover, it links spatial patterns of parasite populations with basic epidemiological models and suggests new avenues of research to advance our knowledge of metapopulation structure and dynamics of both marine parasites and their hosts.

A chapter by A. Kostadinova and A. Pérez-del-Olmo (Spain) [chapter No. 6] focuses on the insights into the systematics of the platyhelminth class Trematoda resulting from the advanced understanding of both morphological and molecular data over the past 15 years. The chapter outlines a framework for robust estimates of trematode phylogeny highlighting the application of advanced morphological and molecular approaches and provides a critical look at the problems resulting from limited taxon sampling.

5.2.7. *Review articles and large-scale surveys*

A survey of bothriocephalidean tapeworms (Cestoda) parasitising African freshwater fish was provided. Based on critical evaluation of type specimens and extensive, newly collected material, only the following seven species, instead of 19 taxa listed in the literature, are considered to be valid and their redescriptions are provided. In addition, one new species is described and a new genus is proposed to accommodate already known species from a clariid catfish. All but one species exhibited narrow host specificity, being limited either to one host species (oioxenous host specificity) or one host genus (stenoxenous specificity). Molecular data based on *lsr*DNA show monophyletic position of all five African taxa analysed [paper No. 106].

A review article was published, summarising the current knowledge of philometrids (Philometridae), the most important, species-rich group of dracunculoid nematodes parasitizing freshwater, brackish-water and marine fishes worldwide. Especially the male surface ultrastructure studied by SEM provided new taxonomically important features for species distinction, which resulted in a 40% increase of the number of known species within a short period (2007–2015). New data were obtained on the biology and pathogenicity of several species belonging to four philometrid genera. A contemporary list of valid philometrid species (162) by continent is provided [paper No. 167].

The current knowledge on the occurrence and distribution of swimmer's itch was summarised, with a focus on Europe. Relevant studies from the past decade were analysed to reveal an almost complete set of ecological factors as a prerequisite for establishing the life cycle of bird schistosomes. Based on both records of the occurrence of the parasite infective agents and epidemiological studies that investigate outbreaks of swimmer's itch, this review concentrates on the risk factors for humans engaged in recreational water activities [paper No. 214].

5.2.8. *Fish-borne helminthoses as emerging human diseases*

Recent (re-)emergence of human infections with helminth parasites transmitted by fish (fish-borne parasitic diseases) calls for more attention to be paid to the biology and diagnosis of their causative agents. As part of the project focused on cestode order Diphyllobothriidea Kuchta, Scholz, Brabec et Bray, 2008, new data on several human-infecting species of *Diphyllobothrium* have been accumulated [paper No. 186], including re-identification of the first human cases caused by the Pacific seal tapeworm, *D. pacificum*, from Europe (Spain) [paper No. 225].

5. **IMPACT OF TEAM ACTIVITIES ON THE SCIENTIFIC COMMUNITY**

Probably the most important measure of the actual impact of a researcher on the scientific community is the number of citations of his/her articles and other publications such as monographs measured as Science Citation Index (SCI). Therefore, a brief overview of SCI of team members with average FTE (average working capacity during the evaluated period) at least 0.4 is provided, reflecting the fact that the number of citations (and Hirsch index, too) is cumulative and thus older researchers are favoured in this parameter.

Science Citation Index (without primary autocitations; from 1990 to 2013)

Name	PhD	Total SCI	Last 5 years	h-index
Alama-Bermejo	2011	25	25	3
Bartošová-Sojková	2010	68	68	6
Blasco-Costa	2009	39	39	4
Brabec	2012	154	154	6
Dyková	1971	1810	489	20
Faltýnková	2006	121	121	7
Fiala	2006	412	293	12
Hartigan	2012	34	34	4
Holzer	2004	163	125	8
Jirků Miloslav	2008	149	142	9
Kostadinova	1994	287	269	11
Kuchta	2007	347	278	9
Moravec	1970	2621	1053	24
Scholz	1989	2089	1107	26
Soldánová	2011	11	11	4

6. **PRACTICAL OUCOMES**

The mission of the Institute and its research teams is basic research and thus practical outcomes are not their first priority. Nevertheless, parasite groups studied are usually chosen based on their veterinary importance as potential pathogens in cultured and feral fish. For example, the Asian fish tapeworm (*Bothriocephalus acheilognathi*) is one of the most successful invasive parasites and was even diagnosed in man [chapter No. 4 and paper No. 178]. In this context, correct identification of pathogens by reliable diagnostic tools is of uttermost importance. Diagnostic assays can be used to screen fish populations in the wild and in culture environments (e.g. sea cages), as well as in the water column between culture sites. Thus, such diagnostic tools are considered an important practical outcome.

In addition, while governmental research funding gets more and more competitive, there is a general need for obtaining alternative funds for research from the private sector, in this case the pharmaceutical and aquaculture industry. These partners strive to design and perform applied research approaches in collaboration with academic expertise. Such applied approaches can result in important patents and effective antiparasitic strategies for the fish farming community and the aquaculture industry and are thus of great importance for lowering the effects of parasitic infections on aquaculture production and economy.

The following diagnostic tools designed and data produced by the team are of potential practical importance:

1. Single and multiplex PCR assays, quantitative PCR assyas as well as *in situ* hybridisation protocols for causative agents of important myxozoan fish diseases such as swimbladder inflammation of common carp (*Sphaerospora dykovae*), skin and gill sphaerosporosis of common carp (*S. molnari*), severe glomerular disease of grey snapper (*S. motemarini*), ceratomyxosis in sharpsnout sea bream (*Ceratomyxa puntazzi*), and others.

2. To facilitate differential identification of morphologically indistinguishable human-infecting broad fish tapeworms (*Diphyllobothrium* spp.) in clinical samples, a new diagnostic method has been developed and optimised in collaboration with Swiss researchers. The method is based on a multiplex PCR amplification of a selected gene (*cox*1), does not involve sequencing and thus represents a significantly cheaper, more straightforward and easily interpretable approach to be utilised routinely mainly by medical diagnostic laboratories. [Paper No. 38].
3. Identification keys and species-specific genetic markers (sequence database), which will facilitate routine diagnostics of potentially pathogenic parasites.
4. Definition of species boundaries in model groups will help in proposing reliable markers to identify parasites on the basis of morphological, ecological and genetic data.

In addition to designing diagnostic assays and producing data, which are useful for pathogen indentification by a wider public, we also provide professional consultation on fish parasites, e.g. information on pathology status or treatments and management strategies. This service is used by fish pathologists, veterinarians, home aquaculturists, large public aquaria (e.g. Oceanografico, Valencia, Spain) and the private aquaculture industry (e.g. Mote Marine Laboratory, Florida, USA; Marine Harvest, UK). We have furthermore elaborated applied studies with an important industry partner, Skretting (Norway), which is the largest producer of fish and shrip food worldwide and is also the global leader in providing innovative and sustainable nutritional solutions for the aquaculture industry. The Skretting Aquaculture Research Centre financed two research contracts on *Paramoeba* spp. that focused on: (i) the identification and cultivation of amoebic gill disease agents from various Atlantic salmon culture sites in Scotland; and (ii) testing of potential amoeboicidal substances in cell cultures of *Paramoeba* spp. as well as determining the survival of cultured amoebae when exposed to mucus samples from salmon that had received different in-feed treatments. The results form an important basis for developing antiparasitic diets for Atlantic salmon. Skretting is also interested in further projects with the Fish Protistology Lab, aiming at using the *in vitro* myxozoan model we are currently developing and optimising.

7. **SUMMARY**

Based on scientific outcomes (> 230 IF articles, 2 monographs and 6 chapters in books within 5 years), the impact of results on the scientific community (reasonably high SCI with up to 300 citations per year/person), international reputation (invited lectures and reviewing PhD thesis abroad) and the key role that team members play within the ichthyoparasitological scientific community, it does not seem to be an exaggeration to state that Czech fish parasitology, in which the the team of the Institute of Parasitology (IPCAS) represents the most important group, occupies the top position in the world.

In the complexity of research covering field sampling, laboratory evaluation of material and analyses of data, the ability to apply successfully an integrative approach to the systematics, and strong influence to the international community of fish parasitologists, the team from IPCAS represents a unique working group incomparable with any other fish parasitology team globally. The application of updated, cutting-edge methodological approaches and current focus on evolutionary and veterinary important groups of parasites of teleost fish and other aquatic hosts, together with public consulting services and industrially funded research, as well as well-blended personal and age structure of the team, represent aspects that will enable us to keep that leading position at the international level also in the forthcoming years.

Research Report of the team in the period 2010–2014

Institute	Biology Centre of the CAS, v. v. i.

Scientific team	Opportunistic parasitic diseases

Team genesis

The evaluated team comprises two laboratories: Laboratory of Veterinary and Medical Protistology and Laboratory of Parasitic Therapy. The Laboratory of Veterinary and Medical Protistology was established in 2009 by combining the Laboratory of Opportunistic Parasites and the Laboratory of Coccidia, consisting of 7 research scientists (mean FTE 5.1). In 2010, the team was reduced to 3 research scientists (M. Kváč, B. Sak, and D. Modrý, the latter with partial-time 0.2; mean FTE 2.2) based on the Institute reorganization and unsatisfactory scientific productivity of some of the previous staff members. The Laboratory of Parasitic Therapy (1 researcher – K. Jirků-Pomajbíková; FTE 1) joined the team in October 2013.

The team currently consists of **M. Kváč** (FTE 1), **B. Sak** (FTE 1), **K. Jirků-Pomajbíková** (FTE 1), and **D. Modrý** (FTE 0.2; his main research activities are at the University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic).

Comparison of scientific outputs between period 2005–2009 and 2010–2014.

During the period 2005–2009, a total of 86 papers in journals with impact factor were published (3.4 outputs per FTE per year) and 3 research projects were achieved (2 of them were postdoc projects of the current head of the team (M. Kváč)). In the period 2010–2013, the Laboratory of Veterinary and Medical Protistology produced 73 IF manuscripts (8.3 outputs per FTE per year). In the most prolific year 2014, the team published 23 IF outputs (7.2 outputs per FTE per year). The number of outcomes and cumulative impact factor per team is provided below in Table 1. Eight grant projects from national and international agencies have been approved (see the List of funded projects in 2010–2014).

Table 1. Number of outcomes and cumulative impact factor per team

2010			2011			2012			2013			2014			Total		
M	B	CIF	M	B	CIF	M	B	CIF	M	B	CIF	M	B	CIF	M	B	CIF
14	0	42.3	20	0	83.9	15	0	51.4	23	1	93.9	23	1	84.2	95	2	355.6

M – IF manuscripts; B – books or book chapters; CIF – cumulative impact factor;

List of funded projects in 2010–2014

- Ticking time-bomb of latent microsporidiosis: hidden threat for human health. 2014–2016. Czech Science Foundation. P.I.: **B. Sak**.
- Prevalence, genotypic characterization and clinical effects caused by *Blastocystis hominis* in patients with HIV and AIDS. 2013–2014. Polish Society for AIDS Research. Contractor: **M. Kváč**.
- Clinical, immunological and molecular profile of microsporidiosis and cryptosporidiosis in patients living with HIV in the population of Lower Silesia. 2013–2014. Polish Society for AIDS Research. Contractor: **M. Kváč**.

- The application of molecular methods to identify and characterize Microsporidia in immunocompetent and immunosuppressed patients with kidney disease and evaluating the impact of selected drugs on the process of Microsporidia invasion in *in vitro* research. 2013-2017. 2012/05/D/NZ6/00615. National Science Centre, Poland. Contractor: **M. Kváč**.
- Development of the scientific team and laboratory for infectious diseases common to humans and great apes. 2012–2015. CZ.1.07/2.3.00/20.0300. P.I.: D. Modrý, Co-I.: **M. Kváč**.
- Anti-inflammatory activity of extracts isolated from selected Indonesian plants and their effect on opportunistic parasitoses. 2011–2015. 505/11/11635. Czech Science Foundation. Co-I.: **B. Sak**.
- Diversity, biology and phylogeny of *Cryptosporidium* spp. parasiting in rodents. 2011-2014. KONTAKT LH 11061. PI.: **M. Kváč**.
- Impact of increased contact with humans on diversity and ecology of protozoan parasites of African great apes. 2009-2011. 206/09/0927. Czech Science Foundation. PI: **D. Modrý**, CoI.: **M. Kváč**

Main research priorities

Laboratory of Veterinary and Medical Protistology

Human and animal health is threatened by various infectious diseases, including both apparent and inapparent infections. The latter represent the actual danger from the medical point of view, since the manifesting diseases could be, and in majority cases are, successfully treated, but the hidden infections may have serious health consequences and various non-specific pathologies prior to being detected, making the disease difficult to treat.

The staff perform research on human and animal parasites belonging mainly to the genus *Cryptosporidium* and the phylum Microsporidia (genera *Encephalitozoon* and *Enterocytozoon*). We have focused on all aspects of parasite structure, biology, life cycles, host-parasite relationships including course of infection, host-, age- and gender-specificity, epidemiology, immune response, phylogeny and evolution.

Laboratory of Parasitic Therapy

Over the last decades, the Western life style has reduced our contact with microbes through adoption of highly hygienic habits, access to clean food and water, and over-use of antibiotics. However, contact with antigens is required for proper immune system development and immunoregulation. This predisposes the people to hypersensitivity and autoimmune diseases mediated by the immune system, such as various allergies, asthma, type 1 diabetes, multiple sclerosis, and inflammatory bowel diseases (IBD).

Abundant evidence drawn from studies of bacteria and various eukaryotes (including protists and helminths) demonstrates that they are centrally involved in the development of IBD. The main lines of this laboratory are focused on the investigation of an impact of the commensal gut eukaryotes on the IBD, identification of additional organisms for prevention and therapy of IBD, and novel therapeutic approaches.

International cooperation in the evaluation period

- CDC, Division of Parasitic Diseases, Atlanta, USA
- Center for Food Safety, University of Georgia, Griffin, USA
- Christchurch Science Centre, Christchurch, New Zealand
- CNRS-Institut des Sciences de l'Evolution de Montpellier, Montpellier, France
- Durham University, Durham, United Kingdom

- Higher National School of Veterinary, EL Harrach, Algiers, Algeria
- Parasitological Institute of the Slovak Academy of Sciences, Košice, Slovakia
- Karisoke Research Center and the Rwanda Development, Rwanda
- Montana State University, Bozeman, USA
- North Dakota State University, Fargo, USA
- Rwanda Development Board (RDB), Kigali, Rwanda
- Staten Serum Institute, Copenhagen, Denmark
- University of British Columbia, Vancouver, Canada
- University of Kent, Canterbury, United Kingdom
- University of Ottawa, Ottawa, Ontario, Canada
- Wroclaw Medical University, Wrocław, Poland
- WWF, Dzanga Sangha Protected Areas, Bangui, Central African Republic

Main results achieved in individual research areas (2010–2014)

A) Human parasitoses with emphasis on cryptosporidiosis and microsporidiosis

Numerous gastrointestinal pathogens have emerged in recent decades, including species of *Cryptosporidium* and microsporidia. They are ubiquitous pathogens infecting a wide spectrum of vertebrates and the symptoms in immunocompetent hosts are usually mild and self-limiting. However, human cryptosporidiosis and microsporidiosis emerged as important opportunistic diseases when AIDS became pandemic.

The research team contributes to elucidate the epidemiology of microsporidiosis, a generally neglected disease, in immunocompetent persons. A series of surveys using one-shot sampling methodology revealed a common occurrence of *Encephalitozoon* spp. and *E. bieneusi* exceeding 40% in healthy populations. Given the life-long exposure to ubiquitous infectious stages, the prevalence increased with age, reaching 100% cumulative prevalence. Infected humans are predominantly asymptomatic carriers. However, microsporidia can be responsible for life-threatening diseases such as encephalitis and meningitis in immunocompetent patients; these diseases may be erroneously considered as illness of unknown etiology due to inadequate methods of diagnosis. In connection with our research, we documented a case of brain abscess caused by the *E. cuniculi* genotype I in an immunocompetent patient, whose life was saved by our prompt specific diagnosis. Moreover, our diagnosis elucidated the health complications of renal transplant recipients.

During the period 2010–2014, we screened more than 3,000 immunocompetent persons with gastrointestinal diseases for the presence of species of *Cryptosporidium*. In addition to *C. parvum* and *C. hominis*, which cause more than 90% of human cryptosporidiosis, we reported unusual human infection with *C. tyzzeri* (mouse-specific species), *C. erinacei* (hedgehog-specific species) and *C. muris* (rodent-specific species) and provided a detailed description of the course of human infection and molecular subtyping of its causative agent. We also contributed to the design of specific primers for subtyping the zoonotic *C. ubiquitum*, which will help identify the sources of human infection.

In the study focused on detection of *Plasmodium* spp. in human faeces, we showed that similar to apes, infected humans shed a detectable amount of *Plasmodium falciparum* in their faeces, which correlates with the results obtained by PCR. For comparison, Southern blotting slightly enhanced the sensitivity of the PCR. We conclude that faeces are as suitable as blood for diagnostics of human malaria.

We published a review article that focuses on the self-infections with parasites and their re-interpretations, with the aim to change our view on some non-pathogenic parasites that may have positive effect on the human health, especially immune-mediated diseases. This article points out that self-infections with parasites also shed the light on the life cycles, host specificity or epidemiology of many parasites. This review is associated with the research addressing the impact of helminths on the human immune-mediated diseases including hypersensitivity (allergies, asthma) and autoimmune diseases (such IBD, rheumatoid arthritis or multiple sclerosis, etc.). We also evaluated the criteria for helminths already tested on humans allowing us to identify other protist and helminth non-pathogenic parasites as candidates that can be tested for their suitability in therapy of human autoimmune diseases.

Total number of outputs: 14

Contribution of the team: Team collaborated with several institutions in the Czech Republic, Slovak Republic, Poland, USA, Uganda and Central African Republic. Out of 14 outputs, 6 were 100% done by team members and others were prepared by collaborative effort, with the team involvement exceeding 50%. The collaborators helped with sequencing, sharing the costs, sample collection, analyses and manuscript preparation.

B) Parasites of non-human primates

Increased anthropogenic pressure on populations of wild and captive great apes and their environment can result in substantial changes in communities of parasites, but also in increased exchange of parasites between humans and apes. Parasite infections are common in wild and captive individuals often without direct observable pathogenicity, however, the anthropogenic disturbance and pressure can result in a suite of alternations in host ecology and its environment. In this regard, we analyzed several thousands of faecal samples of African great apes obtained from several African wild sites and sanctuaries. For comparison, we collected the faecal samples from the European zoological gardens and facilities, including several Czech zoos, of which some were key places for our experimental work. This research has three main lines: (i) to monitor the diversity of the parasite fauna of wild-ranging African great apes, (ii) to study the distribution, host specificity, epidemiology, molecular diversity and phylogeny of gastrointestinal protists, and (iii) to study the distribution, molecular diversity and phylogeny of the gut ciliates.

We described the results of monitoring of the intestinal parasite fauna in three unique wild chimpanzee populations in Africa, namely the introduced chimpanzee population in the Rubondo Island in Tanzania, the wild chimpanzee population in Guinea Bissau characterized by intensive anthropogenic fragmentation, and the population of savannah chimpanzees living in extremely dry habitats in Tanzania. Overall, the spectrum of parasites was similar to wild chimpanzee populations elsewhere, but each of the populations studied was characteristic by a high prevalence of specific or unusual parasites. The differences observed between the parasite fauna of such heterogeneous ape populations contribute to our understanding of the ecology of parasitic infectious diseases and have the potential to contribute to conservation polices.

Protists represent a neglected part of gut parasite communities of apes and provide several advantages for the research focused on the pattern of zoonotic transmissions. In this regard, we used molecular tools for evaluation of the real prevalence and genetic diversity of protists with possible zoonotic potential (trichomonads, microsporidia, *Cryptosporidium*, *Giardia*, *Blastocystis*) throughout several captive and wild

populations of African great apes. Microsporidia were detected in all captive (European zoos and African sanctuaries) and wild populations of apes, where several new genotypes were found. However, prevalence was always highest in African sanctuaries and in the case of wild nature in the groups of individuals under intensive anthropogenic pressure (research and tourism). Similarly, *Giardia* assemblages and *Cryptosporidium* species were always found especially in the groups of apes at the higher level of habituation. These results emphasize a potentially negative impact of habituation on selected pathogens that might occur as a result of the more frequent presence of humans in the vicinity of habituated great apes. In the case of trichomonads, we conducted the monitoring of their presence only in captivity and our results suggest that captive primates possibly may be infected by intestinal trichomonads of other vertebrates.

Our last main research area was focused on the intestinal ciliates of African great apes, the symbiotic ones and also pathogenic species. We described a new species of ciliate found in wild chimpanzees (*Troglocorys cava*) and conducted a survey of the distribution of two entodinimorphid ciliates (*Troglodytella abrassarti* and *T. cava*) and *Balantidium coli* in captive and wild-ranging African great apes. *Troglodytella* was found in both types of populations, whereas *Troglocorys* only in wild populations and *Balantidium* in captivity, probably as a result of a close contact with reservoir hosts and starch rich diet. Furthermore, we experimentally confirmed the effect of dietary starch on the population of *Balantidium* in the gut of captive chimpanzee and concluded that this kind of diet might predispose great apes to clinically manifested balantidiasis. To evaluate the genetic diversity of *Balantidium*, we chose two genetic markers and our phylogenetic trees showed the polyphyletic character of the genus *Balantidium*. By contrast, we proved the active participation of *Troglodytella* on the hindgut digestion based on the analyses of the specific activities of their enzymes against different polysaccharides. For experimental purposes, we validated the quantification method for the above studied intestinal ciliates of apes.

Total number of outputs: 21

Contribution of the team: All outputs were made under collaborations with several institutions, zoological gardens and sanctuaries from Czech Republic, Central African Republic, Uganda, Kenya, USA, with the involvement exceeding 30–70%. The collaborators helped with the sample collection, data analyses and preparation of manuscripts.

C) Porcine cryptosporidiosis and microsporidiosis

The research was designed to address a critical gap in the knowledge of the diversity of species of *Cryptosporidium* and microsporidia, and it led to an increased understanding of how genetic diversity is related to biological diversity.

On the basis of discrepancies between field research and general biology of members of the genus *Cryptosporidium*, we performed comprehensive surveys and experimental studies of cryptosporidiosis in pigs and wild boars. A major outcome was the description of a novel species, *Cryptosporidium scrofarum*, based on unique morphological, biological and molecular characteristics. Experimental studies surprisingly showed that *C. scrofarum* is infectious only for older pigs; this age specificity is unique among all *Cryptosporidium* species. We also provided evidence that the system of *Cryptosporidium* detection in pigs using PCR can produce misleading results and proposed a new methodology to improve reliability of diagnostics. The proposed method was validated on more than 2,500 domestic and wild pigs and under experimental conditions. The epidemiology and diversity of

Cryptosporidium and microsporidia were analyzed. Due to cumulative prevalence of zoonotic microsporidia reaching up to 90% in both domestic pigs and wild boars, these hosts represent major natural reservoirs of these pathogens.

The non-susceptibility of pigs to species of *Cryptosporidium* from rodents was verified under experimental conditions. It is necessary to highlight that the performed experimental infections are unique within research community.

Total number of outputs: 8

Contribution of the team: Work was completely (100%) done by team members. The collaborators helped with preparation of manuscripts.

D) Parasites of other wild and domestic ungulates

At the beginning of the evaluated period, the team finished the long-term project focused on cryptosporidiosis in livestock. The main result of this project was the determination of age-specificity and management-depended distribution of *Cryptosporidium* infection in cattle.

However, the main focus in this research stream during 2010-2014 was targeted on domestic equines. Although both horses and donkeys are used worldwide for work, food and social activities, the knowledge of *Cryptosporidium* and microsporidia diversity in these economically important hosts is poor compared to other domestic animals. As a result of our screening, the number of *Cryptosporidium* species known to naturally infect horses has increased from 2 to 6. However, the sources and course of infection, and the pathogenicity of most taxa remain unknown. Novel natural hosts of *C. muris* were detected in a survey targeted on *Cryptosporidium* infections of wild and captive (zoos) ungulates. Based on our data, a new gastric *Cryptosporidium* species from mammals will be described.

In the case of microsporidial infection, we described equine specific genotypes of *E. bieneusi* and published the first report of the immune response and tissue distribution of *E. cuniculi* in horses. Compared to the data from birds and rodents (see below), we showed a single strain distributed throughout the host, independently of the type of host.

In addition, PCR assays for detection of *Babesia caballi* and *Theileria equi* were validated by studies targeted on equine piroplasmosis in various part of the world, including the original homeland of Przewalski horses, revealing that camels and dogs do not share piroplasms with horses. A novel genotype of *B. caballi* was reported.

Total number of outputs: 9

Contribution of the team: Completely (100%) done by team members, except for studies performed under collaboration with colleagues from Algeria, Mongolia, Jordan, Romania with the involvement of team members being 20–90%. The collaborators helped with samples collection, analyses, and preparation of manuscripts.

E) *Cryptosporidium* and microsporidia of small mammals

Cryptosporidium and microsporidia are considered to be opportunistic parasites and an infected immunocompetent individual is able to control the disease by immune response (self-limited disease). Adaptive immune responses, especially cell-mediated ones, play a key role in protective immunity against both pathogens. Generally, it has been suggested that CD4+ T cells and IFN-ɣ play an essential role in protective immunity against intestinal cryptosporidiosis. Our results firstly demonstrated the involvement of activated CD8+ T lymphocytes in the protection of mice against gastric cryptosporidiosis.

Microsporidia cause severe infections with lethal outcome in immunocompromised hosts. Although the protective role of T-cell adaptive immunity has been previously reported with CD8+ T cells playing a critical role, using modern methods, microsporidia recently have been found in various immunocompetent hosts, including humans, supporting the hypotheses that microsporidia cannot be effectively eliminated by the host immune system. The results of our studies on experimentally infected murine (and equine and avian, see part D) hosts demonstrated that microsporidia can successfully survive in organs of immunocompetent hosts and can reactivate from undetectable levels and spread within these hosts after induction of immunosuppression. Moreover, our studies showed limited effectiveness of chemotherapy. These findings stress the danger of latent microsporidiosis as a life-threatening risk factor, especially for individuals undergoing chemotherapy and recipients of organs originating from infected donors (see part A).

The members of the team collaborated on the development of multilocus sequence tool for typing gastric species of *Cryptosporidium* from mammals. Our surveys showed the presence of various subtypes of *C. muris* and *C. andersoni* and the existence of cryptic species within *C. muris*. Subsequent studies focused on the biology of different strains of *C. muris* under experimental conditions confirmed the hypothesis of cryptic species. In addition, we proposed a novel laboratory host suitable for long-term propagation of *C. andersoni*, which will facilitate future reseaech on this zoonotic species.

Within the framework of long-term cooperation, we described in detail the life cycle and course of infection with *C. muris* TS03, which most likely represents a separate, still undescribed species, as indicated by its biological and molecular characteristics. This is the most completely documented life cycle of any species of *Cryptosporidium* so far.

In a cutting-edge study carried out on the European house mouse hybrid zone, we showed that two house mouse subspecies serve as hosts to genetically, morphologically and biologically distinct subtypes of *C. tyzzeri*, and that *C. tyzzeri* coevolved with these house mouse subspecies separately for 500,000 years before the subspecies re-established contact in the hybrid zone. This study provided important insight into the early stages of parasite speciation. In contrast, no host speciation was observed in microsporidia of both house mouse subspecies. However, the common presence of zoonotic microsporidia in wild rodents stressed the importance of synantropic rodents as a potential source of human microsporidial infection.

With regard to microsporidia, the team cooperated on the collection and maintainance of various *E. cuniculi* strains, including those that are highly virulent for small rodents. Certain isolates were subsequently subjected to genome sequencing for comparison of intraspecific heterozygosity and a typical signature of a diploid nuclear state.

Last but not least, a study of hedgehog cryptosporidiosis resulted in detailed morphological, biological and molecular characterization of the *Cryptosporidium* hedgehog genotype followed by a description of this genotype as a new species, *Cryptosporidium erinacei* n. sp.

Total number of outputs: 12

Contribution of the team: Out of 12 outputs, 8 were 100% done by team members and others were made in collaborations with the institutions from the Czech Republic, USA, New Zealand and Poland with the involvement of team members being 20–

80%. The collaborators helped with samples collection, sequencing, sharing costs, data analyses and manuscript preparation.

F) Others (2010-2014)

In addition to the above mentioned outputs, team members cooperated on different small projects focused on the various parasites of wild animals and showed a broad portfolio of their scientific activities. Of the many outputs, the following representative examples could be selected: (i) research on the monoxenous coccidian species in birds, turtles, pangolins or cottontails including descriptions of new species or assessment of interrelationships of these coccidia; (ii) a study on the prevalence and diagnostics of filariosis in dogs in Kenya; (iii) a molecular phylogenetic study of schistosome from forest elephants; (iv) an elucidation of the life cycle of helminths circulating between fish and snakes; and (v) a study on the role of tortoise ticks in the epidemiology of Q-fever.

Total number of outputs: 31

Contribution of the team: All outputs made under collaborations with several institutions, with the involvement exceeding 20%.