

Final Report Leibniz Competition Fund
Powering endurance: Fuel selection in migratory bats (BatFuel)

Final report

Project title: Powering endurance: Fuel selection in migratory bats (BatFuel)

Project number: K101/2018

Leibniz Institute in charge: Institute for Zoo and Wildlife Research

Project leader: PD Dr. Christian C Voigt

1. Executive Summary

Migratory bats perform long distance endurance flights to reach their wintering grounds, yet little is known about the physiological mechanisms that enable them to do so. For mammals, endurance exercise is powered primarily by glycogen but when reserves are depleted and no food is available, individuals rapidly fatigue. In contrast, migratory birds overcome this by fuelling extended flights via fatty acid metabolism. As capital migrants, birds have evolved highly efficient transport enzymes to facilitate the intracellular transport of hydrophobic fatty acids in the pectoralis and heart muscle. Evidence is inconclusive as to whether migratory bats use primarily exogenous nutrients, such as insects, to fuel sustained migratory flights (income migrants), a mixture of endogenous and exogenous fuels, or primarily endogenous lipid fuels. Our aim was to understand whether convergent selection pressures have led to adaptations in migratory bats that enable them to utilize fatty acid metabolism during endurance flight in a similar manner to birds. To this end, we conducted comparative studies on the physiology, transcriptomics and metabolomics of two model organisms, the long-distant migratory bat *Pipistrellus nathusii* and the similar-sized migratory bird *Sylvia atricapilla*. Pertaining to the assessment of bioenergetics, we aimed to gain a deeper understanding of mitochondrial functionality, respiratory capacity and adaptations of mitochondrial metabolism in the flight muscle of bats and birds.

We collected biological samples of *P. nathusii* and *S. atricapilla* over two migration seasons for use in transcriptome and metabolome analyses. Further, we completed experiments under controlled wind tunnel conditions to determine the physiological capacity of bats and birds during rest, post-flight, and recovery state. After experiments, blood samples were taken to compare transcriptome and metabolome profiles between states and species. In addition, pre- and post-flight respirometry were conducted to assess oxidative fuel use based on respiratory quotient; the ratio between oxygen and carbon dioxide in exhaled breath.

In response to requirements for non-lethal sampling, we optimized the protocols for RNAseq and metabolomics for blood samples of small volumes. We have also successfully developed and optimized a methodological protocol for high resolution mitochondrial respiration assays of permeabilized muscle fibres, which was planned to be used to compare mitochondrial capacity in tissues from migrating bats and birds. We have performed metabolite analysis following a non-targeted metabolomic approach by LC-MS/MS using HILIC chromatography to separate and detect polar metabolites in a range of 50-1000 Dalton. First, we evaluated the impact of different blood collection types on metabolite patterns, including whole blood, plasma, and serum. Afterwards, we measured metabolites of whole blood and applied multivariate and univariate statistical analyses to identify those metabolites that are sensitive to the state of exercise.

As part of a newly established collaboration with the Max Planck Institute (MPI) of Molecular Cell Biology, we generated PacBio long reads and HiC long range data to assemble a reference-quality chromosome level genome for *P. nathusii*, which is close to become annotated using newly generated transcriptome data from short (generated by the BatFuel project) and long reads (generated by collaborators). The final primary assembly yielded a length of ~1.8 Gb consisting of 153 scaffolds with a scaffold N50 of ~97 Mb. The primary assembly has a BUSCO completeness of 96.1%, which indicates a high contiguity and completeness of the sequences genome. The new reference genome will be used to cross reference RNAseq results from samples and provide an important reference for the Bat1K project.

Due to the COVID-19 pandemic and the war in Ukraine a number of aspects of our project have been put on hold or were delayed. Most importantly, fieldwork was impaired by covid-19 related travel restrictions, and the onset of a newly established collaboration with Russian scientists was stopped after the Russian invasion into Ukraine.

1. Achievement of objectives and milestones

Focal point (FP) 1- Physiology of bats and birds exercising in wind tunnel: We compared flight metabolic and respirometry data of whole animals and conversion efficiencies in animals flying in a wind tunnel. Further, we aimed to target the mitochondrial capacity of isolated muscles, focusing on lipid transport, gluconeogenesis and mitochondrial oxidative phosphorylation.

Comparison of exercise metabolism and oxidative fuel use (M1): We conducted short flight experiments in birds and bats, using an altered methodological protocol, specifically, we used the ¹³C-labeled Na-bicarbonate (NaBi) method to measure flight metabolic rates (power input) in conjunction with tomographical particle image velocimetry (TomoPIV) that yield power output. We collected respirometry data from 16 bats and 9 birds in 'rest-exercise-recovery' experiments to determine respiratory exchange ratios and thus uncover fuel use during flight. Further, we collected blood samples for analysis in (M3) and (M5).

Comparison of mitochondrial capacities (M2): Mitochondria as the powerhouse of the cell cover the majority of ATP demand for metabolism by oxidative phosphorylation (OXPHOS). Due to the small size of muscle biopsies, isolation of mitochondria was not an option. Instead, we measured OXPHOS rates of mitochondria in permeabilized muscle fibers using high-resolution respirometry (OROBOROS Oxygraph). To establish and optimize the method, the soleus muscle (red fiber type) from mice was dissected into small fibre bundles and permeabilized with saponin from quillaja bark. Respirometry was performed in a closed-chamber-respirometer with oxygen enriched buffer. A Substrate-Uncoupler-Inhibitor-Titration (SUIT) protocol was applied which allows quantitation of several respiratory states. Unfortunately, we were not able to gain data from wild animals as explained in section 2.

FP2- Profiling metabolite and gene expression alterations of high intensity endurance exercise: In FP2, we describe metabolite and transcriptome alterations for 'rest-exercise-recovery' experiments to identify metabolic pathways and genes in bats and birds

Profiling metabolite and transcriptome alterations in exercising animals (M3): Based on samples collected in M1 we performed metabolite analysis for profiling metabolite alterations in 'rest-exercise-recovery' experiments under controlled conditions as well as exercising under wild conditions in migratory animals. We optimized blood collection and handling of small volume sampling based on previously collected samples. We observed that whole blood collection tubes were suitable for metabolomics, recovering similar metabolites compared with plasma and serum collection procedures. We then conducted a comprehensive metabolomics analysis by HILIC LC-MS/MS, which was developed and optimized in-house to study polar metabolomes.

Network analysis (M4): We are currently identifying relevant metabolites (by-products of metabolic pathways typical for income migrants or capital migrants), such as lipids, amino acids, carnitines or fatty acids using multivariate statistics and novel adapted tools (mass difference enrichments, network correlations, neuronal network) developed in-house at the Technical University of Munich for compositional and metabolite annotation. Here, we focus specifically on the most important metabolites to quantify these for further interpretation. Preliminary analysis showed that levels of acylcarnitines and N-acyltaurines increased in exercising bats compared to non-exercising bats. In wild bats, concentrations of several amino acids were elevated, yet both in migration and non-migration season. Exercising non-migrating bats showed also higher levels of acylcarnitines.

Comparing the expression of specific metabolic genes (M5): Due to the small sample volume obtained in a non-lethal way from wild animals and the broader and more promising results of large-scale transcriptomics analysis, we shifted our focus from the analysis of gene-specific expression patterns to transcriptomics, which also helped in annotating the genome.

FP3- Physiological, bioenergetic and gene expression profile of bats and birds under natural conditions: Our aim was to survey the seasonal changes in the body composition of bats to evaluate the size of fat deposits and digestive organs, and validate findings from M3 and M5 by comparing results between captive and wild conspecifics.

Bioenergetic and gene expression profile of animals under natural conditions (M6): Specifically, we obtained blood and faecal samples from 60 birds and 60 bats each during two migration seasons. These samples are used to assess the metabolite and transcriptome profiles. Samples for the non-migratory season were collected in June 2021 and during migration at the Baltic Sea coast during August-Sep 2020 and 2021.

Body composition changes between seasons and between resident and migratory bats (M7): In this work module, we collected carcass material at wind turbines to compare the body composition (fat content of bats and dry masses of organs) between *P. nathusii* (migrating) and *P. pipistrellus* (only resident populations).

2. Activities and obstacles

The consortium partners met regularly to discuss and plan the project's research agenda, in particular to assess and solve potential challenges and complications ahead. Publications were usually accompanied by corresponding press releases, including radio and newspaper interviews.

The research activities were overshadowed by four unforeseen challenges. First and foremost, our research activities were severely affected by the covid-19 pandemic. The covid-19 pandemic affected virtually all working modules, but most importantly field (M1 and 7) and laboratory work (M3 and 5). Specifically, we faced a restricted access to institutes and laboratories during the peak of the covid-19 pandemic. Additionally, our fieldwork was severely hampered by international travel restrictions. For example, reaching our field sites in the spring and autumn was highly challenging due to regional and national travel restrictions. Second, due to unforeseen circumstances regarding the governance of the wind tunnel facility at the MPI in Seewiesen and an overall restructure of their institutional management, we were forced to sever our previously established collaboration and seek an alternative location for the wind tunnel experiments. This was found with our new collaboration partner, Prof Anders Hedenström of Lund University who researches the aerodynamics of flight in migratory birds. Accordingly, we had to move our instruments to Lund University. However, we could not achieve this for the instrument used to measure the oxidative fuel in mitochondria. In response, we sought to establish a new collaboration with the Rybachy Biological Station and researchers from St Petersburg State University where we could make use of samples obtained from migratory birds and bats, which were sacrificed for another project. This collaboration was put on hold by the third challenge we faced, the Russian invasion of Ukraine. As a result, we stopped working with Russian institutions. As a result from this sequence of challenges, no results were obtained for M2. Finally, in response to emerging animal welfare and conservation regulations, we refrained from sacrificing animals as part of our project. Instead, we switched to non-lethal sampling, which limited our analytical approach as non-lethal sampling resulted in very small sample volumes. Accordingly, we had to adjust our laboratory protocol, which required additional effort and time. Finally, although we found a large number of carcasses under wind turbines to analyse seasonal differences in the body composition of migratory bats, we were unable to find sufficient numbers of fresh carcasses of the target species. Both target species were small (4-8 g) and dissection revealed rapid decomposition of organs, particularly in the digestive tract. As a result, we are still in the process of collecting specimens from wind turbine fatalities to obtain a sufficient number of individuals in acceptable condition for comparative analysis.

3. Results and successes

FP1 has yielded a number of scientific papers in peer-reviewed journals. As part of our collaboration with Prof Anders Hedenström, we became engaged in a study on the power input, power output and energy conversion efficiency in small migratory passerines. In this comparative study, we established that the NaBi method is a valuable approach to study the energetics of un-instrumented vertebrates (Hedh et al. 2020). We then used the NaBi method in combination with particle image velocimetry (PIV) to compare metabolic and mechanical

power in *S. atricapilla* flying in a wind tunnel. We observed a U-shaped power curve for the metabolic power of flight and confirmed a relatively high conversion efficiency for passerines (Hedh et al. 2020). We then conducted similar experiments in Nathusius' pipistrelle bats, the other focal species of our project. The results of our study suggest that peak conversion efficiency in this species occurs near maximum range speed, the speed at which these bats fly during migration and where the cost of transport is minimized (Currie et al. 2023). A meta-analysis of 16 bird and 8 bat species revealed a positive scaling relationship between estimated conversion efficiency and body mass, with no discernible differences between bats and birds (Currie et al. 2023). This has profound consequences for modelling flight behaviour as estimates assuming 23% efficiency underestimate metabolic costs for *P. nathusii* by almost 50% on average (36–62%). Our findings suggest that conversion efficiency may vary around an ecologically relevant optimum speed and provide a crucial baseline for investigating whether this drives variation in conversion efficiency between species.

As a result of our activities in the field of bat migration, we contributed to other scientific projects that were led by national and international partners. For example, we confirmed the long-distance migration of Nathusius' pipistrelles as part of our collaboration with Latvian and Spanish colleagues (Alcalde et al. 2020). In addition, we contributed to a consortial project that demonstrated offshore and coastline migration of Nathusius' pipistrelles (Bach et al. 2022). As a first output of FP2, we have reviewed the current literature and knowledge about the foraging behaviour of migratory bats (Voigt et al. 2023). Lastly, as a result of FP3, specifically, the carcass searched on in M7, we contributed to two papers in conservation journals, highlighting the problem of high bat fatalities at wind turbines. (Voigt et al. 2022, Scholz et al. 2023).

4. Equal opportunities, career development and internationalisation

Gender and career development

Dr Camila Mazzoni is one of one of the primary investigators on this project. She is an international researcher with connections to Latin America, who has established herself here in Germany. The result of this project will add to her ongoing Habilitation at the Freie Universität Berlin, which is an important step towards her qualifying for a full professorship here. As a result of work on this project she has developed new collaborations within Germany, i.e., with Prof Hiller in order to assemble and annotate the genome of *P. nathusii*. Importantly, Dr Mazzoni was selected **first chairperson of the European Referenc Genome Atlas (ERGA) Network**, a pan-European scientific community of experts in genome sequencing, which coordinates the generation of reference-quality genomes for all eukaryotic European species.

Dr Shannon Currie, the postdoctoral researcher and project manager has been involved in planning and execution of all focal points of the project and as such has learned a number of new techniques. In order to enable her to conduct animal experiments within the EU she attended the FELASA B certification course, essential to her continued work in Europe on small mammals. After her employment at the Leibniz-IZW, Dr Currie was hired as an assistant researcher by the University of Hamburg. In the meantime, she has accepted an offer for a **lecturer at the University of Melbourne**, where she will start in January 2024.

Internationality:

All of the fieldwork and experimentation took place overseas. Accordingly, we have established a number of international collaborations. **Prof Gunārs Pētersons** is our collaborator for the fieldwork conducted in Latvia, as listed in the original proposal. As our initial planned collaboration with the MPI for Ornithology in Seewiesen was severed, a new collaboration at Lund University in Sweden was established with **Prof Anders Hedenström**. In 2021, we reached out to the Biological Station Rybachy and researchers at St. Petersburg State University with **Dr Nikita Chernetsov** to collect samples from sacrificed birds and bats during migration. This inquiry was put on hold because of the war in Ukraine.

5. Structures and collaboration

The IZW has a long-standing collaboration with the Pape field station, sealed by a formal collaboration agreement involving our Latvian colleague Prof. Gunārs Pētersons from the University of Jelgava. In addition, collaboration is ongoing with Dr Oskars Keiss from the University of Riga with regard to the ornithological aspect of our study and collection of data from Engure, Latvia during the non-migratory season.

Due to unforeseen circumstances regarding the governance of the wind tunnel at the Max Plank Institute in Seewiesen and an overall restructure of their institutional management we were forced to seek an alternative location for the wind tunnel experiments. This was found in our new collaborative partner, Prof Anders Hedenström of Lund University.

In addition, we aimed at establishing a collaboration with Dr Nikita Chernetsov of St Petersburg State University who is conducting research on navigation during migration. However, we cancelled our initial contact during the onset of the war in Ukraine following official recommendations by the Leibniz Society and the Leibniz-IZW.

*A new collaboration with Prof. Michael Hiller, from the Loewe Centre for Translational Biodiversity Genomics and Goethe University Frankfurt a.M. was established. Michael Hiller has been deeply involved in the Bat1K project and will assist us with his expertise on genome annotation on comparative genomics. A liver sample of the *P. nathusii* individual previously sequenced on an Illumina platform was send to the Max Plank Institute of Molecular Cell Biology in Dresden to sequence ~30x coverage of PacBio HiFi reads. These long reads are used to assemble a highly contiguous version of the *P. nathusii* genome. This assembly will serve as a reference for the RNA-Seq analysis (which in turn will serve to improve the annotation) as well as it will lay the basis of the comparative genomics where the *P. nathusii* genome will be compared to other high quality bat and bird genomes in regards to gene content and structure. High quality assemblies of multiple bat and bird species have recently been published by the Bat1k, Bird10k and Vertebrate Genome Project (VGP) and will be used to compare to the *P. nathusii* assembly.*

6. Quality assurance

The consortium has followed the DFG guidelines of good scientific practice. Prof Dr Fickel, one of the PI of the consortium serves also as the Ombudsperson for the Leibniz-IZW. We held regular meetings, almost exclusively in a digital format that also helped to ensure quality standards. During meetings, we updated each other on the current state of work, achievements and challenges. We have agreed on publishing manuscripts solely in peer-reviewed journals, preferably in an open access format.

In order to mitigate animal welfare concerns, we have altered our initial project proposal, which called for the euthanasia of bats. We have opted for a less lethal approach and have collected blood and faeces for the analysis of transcriptomics and metabolomics, and added stable isotope analysis of breath samples. However, the assessment of mitochondrial respiration (FP1- M2) cannot be completed without the sacrificing of individuals. In line with this we tried to establish a new collaboration with partners who are already collecting specimens for alternative purposes that are complementary to our work (see Section 5), yet these efforts were put on hold because of the Russian invasion of Ukraine.

We plan to publish as much as possible in journals with the option for open access. As part of this strategy, we have already published two papers open access in *Proceedings of the Royal Society B*, in *Conservation Science and Practice* and in *Global Ecology and Conservation*. Moreover, we plan to present our data beyond the funding period.

7. Additional resources

Most 'in-kind' services received in the context of the project were financed from the Leibniz-IZW for consumables and travel costs. For example, the Leibniz-IZW contributed to the maintenance of our Latvian field site and its infrastructure from institutional money as outlined in the collaborative contract between the University of Riga and the Leibniz-IZW.

8. Outlook

Our project has made good progress considering the aforementioned difficulties. Our research has large implications for basic research with respect to the regulation of oxidative fuels and also for conservation management of endangered migratory bats.

Identification of stopover sites and flyways during migration that provide fuel for bats during their journey: The flyways that bats take to travel along their migration journey are as yet unknown. It is important to understand and conserve the paths they take. Alongside this, as bats are strictly nocturnal the stopover sites along their journey are absolutely essential from both an energetic and predator avoidance perspective. As such identification of stopover sites and assessing the quality of these sites in terms of food availability and roosting locations is important. At present a radiotracking network is being developed along the coastline of the Baltic Sea which should enable us to attach very small, light-weight radio transmitters to these bats and track their movements (Bach et al. 2022).

Migration phenotype and gene expression during endurance flights: Some bat species in Europe express a level of flexibility in their migration patterns, much like migratory birds. These individuals can be classified as long or short distance migrants and may also remain sedentary within their range. It would be interesting to investigate how gene expression associated with endurance flight varies between these individuals and whether or not this is a function of migratory phenotype.

Metabolomics: Our studies showed the effect of exercise, migration, collection year and sex. Metabolites were altered by different factors but in general acylcarnitines and n-acyl taurines were most promising candidates, influenced by exercise under controlled conditions. We are planning to confirm gene expression levels of carnitine palmitoyltransferase I and fatty acid amide hydrolase by transcriptomics data. We will describe the effect of body condition (ratio between body mass and forearm length) on metabolite levels. We are targeting to publish two manuscripts within this project with following tentative titles: 1) A comparison of whole blood, plasma and serum collection tubes for non-targeted LC-MS/MS based studies; 2) The impact of exercise on whole blood metabolites in migrating bats.

Appendix

Peer Reviewed Publications:

- Alcalde, J.T., Jiménez, M., Brila, I., Vintulis, V., Voigt, C.C., Pētersons, G., 2020. Transcontinental 2200 km migration of a Nathusius' pipistrelle (*Pipistrellus nathusii*) across Europe. *Mammalia*. 85(2), 161-163 (Impact Factor: 1.0)
- Bach, P., Voigt, C. C., Göttsche, M., Bach, L., Brust, V., Hill, R., ... & Seebens-Hoyer, A. (2022). Offshore and coastline migration of radio-tagged Nathusius' pipistrelles. *Conservation Science and Practice*, 4(10), e12783. (Impact factor 3.2)
- Currie, S. E., Johansson, L. C., Aumont, C., Voigt, C. C., & Hedenström, A., 2023. Conversion efficiency of flight power is low, but increases with flight speed in the migratory bat *Pipistrellus nathusii*. *Proceedings of the Royal Society B*, 290(1998), 20230045. (Impact factor 4.7)
- Hedh, L., Guglielmo, C.G., Johansson, L.C., Deakin, J.E., Voigt, C.C., Hedenström, A., 2020. Measuring power input, power output and energy conversion efficiency in un-instrumented flying birds. *Journal of Experimental Biology* 223, jeb223545. (Impact Factor: 2.8)
- Scholz, C., Ittermann, L., Brunkow, M., Voigt, C.C. 2023. Fehlende Betriebssteuerungen an alten Windenergieanlagen können hohe Schlagopferzahlen bei Fledermäusen verursachen. *Naturschutz und Landschaftsplanung*. Doi 10.1399/NuL.2023.08.02. (Impact Factor 0.12)
- Voigt, C.C., Kaiser, K., Look, S., Scharnweber, K., & Scholz, C. 2022. Wind turbines without curtailment produce large numbers of bat fatalities throughout their lifetime: A call against ignorance and neglect. *Global Ecology and Conservation*, 37, e02149. (Impact factor 3.9)
- Voigt, C.C., Currie, S.E., McGuire, L. 2023. Bat migration and foraging: Energy-demanding journeys on tight budgets. In: *Bat Foraging* (eds. Russo, D. & Fenton, B.). in press. (no Impact factor)

Completion of Master's theses:

- Emma Selbherr (2020) "High-Resolution Respirometry on permeabilized muscle fibres" supervised by Prof Dr Martin Klingenspor
- Thea Yunhui Petermann (2023) "Fuel use and energy conservation in migratory birds accessed via respirometry and ¹³C stable isotopes in breath" supervised by Dr Shannon Currie and PD Dr Christian Voigt