FACULTY OF BIOLOGY
JAGIELLONIAN UNIVERSITY

Methodological Workshop in Evolutionary Biology

GRANT WRITING PART

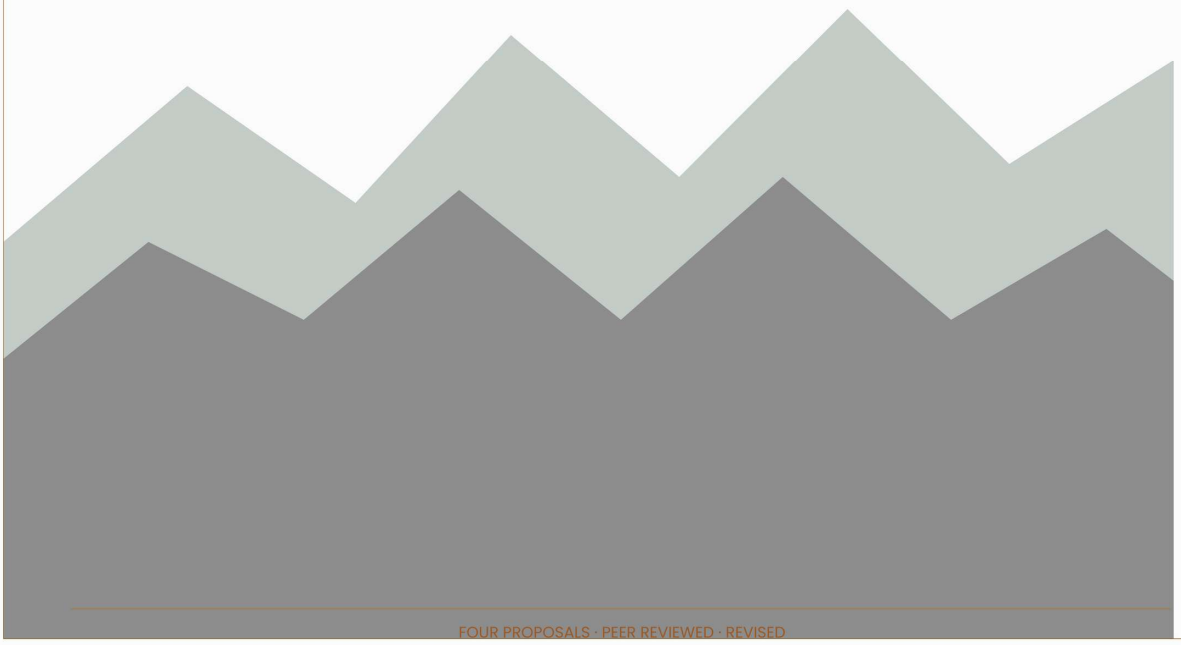
CHIROPTERA
echolocation & the city

CLADOSPORIUM
a pioneer on Mars

ATTACUS ATLAS
wings that speak

SALMO SALAR
the colour of stamina

OCHOTNICA GÓRNA · GORCE MOUNTAINS
APRIL 2025



FOUR PROPOSALS · PEER REVIEWED · REVISED

Table of Contents

General Information

This report documents the grant-writing part of the Methodological Workshop in Evolutionary Biology for PhD students, held in Ochotnica Górna in April 2025. Participants worked in small teams to prepare a research grant proposal on a topic of their choice, following the format of a national funding agency call. Each proposal was reviewed by peer participants (cross-team review), using a simplified grant-proposal review form covering scientific quality, potential impact, feasibility, and budget justification. Authors then revised their proposals in light of the reviews, producing a final version.

Participants

Team	Participants
Bats	Kinga Szafrańska, Failasuf Aulia Nugroho
Mars	Aya Dridi, Patrycja Wąchała
Moth	Alice Ballabio, Adam Solecki, Antonii Bakai
Salmon	Napat Emdee, Rama S. Krovi, Jakub P. Płachta

Organizer

Prof. dr hab. Joanna Rutkowska — Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University

Reviewers

Each proposal was reviewed by fellow participants from other teams (cross-team peer review), with additional reviews contributed by Dr hab. Aneta Arct and Dr hab. Joanna Sudyka (Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University).

1. Ecological and behavioral functions of bat echolocation pattern in changing landscapes

Team: Kinga Szafrańska, Failasuf Aulia Nugroho

Project (initial proposal)

Title: Ecological and behavioral functions of bat echolocation pattern in changing landscapes

Authors: Kinga Szafrańska, Failasuf Aulia Nugroho

Summary

The process of urbanization has a significant impact on the natural environment, leading to the loss of natural habitats and increased environmental pollution from light and sound. Such environmental disturbances are particularly relevant for echolocating species, whose sensory systems may be disrupted or forced to adapt under urban conditions. It is still not fully understood what determines the echolocation patterns of bats in different environments and what factors influence these patterns. The aim of this project is to fill this knowledge gap and assessment of echolocation patterns of bats occurring in different types of environments differing in the intensity of disturbances such as sound and light pollution and building density. Thanks to the comprehensive approach examining not only acoustic analysis but also environmental variables, we will be able to define the specific environmental factors that influence echolocation patterns in habitats with varying degrees of disturbance. The project will contribute to a better understanding of how bats adapt their **echolocation strategies in response to environmental changes related to urbanization**. In addition, the project has the potential to increase public awareness of urban biodiversity and promote sustainable city design that is more friendly to wildlife.

1) Scientific goal of the project

Echolocation gives bats their extraordinary ability to creat detailed 3D maps of their surroundings through sound waves and echoes (Diebold et al. 2020). This remarkable adaptation serves multiple functions from hunting to navigation, but i**s being challenged by human-altered environments.** Urban development introduces novel stressors: artificial light, noise pollution, and habitat fragmentation. While some bat species adapt to these changes (Lowry and Wong 2013) – potentially by modifying their echolocation calls – the specific mechanisms remain poorly understood. Research suggests bats might employ strategies similar to birds that shift call frequencies in noisy environments (Hans and Peet 2003), but how echolocation patterns evolve to cope with environmental stressors requires further investigation. Here, we would like to **investigate the the pattern of acoustic behavior of free ranging bats in different landscape** assuming different proximity of anthropogenic stressor. We hypothesize that:

a) **Bats in urban areas would alter their echolocation pattern.** The pattern of objects in urban areas differ to the natural areas. We assume that bats living in the urban area will produce different echolocation calls with higher frequency and long amplitude in consequence of noise pollution and habitat alterations.

b) **Conspesific bats, in a given environments, have the same echolocation pattern.**

We expect that bats living in the natural areas for example emit a long duration of calls and greater interval, to adapt with areas which has cluttered environment.

c) **Species-specific echolocations evolve as adaptations to different habitats.** We predict that species with large echolocation plasticity would likely be found in different habitats, so these species would adapt better to different areas.

2) Significance of the project

Urbanisation is a major land use change proce20ss that has significantly transformed the habitats and landscapes available to wildlife. Many researchers have shown that urbanisation can negatively affect animal species and their communities. The most commonly cited impacts include habitat loss and fragmentation (Scolozzi and Geneletti 2012), road mortality and the creation of ecological barriers (Baker and Harris 2007), as well as chemical and physical pollution, including anthropogenic noise and artificial lighting (Perugini et al., 2011; Francis and Barber 2013; Stone et al., 2009). Direct human interference is also not insignificant (Markovchick-Nicholls et al., 2008). Despite this, it is also known that urbanisation may favour some species that are able to adapt to human-altered environments for example, by benefiting from the higher temperatures characteristic of urban areas (Pikett et al., 2001) or by using the city as a refuge from large predators (Baker and Harris 2007). This provides a basis for understanding **how urbanisation affects specific species**. There is growing evidence that bats are deeply affected by urbanisation, showing responses in terms of altered diversity, population size and behaviour (Russo and Ancillotto 2015). However, it is still unclear what determines the echolocation patterns of these animals in different habitats and what factors can change these patterns. The present study provides insight into how differences in echolocation patterns in different bat species are shaped by diverse landscape features. This approach enables the evaluation of how varying intensities of urban stressors influence bat activity, particularly their echolocation strategies, and how bats might adapt their echolocation behaviors in response to different urban environments. Furthermore, this study represents a more comprehensive approach than previous studies, as **it takes into account not only acoustic analysis but also other environmental variables** (light pollution, sound pollution, building density). The outcomes of this project contribute to a better un derstanding to see how bat's strategy to cope with habitat change in urban areas using their echolocation ability. This will provide **detailed insight into how environmental variables shape bat the pattern of echolocations across different habitat** types and different stressor intensity (i.e light intensity, noise intensity). In addition to its scientific relevance, the project has the potential to engage public interest by raising awareness of urban biodiversity and promoting the **importance of designing cities** that are more compatible with the needs of wildlife.

3) Concept and work plan

General work plan

The experiment will be **conducted over a period of 24 months**. In Poland, 24 bat species have been recorded, with the number of species increasing in the Carpathians (excluding the Tatras) and the warm, karstic Kraków-Częstochowa upland (Sachanowicz et al., 2006). For this research, we will establish three different habitat types: urban areas, rural areas, and natural areas. Each habitat will contain 10 sampling sites where all echolocation signals will be recorded using ultrasound detectors equipped with ultrasonic microphones to ensure full spectrum recording. Environmental data will be collected using data loggers placed at each sampling site. Noise levels will be measured using sound detectors also placed at each site. Data collection will begin at the start of spring and continue through early and late summer.

Spesific Research Goal

This research aims to:

1. Measure echolocation pattern of bats across urban, rural and natural areas.
2. Assess the echolocation patterns among individuals of the same bat species to determine call adaptation and it's echolocations plasticity in different habitats.
3. Evaluate specific environmental factors (e.g noise pollution, light level, habitat alterations) directly influence bat echolocation parameters in each habitat.

Results of preliminary research

Poland is home to at **least 24 documented bat species**, distributed across eight genera: Barbastella, Plecotus, Nyctalus, Pipistrellus, Eptesicus, Vespertilio, Myotis, and Rhinolophus. Species richness follows a pronounced south-north gradient, with **diversity increasing toward the southern regions** (reflecting the transition from lowlands/lakelands to mountains/uplands). A secondary, less prominent east-west gradient is also observed, corresponding to the shift from continental to Atlantic climate influences (Sachanowicz et al., 2006). These bats presence in diverse environments, including fortifications, caves, and buildings (Eurobats, 2025), with recent research documenting their successful adaptation even to urban landscapes (Kohyt et al., 2024).

Gannt chart

Table 1. **Time schedule for the project**, grey color indicates task implementation time

Stage	Project Task	2026				2027			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Preparation	Permission from "Generalna Dyrekcja Ochony Środowiska"								
	Team training								
	Site sampling determination								
Field Work	Województwo dolnośląskie area sampling (three different habitat, each 10 sites)								
	Województwo małopolskie area sampling (three different habitat, each 10 sites)								
	Environmental data collection								
Data Analysis	Echolation parameter analysis in software								
	Consultation with echolocation expertise at University of Monachium								
	Statistical analysis								
	Comprehensive elaboration of the results								

Risk analysis

All known risk factors have been identified and thoroughly discussed with experts specializing in bat ecology and acoustic monitoring. Due to the nature of the planned fieldwork, which involves the use of sensitive and potentially vulnerable equipment, we intend to invest in high-quality recording devices to minimize the risk of malfunction or damage. As these devices will be deployed in outdoor locations where theft or vandalism may occur, we plan to employ trained personnel to regularly monitor and safeguard the equipment throughout the study period. Although fieldwork may involve certain risks related to working at night and in remote areas, appropriate safety measures have been taken. The research team has undergone first aid training and will carry a well-equipped medical kit during all field activities. All procedures have been carefully planned to ensure maximum safety for all team members. In order to minimize the risk of analytical errors in echolocation data processing, we intend to seek expert consultation with a researcher from the University of Munich specializing in bat bioacoustics.

4) Research methodology

Study area

Our research will be conducted in the Polish cities in which bats richness are high (Sachanowicz et al., 2006). We studied bats in three different environments: urban areas, rural area, and natural habitats. We select 10 study sites in each environment type. To make sure our samples were independent, we place each study site at least 1 kilometer apart from others. Before starting our main study, we conducted preliminary acoustic surveys to confirm the presence of bats to determine the study sites.

Table 2. Study locations

No	Urban areas	Rural areas	Natural areas
1	City center of	Areas of Czarny Bór	Góry Stołowe National Park

No	Urban areas	Rural areas	Natural areas
	Wałbrzych (województwo dolnośląskie)	(województwo dolnośląskie)	(województwo dolnośląskie)
2	City center of Krakow (województwo małopolskie)	Areas of Zabierzów (województwo małopolskie)	Ojcowski National Park (województwo małopolskie)

Details and definitions: **Urban area** is characterized with high building density, pavement, artificial light, anthropogenic activity; **rural area** is an areas which include agricultural landscape, settlement, and vegetation patches; **natural area** is a mature forest with understory and canopy structure.

Preparation

Data collection will occur from **May through October 2026**, capturing temporal data across spring through late summer seasons. Preliminary random observations will be conducted in each habitat type to determine appropriate sampling sites and confirm bat presence. Sites where bat presence is detected will be marked using a GARMIN 010-02451-11 GPS device. After the ten sampling sites are determined, we document environmental factors like vegetation, building density and other possible anthropogenic activities (e.g pavement, field areas).

Field work

Field data collection will start with the installation of monitoring equipment at each location. A Pettersson D500X ultrasound detector equipped with an ultrasonic microphone will be positioned at a height of approximately 1.5 meters above ground level. **Environmental parameters** will be monitored using a HOBO U30 USB Weather Station data logger, which will record temperature, relative humidity, and light intensity. Ambient noise levels will be measured using a PCE-322-SC43 sound level meter installed adjacent to the data logger. Each monitoring station will be supervised by a dedicated **observer responsible for equipment** setup and operation verification. **Recording sessions will begin 30 minutes after sunset and continue for a 4-hour duration.** Each site will be sampled for **two consecutive nights**, with weekly random sampling rotated among urban, rural, and natural habitat types. Simply, **we will conduct one time measurement (in both województwa) in each month**.

Data analysis

To identify echolocation patterns, data from the Pettersson D500X ultrasound detector will be extracted and analyzed using Batscope software (Obrist, M.K. & Boesch, R., 2018). Batscope will automatically analyze and classify calls to species level. The data will also be analyzed using Avisoft-SASLab Pro software to measure highest and lowest frequencies, frequency modulation patterns, and signal duration. Subsequently, the data will be analyzed by comparing frequency patterns with those in the Avisoft Bioacoustic database to classify patterns into social calls, distress calls, and feeding buzzes. **To strengthen this**

data analysis, we will consult with echolocation experts in Europe from the University of Monachium in Germany.

Statistical analysis

Multidimensional statistical analyses will be used to determine the relationship between echolocation patterns and the intensity of the environmental factors studied (light pollution, noise pollution, and built-up area). Data will be analyzed using ANOVA to measure bat echolocation patterns, repeated measures ANOVA to assess echolocation patterns among individuals of the same species across different habitats, and PCA analysis to evaluate the influence of environmental factors on echolocation parameters. All data will be done in R application.

5) Composition and qualifications of the research team

PI 1: Kinga Szafrańska

- Completed Master's degree at Jagiellonian University (UJ) with distinction (2024)
- Presented research findings at an international conference (2024)
- Experience in four distinct projects investigating the impact of various environmental factors on lichen communities and biological soil crusts

PI 2: Failasuf Aulia Nugroho

- Completed Master's degree at Jagiellonian University (UJ) with distinction (2020)
- Published author of two scientific publications:

  Second author publication about bats distribution in Indnesia (2024), first author about microplastics (2020)
- Experience conducting bat distribution surveys across Indonesia as part of a national survey initiative

6) Project literature

Baker, P.J., Harris, S., 2007. Urban mammals: what does the future hold? An analysis of the factors affecting patterns of use of residential gardens in Great Britain. Mammal Rev. 37, 297–31

Diebold, C. A., Salles, A., & Moss, C. F. (2020). Adaptive echolocation and flight behaviors in bats can inspire technology innovations for sonar tracking and interception. *Sensors*, *20*(10), 2958.

Eurobats, 2025. Conservation of Key Underground sites: the database. Accessed on 13/04/2025 at 12:00. https://www.eurobats.org/activities/intersessional_working_groups/underground_sites

Francis, C.D., Barber, J.R., 2013. A framework for understanding noise impacts on wildlife: an urgent conservation priority. Front. Ecol. Environ. 11, 305–313

Hans, S., & Peet, M. (2003). Ecology: birds sing at a higher pitch in urban noise. *Nature*, *424*(6946), 267.

Kohyt, J., Karczmarz, J., Pereswiet-Soltan, A. et al. Spatiotemporal use of urban rivers by local bat populations in a large city (Cracow, Southern Poland). Urban Ecosyst 27, 1663–1673 (2024). https://doi.org/10.1007/s11252-024-01545-x

Kohyt, J., Pierzchała, E., Pereswiet-Soltan, A., & Piksa, K. (2021). Seasonal activity of urban bats populations in temperate climate zone—A case study from southern Poland. *Animals*, *11*(5), 1474.

Lowry, H., Lill, A., & Wong, B. B. (2013). Behavioural responses of wildlife to urban environments. *Biological reviews*, *88*(3), 537-549

Markovchick-Nicholls, L., Regan, H.M., Deutschman, D.H., Widyanata, A., Martin, B., Noreke, L., Hunt, T.A., 2008. Relationships between human disturbance and wildlife land use in urban habitat fragments. Conserv. Biol. 22, 99–109

Obrist, M.K., Boesch, R. (2018) BatScope manages acoustic recordings, analyses calls, and classifies bat species automatically. Can. J. Zool.(96): 939-954. doi: 10.1139/cjz-2017-0103.

Perugini, M., Manera, M., Grotta, L., Abete, M.C., Tarasco, R., Amorena, M., 2011. Heavy metal (Hg, Cr, Cd, and Pb) contamination in urban areas and wildlife reserves: honeybees as bioindicators. Biol. Trace Elem. Res. 140, 170–176

Pickett, S. T., Cadenasso, M. L., Grove, J. M., Nilon, C. H., Pouyat, R. V., Zipperer, W. C., & Costanza, R. (2001). Urban ecological systems: linking terrestrial ecological, physical, and socioeconomic components of metropolitan areas. *Annual review of ecology and systematics*, *32*(1), 127-157.

Russo, D., & Ancillotto, L. (2015). Sensitivity of bats to urbanization: a review. *Mammalian Biology*, *80*(3), 205-212.

Sachanowicz, K., Ciechanowski, M., & Piksa, K. (2006). Distribution patterns, species richness and status of bats in Poland. *Vespertilio*, *9*(10), 151-173.

Scolozzi, R., Geneletti, D., 2012. A multi-scale qualitative approach to assess the impact of urbanization on natural habitats and their connectivity. Environ. Impact Assess. Rev. 36, 9–22

Stone, E.L., Jones, G., Harris, S., 2009. Street lighting disturbs commuting bats. Curr. Biol. 19, 1123–1127

7. Table with budget of the project.

	Amount in PLN
Direct costs, including	654 610
- personnel costs and scholarships	240 000
- research equipment/device/software cost	388 410
- other direct costs	26 200
Indirect costs, including:	11 200

	Amount in PLN
- indirect costs of OA	10 000
- other indirect costs	1 200
Total costs	**665 810**

8. Breakdown of project costs including justification and relevance for the tasks in the project.

a. **Personal salaries**

Position/Contributor	Salary per month (PLN)	Salary collection period [in months]	Total (PLN)
PI 1	4000	24	96 0000
Contribution to the project	Research planning,coordination, field work, participation in data collection, data analyses and writing manuscripts		
PI 2	4000	24	96 0000
Contribution to the project	Research planning,coordination, field work, participation in data collection, data analyses and writing manuscripts		
Field technician 1	1000	6	6 000
Contribution to the project	Field work		
Field technician 2	1000	6	6 000
Contribution to the project	Field work		
Field technician 3	1000	6	6 000
Contribution to the project	Field work		
Field technician 4	1000	6	6 000
Contribution to the project	Field work		
Field technician 5	1000	6	6 000
Contribution to the	Field work		

Position/Contributor	Salary per month (PLN)	Salary collection period [in months]	Total (PLN)
project			
Field technician 6	1000	6	6 000
Contribution to the project	Field work		
Field technician 7	1000	6	6 000
Contribution to the project	Field work		
Field technician 8	1000	6	6 000
Contribution to the project	Field work		
Total			**240 000**

b. **List of equipment to be purchased**

Name of apparatus	Year of purchase	Unit Cost (PLN)	Amount	Cost (PLN)
GPS	2026	1699	10	16 990
Justification of purchase	A GPS receiver was used to pinpoint the location on the map, allowing for precise mapping of observation locations.			
Ultrasound Detector	2026	7600	10	76000
External Microphone	2026	1806	10	18 060
Justification of purchase	The use of an ultrasonic detector with a microphone made it possible to record the echolocation voices of bats in the field.			

Name of apparatus	Year of purchase	Unit Cost (PLN)	Amount	Cost (PLN)
Weather Station data logger	2026	15000	10	150 000
Justification of purchase	Essential for measuring temperature, humidity and light intensity			
Sound level meter	2026	11 586	10	115 860
Justification of purchase	Necessary for measuring noise level			
Laptop with programme licence Office and EndNote	2026	5000	2	10 000
Justification of purchase	Computers for every day work, planning the studies, data analyses, writing manuscripts for PI			
Portable drive	2026	500	3	1 500
Justification of purchase	Disc used for backups and data archivization for PI			
Total				**388 410**

c. **Other costs justification**

Materials

Materials used to install survey equipment in the field : **10 000 PLN**

Office materials including printer cartridge: 600 per year. Cost for 2 years: **1 200 PLN**

Visits and consultations

(travel expenses / travel expenses by external collaborators and/or consultants and costs of meetings)

Cost: **15 00 PLN**

Visit of a bat echolocation sound specialist at the University of Munich for both PI

Other costs

(such as do not fit into other categories, including the cost of disseminating the results)

Cost: **11 200 PLN**

Costs of publishing (this includes figure artwork and publication fees, such as extra-page charges): **10 000 PLN**

Cost of purchasing the qualified signature (valid for 4 years): **1 200 PLN**

Authors' Declaration

We declare that AI has been used in the preparation of this proposal for translation purposes and sentences improvement.

Reviews

Adam Solecki

Simplified form for grant proposal review

Title of the project: Ecological and behavioral functions of bat echolocation pattern in changing landscapes

1. **Assessment of scientific quality of the research project**

The project represents a novel approach to the studies of bats ecology in the landscape of differentiated antropopression. It tackles the underexplored field and possess question on whether the antropogenic habitat alters the echolocation patterns within species. Which may lead to confusion during navigation and stand for an additionally source of mortality in ecosystem that is already challenging due to other factors. It may also add to the understanding of the differences between species composition of chiropterofauna in urbanized, rural and natural areas as those types of habitats are going to be studied. Its also interesting to se the idea of multi-envronmental variable capable dataloggers being proposed as a mean to asses the properties of the habitat.

Although the main idea is promising and tackles important problem, the choice of the experimental locations is not convincing. Some of the areas designated as rural and natural are similar in the urbanization level and forest coverage, also all of the chosen areas differ in the types of available hideouts which might significantly affect the species composition and the authors didn't recognize that problem, this differences might not be based on the type of habitat but rather geological or historical property of the area and microhabitat type in which the experiment is going to be setup. The other problem is the lack of information on the amount and structure of equipment placement in the each site, which may lead to bias in obtained data.

Overal the scientific goal of the project is worth recognition but the methodes of sampling requires additional polishing. I would also consider alteration of the study site choice in a

way that would enable comparison of species that prefers given microhabitat e.g. water reservoirs or rivers in the propose urbanization gradient.

2. **Assessment of potential impact of the research project**

The results of the study would enable further analyses on the impact of urbanized landscapes on chiropterofauna. Proposed hypothesis might offer us an insight into the effect of human-bat coexistence in the bat populations across the world and possibly add different perspective enabling for the designation of the species most vulnerable to urbanization. This in turn would enable us to prioritize certain areas and habitats in the context of the chiropterofauna protection. And possibly to promote better, more bat friendly infrastructure.

The field of human – wildlife coexistence is relatively new and develops rapidly, gaining recognition around the world. Which should enable good opportunities for publication. Also all the insights to echolocation mechanism are valuable not only to ecologists but also to engineers developing radar technologies.

3. **Assessment of feasibility of the research project**

With current, proposed methodology project is definitely feasible but the goals might not be achieved in particular the study site selection is doubtful due to similarities and possible impact of microhabitat that was not accounted for.

Bering that in mind the risk assessment analyses was sound and assuring although unspecific.

The proposed equipment is well selected, as well as proposed collaboration in echolocation data analyses.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The PI scholarship exceeds what is offered by Preludium grants.

Other costs are well justified, although with that high number of specialist and costly equipment, one might expect more replicates of the experiment in other phenological periods e.g. summer and autumn.

The authors didn't consider any means of alternate research communication, other than publications.

5. **Strengths of the proposal**

Good summary with well-defined aim and relevance of the project.

Well defined and clear aim and scientific background, hypothesis A and B are well structured and logical.

State of knowledge is presented clearly and elegantly although with few references (understandable due to the time constrains).

Authors were not blind for the possibility that urbanized habitats might be actually beneficial for some species.

Current development in the field was correctly pointed out.

Thorough the proposal the most important points are in bold which helps with understanding of the project.

Outcomes described in the lines 64-68 are well defined.

The planned experiment has two replicates.

Usage of dataloggers capable for measurements of many environmental variables that are going to be relevant for bats and physical properties of echolocation signals.

Good description of planned analyses (data and statistics)

Collaboration with renown foreign researchers

6. **Weaknesses of the proposal**

Authors stated the wrong number of species known for local chiropterofauna.

Only few examples of human related risks to chiropterofauna.

Obvious and poorly defined hypotheses C, generalist usually has to be more plastic in its traits, precisely because they might be found in many areas with differing requirements.

In the lines 58-60 authors mentioned an "approach" that would enable them to perform the experiment but they fail to define what this approach might be.

In the following lines 60-64 the logical sequence was lost.

For the analyses of habitat and urbanization gradient some satellite images or aerial photos should be considered.

Preliminary research based solely on the literature review is kind of a waste here since this information should be included in the state of knowledge.

Small distances between the research sites considering the speed and mobility of bats.

Poor selection of study sites – some selected as "natural" and "rural" are very similar.

Jakub Płachta

rEVIEWER: Jakub Płachta

Simplified form for grant proposal review

Title of the project: Ecological and behavioral functions of bat echolocation pattern in changing landscapes

1. **Assessment of scientific quality of the research project** (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)

I think the problem described in the proposal is worth studying and quite topical given the raise in urban ecology research. Bats are an often-overlooked group in urban environments, so it is nice to see these animals as a focus. While research on bat acoustics in urban environments has been done in the past, a comparison across an urbanization gradient, including completely natural environments was a good, novel idea. Although the additional goal of promoting public awareness and bat-friendly city design is an added practical benefit, it was almost glossed over in the proposal, which is a shame since it is a valuable goal. I would like to see this point expanded on. Also, I saw very little potential reasons given for bats' acoustic pattern adjustment in the cities. "Noise pollution" and "habitat alterations" mentioned in the first hypothesis are very vague, especially the latter. "Light pollution levels" could use some brief explanation as to why they would affect the acoustic signatures.

2. **Assessment of potential impact of the research project** (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)

The proposal tackles both the pure scientific significance of the problem as well as suggests practical implications of the knowledge acquired. However, I think that limiting the scope of research to southern Poland somewhat also limits the project's potential in the international context. While Poland boasts over half of the documented European bat species (45), the landscape of Lower Silesia and Kraków is somewhat distinct. If the authors are already in contact with the University in Monachium, given the equipment, personnel and methodology, I think it wouldn't be difficult to expand the scope of research to Central Europe, but I do not see it as a strict requirement.

3. **Assessment of feasibility of the research project** (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)

   The methodology is appropriate to the research subject, and the equipment well-described and more than adequate for the proposed research. The urban gradient definition, however imprecise, is a welcome addition. Preliminary research ensures that sites of high quality will be chosen. However, 1 km distance between sites might not be enough to ensure independence since bats can fly multiple kilometers while hunting. I like the effort to establish communication with an expert in the field, it shows authors' awareness of their own gaps in knowledge and skills, so does the "team taining" task (very vague, I must point out). The software is well-established in the field and additional expertise provided by the University of Monachium will, in my mind, somewhat compensate for the author's inexperience in acoustic analysis. I noted the lack of either scientific conferences or other ways to disseminate the results to the community of the public, given that popularization and awareness are stated explicitly as goals of the project. In the tasks, not time is given for the publishing process, which is always time-consuming. Also, I would like to see some examples of target journals.

Risk assessment is somewhat vague (despite stating that "All known risk factors have been identified"). It focuses on equipment safety, which would have been understandable if not for a lacking description of risk to personnel, most of which will be hired technicians. Statements like "certain risks" (Line 108) or "appropriate safety measures have been taken" (Line 109) sound weird, like the authors didn't want to state them outright. Hostile human interactions are not unprecedented during research (especially at night in the city). It is even more puzzling, since earlier they identify risks of vandalism and theft, which are inherently dangerous to personnel, should an altercation happen. Even still, I appreciate the mention of personal medical kits and first aid training.
Statistical analysis description is rather vague, failing to mention the explanatory variables, response variables or random factors. The "echolocation patterns", I assume, are the frequencies mentioned in the Data Analysis section but they should be included in the statistics description. I am no expert in acoustic pattern analysis, but from the description it seems like it would require somewhat complex mixed modeling Built-up area is mentioned, but nowhere in the proposal is it stated how will it be assessed (GIS?). Nevertheless, an inclusion of PCA analysis for pattern identification is a good idea, in my opinion, but it should be better explained rather than mentioned.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The budget is presented in detail and accounts for most of the necessary expenses. A major flaw here is that the budget for equipment easily surpasses the 30% direct cost limitation for these proposals. A major reason for this problem might be the very expensive Weather Station data loggers, priced at 15 000 PLN/unit, which to my knowledge is grossly overpriced for the model provided, even including necessary add-ons (~8 000 PLN/unit). Same applies to the sound level meters, with an even bigger margin. Adjusting these costs should substantially reduce the equipment expenses.

5. **Strengths of the proposal**

- Multiple testable hypotheses
- Basic and applied value of the research
- Relevance for public interest
- Clearly described experiments
- Care taken in surveying target areas

Weaknesses of the proposal

- Vagueness in describing the rationale for the hypotheses
- Insufficient statistical description
- Inadequate budget

***Final assessment:**

In total, I think the project is a worthwhile endeavor. The authors tackle an important issue with potential for public support and implementation. The experiments are understandable, and measurements are adequate for the hypotheses. More care should be

put into justifying reasoning and statistical analysis. Perhaps equipment purchases were a bit overzealous, but that can be fixed. I like this proposal.

*Minor comments:
Line 30 – "assuming" – you don't have to assume; you will measure it
Line 37 – this "for example" is unnecessary and sounds unconfident
Line 58 – "This approach" – what approach? In this section so far, you've only provided the state of the art. Our approach? Proposed approach?
Line 74 – You're not establishing the habitats. You're choosing them.
Line 90 – "at least 24 documented bat species" – while technically not wrong, this statement is weird. There are 27 species in Poland, you can find this on the governmental site with citations.

Dr hab. Aneta Arct

Title of the project: Ecological and behavioral functions of bat echolocation pattern in changing landscapes

1. **Assessment of scientific quality of the research project**

The proposed project focussing on very actual questions regarding how bat echolocation patterns respond to environmental disturbances associated with urbanization. This falls squarely within the growing field of urban ecology and behavioural adaptation, and the focus on sensory ecology, particularly echolocation, is of interest. The applicants propose to investigate variation in bat echolocation across urban, rural, and natural habitats by recording ultrasonic calls and correlating them with environmental variables such as noise, artificial light, and building density. Additionally, the project aims to raise awareness of urban biodiversity and promote wildlife-inclusive urban planning, which is a valuable broader impact.

2. **Assessment of potential impact of the research project**

I find that the proposed project addresses an ecologically relevant and increasingly studied topic: the effect of urbanization on bat echolocation behaviour. The focus on echolocation as a behavioural and sensory trait aligns well with the current global interest in urban ecology and animal behavioural plasticity. However, in my view, the potential for substantial international impact is limited by the lack of novelty in the project's conceptual and methodological design. Similar studies on bat acoustics in urban contexts already exist (e.g., Starik & Göttert, 2022), and I am surprised that this important publication is not even mentioned or discussed. It directly relates to the project's core objectives and could have served as a valuable reference or starting point. Furthermore, I believe the absence of physiological, genetic, or experimental components significantly weakens the proposal's potential to generate high-impact, mechanistic insights. Although the topic itself holds promise, I do not see how the current version of the project would produce major international research outputs or publications in leading journals within the field.

3. **Assessment of feasibility of the research project**

The field component of the project is feasible in logistical terms. The use of standard acoustic monitoring devices and environmental sensors is appropriate, and the research team demonstrates experience with fieldwork. However, several key aspects reduce overall

feasibility in a scientific sense. First, the sampling design is limited to short recording periods on a small number of nights, which may not provide enough data to reliably assess variability in echolocation. Second, the project design is based entirely on correlational analysis, with no experimental manipulation or tracking of individual bats. This limits the ability to address the stated hypotheses, particularly those relating to behavioural plasticity. Third, the taxonomic scope of the study is too broad given the resources and goals. The project includes multiple species with varying behaviours and echolocation strategies, yet offers no clear rationale for this diversity nor an analytical plan to deal with interspecific differences. The idea of assessing 'plasticity' in echolocation across environments within species is not convincingly developed, particularly given that there is no method in place to identify or track individual bats. Without individual-level data (e.g., through marking or genetic identification), claims about behavioural plasticity remain speculative. Furthermore, while international cooperation is mentioned (with a bat bioacoustics expert in Munich), the collaboration is informal and not well-integrated into the work plan. Risk management is described in detail regarding field equipment, but there is no consideration of scientific risk- i.e., whether the design is sufficient to detect the effects of interest. Overall, the project is logistically feasible but methodologically weak, which undermines its potential to meet its scientific objectives.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The proposed budget is detailed and well-structured, covering personnel, equipment, and operational expenses. However, I noted the absence of any allocation for conference participation, either nationally or internationally. Given the project's declared aim to contribute to the understanding of urban biodiversity and to engage with the wider scientific community, it is surprising that no funds have been designated for attending or presenting at scientific conferences. Such activities are essential for disseminating research findings, receiving expert feedback, and building international visibility. Without this element, the impact of the project will likely remain limited to local contexts. Furthermore, the proposal includes eight individual field technicians, each listed with identical responsibilities and separate monthly payments. This structure is unusual and unnecessarily detailed for this type of contribution. In the Polish funding system, this form of field support is more appropriately categorized under "wykonawcy zbiorowi" (collective contractors) a standard category for technical or manual work carried out by multiple individuals with the same task.

5. **Strengths of the proposal**

The project addresses a timely topic related to the impact of urbanization on wildlife behaviour. The proposed fieldwork is clearly structured and utilizes well-established acoustic monitoring tools. The team has relevant field experience and demonstrates organizational capacity. The topic has potential for public engagement and science communication on urban biodiversity**.**

6. **Weaknesses of the proposal**

However, despite these strengths, the proposal is limited by a number of important conceptual and methodological weaknesses. Most notably, the study is entirely correlational and lacks any experimental component, which severely limits the ability to

infer causality. This issue is compounded by the fact that the project design relies on a categorical comparison of habitat types (urban, rural, natural) rather than on a continuous environmental gradient. In reality, factors such as light pollution, noise intensity, and habitat fragmentation vary along gradients. A gradient-based design would not only reflect ecological reality more accurately but would also offer much greater analytical resolution and statistical power. As it stands, the chosen classification may obscure important variation and limit the generalizability of the findings. As I mention above the sampling design is also somewhat limited. Furthermore, while basic statistical tools are mentioned, the proposal lacks a detailed or modern analytical framework. There is no mention of mixed-effects models or other techniques needed to account for repeated measures or spatial autocorrelation. The theoretical novelty is limited, and similar studies have already been published. The project does not incorporate molecular, physiological, or endocrinological methods that could increase the impact of the findings.

minor text errors: Line 40: proce20ss

Line 63: un derstanding

Final version

Title: Adapting to the City: Echolocation Strategies of Bats Along an Urbanization Gradient

Authors: Kinga Szafrańska, Failasuf Aulia Nugroho

S**ummary**

The process of urbanization has a significant impact on the natural environment, resulting in the loss of natural habitats and increased light and noise pollution. Such environmental disturbances are particularly relevant for echolocating species, whose sensory systems may be disrupted or forced to adapt to urban conditions. It is still not fully understood what determines the echolocation patterns of bats in different environments and what factors influence these patterns. The aim of this project is to fill this knowledge gap by assessing the echolocation patterns of bats in different types of environment with different levels of disturbance, such as sound and light pollution and building density. By taking a comprehensive approach, looking not only at acoustic analysis but also at environmental variables, we will be able to define the specific environmental factors that influence echolocation patterns in habitats with different levels of disturbance. The project will contribute to a better understanding of how bats adapt their echolocation strategies in response to environmental changes associated with urbanization. In addition, the project has the potential to raise public awareness of urban biodiversity and promote sustainable urban design that is more friendly to wildlife.

1) Scientific goal of the project

Echolocation gives bats the extraordinary ability to create detailed 3D maps of their environment using sound waves and echoes (Diebold et al. 2020). This remarkable adaptation serves multiple functions, from hunting to navigation, **but is challenged by human-altered environments**. Urban development introduces new stressors: artificial light, noise pollution and habitat fragmentation. While some bat species adapt to these changes (Lowry and Wong 2013) - possibly by modifying their echolocation calls - the

specific mechanisms remain poorly understood. Research suggests that bats may use strategies similar to birds that shift call frequencies in noisy environments (Hans and Peet 2003), but how echolocation patterns evolve to cope with environmental stressors requires further investigation. Here, we aim to **investigate the pattern of acoustic behavior of free-ranging bats in different landscapes,** assuming varying proximity to anthropogenic stressors. We hypothesize that:

a) **Hypothesis 1 (H1) - Bats in urban areas would alter their echolocation pattern.** Given the greater acoustic and spatial complexity of urban environments compared to natural ones, bats are likely to adapt their echolocation to improve navigation. Specifically, we predict that urban bats will produce shorter, higher-frequency calls with greater amplitude than individuals living in less urbanized areas, as these modifications would increase spatial resolution and reduce noise interference.

b) **Hypothesis 2 (H2) - Conspecific bats, in a given environment, have the same echolocation pattern.** Assuming that echolocation patterns are adapted to environmental conditions and are consistent within a species, we expect that bats of the same species living in the same environment will use similar echolocation calls. In particular, we predict that their calls will not differ significantly in structure or timing because similar ecological challenges will lead to the same acoustic solutions among conspecifics.

2) Significance of the project

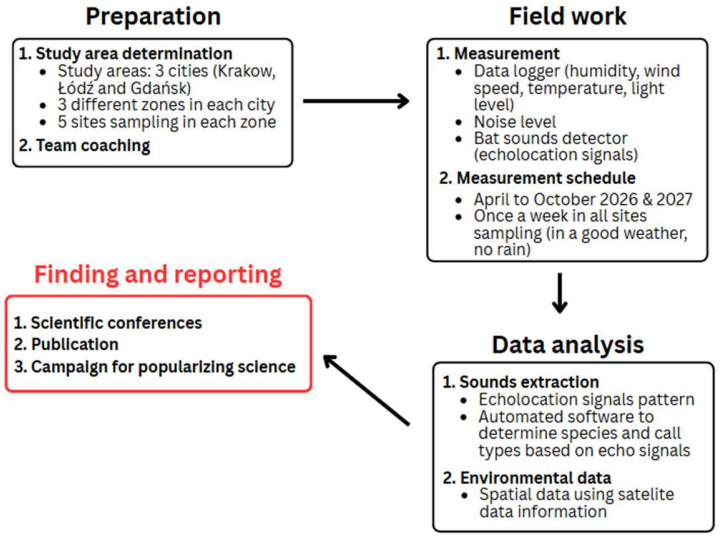
Urbanisation is a major land use change process that has significantly transformed the habitats and landscapes available to wildlife. Many researchers have shown that urbanisation can negatively affect animal species and their communities. The most commonly cited impacts include habitat loss and fragmentation (Scolozzi and Geneletti 2012), road mortality and the creation of ecological barriers (Baker and Harris 2007), as well as chemical and physical pollution, including anthropogenic noise and artificial lighting (Perugini et al., 2011; Francis and Barber 2013; Stone et al., 2009). Despite this, it is also known that urbanisation may favour some species that are able to adapt to human-altered environments for example, by benefiting from the higher temperatures characteristic of urban areas (Pikett et al., 2001) or by using the city as a refuge from large predators (Baker and Harris 2007). This provides a basis for understanding **how urbanisation affects specific species**. There is growing evidence that bats are deeply affected by urbanisation, showing responses in terms of altered diversity, population size and behaviour (Russo and Ancillotto 2015). However, **it is still unclear what determines the echolocation patterns of these animals in different habitats and what factors can change these patterns.** This study provides valuable insights into how the echolocation patterns of different bat species are influenced by different landscape features. By examining variations in echolocation across gradients of urban stressors, it provides a better understanding of how bats respond and potentially adapt to changes in their environment. Unlike many previous studies, **this research takes a more comprehensive approach by integrating acoustic data with key environmental variables** such as light pollution, noise levels and change in land use. **The results contribute to a deeper understanding of how bats use echolocation to cope with habitat change** in urban environments. In particular, this study highlights how environmental factors shape

echolocation behaviour across different habitat types and different levels of urban disturbance (e.g. light and noise intensity). This study provides valuable insights into how the echolocation patterns of different bat species are influenced by different landscape features. In addition to its scientific contributions, this research has practical implications for the management and conservation of urban wildlife. By exploring how bats adapt their echolocation in response to urban stressors, **it raises awareness of their ecological role in cities and highlights the need for urban spaces** that accommodate the behavioural needs of wildlife. These findings can inform strategies to mitigate the effects of urbanisation on bat populations, such as reducing light pollution or minimising noise disturbance.

3) Concept and work plan

General work plan

The research will take place from April to October in 2026 and 2027. The fieldwork will take place in three urban centres in Poland: Krakow, Łódź and Gdańsk. These cities were selected to reflect a north-south geographical gradient and to represent different levels of urbanization and demographic structure (Sas, A. 2025). In each city, 15 green space sites will be selected for sampling. These sites will be located at different distances from the city centre - close to the centre, in the middle (suburbs) and furthest away (rural areas) - to reflect an urban-rural gradient. Data will be collected using ultrasonic detectors with ultrasonic microphones for full-spectrum recording of echolocation signals, while environmental parameters will be monitored using data loggers placed at each site, paying particular attention to light and noise levels as key urban disturbance factors.



A diagram of a diagram AI-generated content may be incorrect.

Figure 1. Diagram of general work plan

Specific Research Goal

Table 1. Specific research goals and method of verification

Hypothesis (H)	Research aims	Method of verification
H1	To assess whether bats in urban areas change their echolocation patterns in response to urban conditions.	Comparison of echolocation parameters (frequency, amplitude, signal length, emission rate) of bats found in urban and rural environments. The analysis will take into account the relationship between echolocation patterns and certain environmental variables: noise levels, intensity of artificial lighting and habitat structure.
H2	Investigate whether conspecific bats living in the same environment show consistent echolocation patterns.	Record the echolocation of multiple individuals of the same species in the same type of environment and analyse the variation between individuals.

Results of preliminary research

According to iNaturalist (Inaturalist, 2024), Poland is home to at least 26 species of bats. Most of them are microchiropterans, which rely on echolocation for navigation, hunting and social interactions. These bats inhabit a wide variety of environments across Poland, including fortifications, natural caves, built structures and urban landscapes. Recent studies by Kohyt et al. (2024) have documented the successful adaptation of certain bat species to urban environments, suggesting that some Polish bats have developed strategies to cope with human-modified environments, despite the challenges posed by artificial light and noise pollution.

Gannt chart

Table 2. Time schedule for the project, grey color indicates task implementation time

Stage	Project Task	2026				2027			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Preparation	Permission from *"Generalna Dyrekcja Ochony Środowiska"*								
	Consultation with echolocation expertise at University of Monachium								
	Team coaching								
	Site sampling determination								
Field Work	Sampling at study sites Kraków, Łódź, and Gdańsk								
Data Analysis	Echolation parameter analysis (data analysis)								
	Urbanization pattern analysis using ArcGIS Pro								
	Writing manuscript								
Milestones									
	Scientific conference								
	Publication								
	Campaign for popularizing science								

Risk analysis

All known risk factors have been identified and thoroughly discussed with experts in bat ecology and acoustic monitoring. Due to the nature of the planned fieldwork, which involves the use of sensitive and potentially vulnerable equipment, we intend to invest in high quality recording equipment to minimize the risk of malfunction or damage. As this equipment will be used in outdoor locations where theft or vandalism may occur, we plan to employ trained personnel to regularly monitor and secure the equipment throughout the study period. Although fieldwork may involve certain risks associated with working at night and in remote areas, appropriate safety measures have been put in place. The research team has received first aid training and will carry a well-equipped medical kit during all field activities. All procedures have been carefully planned to ensure maximum safety for all team members. To minimize the risk of analytical errors in the processing of echolocation data, we intend to seek expert advice from a researcher specializing in bat bioacoustics at the University of Munich.

4) Research methodology

Study area

Our research will focus on cities in Poland with high bat diversity. Bat species richness in Poland varies geographically, with higher diversity observed in the Carpathians (excluding the Tatras) and the warm, karstic Kraków-Częstochowa Upland (Sachanowicz et al., 2006). We will study bats along urbanization gradients in three selected Polish cities. In each city, we will select sampling sites in three distinct zones extending from the city center outwards. Each zone will contain 5 sampling sites, for a total of 15 sites per city (see Fig 1). All sampling sites will be randomly selected in green spaces with the key requirement that each site must include a buffer zone with a 500-meter radius chosen to encompass the typical foraging range of most bat species and to provide an appropriate spatial scale for analyzing bat-habitat relationships (Starik et al. 2024). This buffer zone ensures that we capture the typical foraging range of most bat species and provides an appropriate spatial scale for analyzing bat-habitat relationships across the urban-rural gradient.

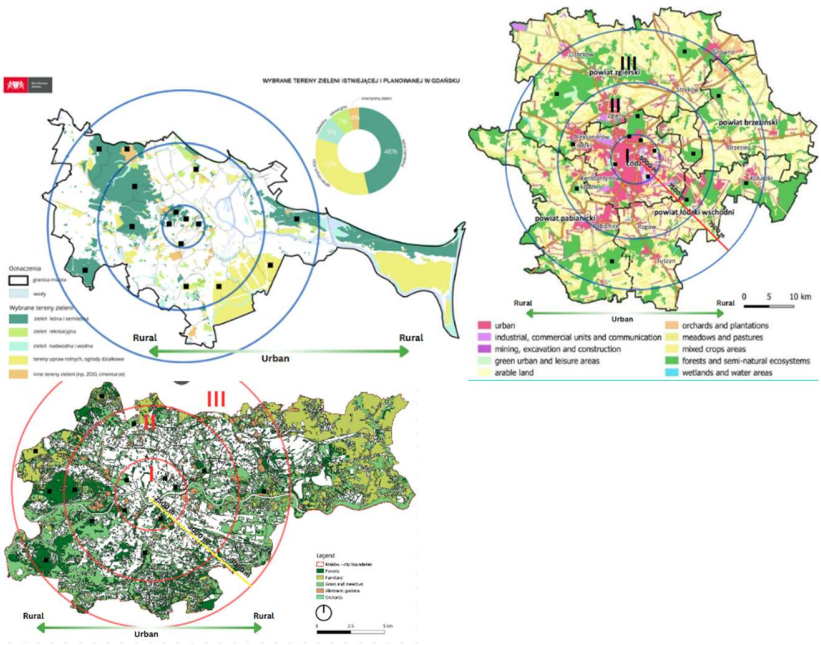


Figure 2. Planned research areas in three Polish cities (Krakow, Łódź and Gdańsk). The maps are adapted from different sources: Krakow's map comes from a sustainability study (Dudzic-Gyurkovich 2023), Łódź's map comes from an H2020 repair project (repair, 2012) and Gdańsk's map comes from the city planning office (Brg 2025). On each city map, circles represent the different urbanization zones we're studying, while black dots indicate our specific sampling locations. The figure shows how our sampling strategy captures the urban-rural gradient in each city.

For clarity, we define our key environmental categories as follows: Urban areas are characterized by high building densities, extensive pavements, artificial lighting and significant human activity. These areas typically have modified habitats that present unique challenges and opportunities for bat populations. Rural areas include agricultural landscapes, small settlements and patches of natural vegetation. These areas generally have less human influence and infrastructure, providing different habitat conditions for bats compared to urban environments. This sampling framework allows us to systematically analyze how bat echolocation patterns can change across these different levels of urbanization.

Preparation

Data collection will take place from April to October 2026 and 2027, collecting temporal data from spring to late summer. In order to assess species-habitat relationships for bats in different areas, we will create spatial data using ArcGIS-pro. This will allow us to create predictor variables that can be used to assess the influence of habitat characteristics (Gallo et al. 2018).

Field work

Field data collection begins with the installation of monitoring equipment at each sampling site. We will use a Pettersson D500X ultrasonic detector equipped with a Pettersson ultrasonic microphone positioned approximately 1.5 metres above ground. Environmental

parameters will be monitored using a HOBO U30 USB Weather Station data logger with an S-LIB-M003 light sensor, which will record temperature, relative humidity and light intensity. Ambient noise levels will be measured using a PCE-322-SC43 sound level meter installed adjacent to the data logger. Each monitoring station will have a dedicated observer who will be responsible for setting up the equipment and checking its operation. Recording sessions will begin 30 minutes after sunset and continue for 4 hours. Data will be collected weekly at all sampling sites. To ensure data quality, sampling will only take place during suitable weather conditions (no rain and wind speed less than 6 m/s as predicted by weather forecasts, following the methods of Martin et al. 2017). In addition, all recording devices will be oriented away from potential sources of clutter (such as foliage, branches, buildings) to avoid echo interference, as recommended by recent research (Starik et al. 2024).

Data analysis

To identify echolocation patterns, we will extract and analyze data from the Pettersson D500X ultrasonic detector using two complementary software approaches. First, we will use Batscope software (Obrist and Boesch 2018) together with batIdent (version 1.5; ecoObs GmbH, Nuremberg, Germany) for automated species identification. Secondly, we will process the recordings using Avisoft-SASLab Pro to measure key acoustic parameters, including highest and lowest frequencies, frequency modulation patterns and signal duration. We will then compare these frequency patterns with the Avisoft Bioacoustic database to manually categorize the calls into functional types: social calls and foraging calls. A major challenge in bat acoustic identification is that closely related species often have very similar echolocation calls with overlapping characteristics. When we cannot confidently identify calls to species level through manual analysis, we classify them into sonotype groupings based on their acoustic characteristics (following the method described in Starik et al. 2024). These sonotypes represent distinct call types that share similar acoustic characteristics, allowing us to analyze diversity and activity patterns even when species-level identification is not possible.

Statistical analysis

We will use Principal Component Analysis (PCA) from the R stats package to evaluate how environmental factors influence bat echolocation parameters. This will help identify key relationships between environmental variables and echolocation characteristics. Additionally, we'll implement generalized mixed effect models from the blme package to assess urbanization impacts while accounting for random effects like sampling site and temporal variation (Starik et al. 2024).

5) Composition and qualifications of the research team

PI 1: Kinga Szafrańska

- Completed Master's degree at Jagiellonian University (UJ) with distinction (2024)
- Presented research findings at an international conference (2024)
- Experience in four distinct projects investigating the impact of various environmental factors on lichen communities and biological soil crusts

PI 2: Failasuf Aulia Nugroho

- Completed Master's degree at Jagiellonian University (UJ) with distinction (2020)
- Published author of two scientific publications:

  Second author publication about bats distribution in Indonesia (2024), first author about microplastics (2020)
- Experience conducting bat distribution surveys across Indonesia as part of a national survey initiative

6) Project literature

Baker, P. J., & Harris, S. (2007). Urban mammals: What does the future hold? An analysis of the factors affecting patterns of use of residential gardens in Great Britain. *Mammal Review, 37*, 297–315.

Diebold, C. A., Salles, A., & Moss, C. F. (2020). Adaptive echolocation and flight behaviors in bats can inspire technology innovations for sonar tracking and interception. *Sensors, 20*(10), 2958.

Dudzic-Gyurkovich, K. (2023). Study of centrality measures in the network of green spaces in the city of Krakow. *Sustainability, 15*(18), 13458.

Francis, C. D., & Barber, J. R. (2013). A framework for understanding noise impacts on wildlife: An urgent conservation priority. *Frontiers in Ecology and the Environment, 11*, 305–313.

Gallo, T., Lehrer, E. W., Fidino, M., Kilgour, R. J., Wolff, P. J., & Magle, S. B. (2018). Need for multiscale planning for conservation of urban bats. *Conservation Biology, 32*(3), 638–647.

Hans, S., & Peet, M. (2003). Ecology: Birds sing at a higher pitch in urban noise. *Nature, 424*(6946), 267.

Kohyt, J., Karczmarz, J., Pereswiet-Soltan, A., et al. (2024). Spatiotemporal use of urban rivers by local bat populations in a large city (Cracow, Southern Poland). *Urban Ecosystems, 27*, 1663–1673.

Lowry, H., Lill, A., & Wong, B. B. (2013). Behavioural responses of wildlife to urban environments. *Biological Reviews, 88*(3), 537–549.

Martin, C. M., Arnett, E. B., Stevens, R. D., & Wallace, M. C. (2017). Reducing bat fatalities at wind facilities while improving the economic efficiency of operational mitigation. *Journal of Mammalogy, 98*(2), 378–385.

Obrist, M. K., & Boesch, R. (2018). BatScope manages acoustic recordings, analyses calls, and classifies bat species automatically. *Canadian Journal of Zoology, 96*, 939–954.

Perugini, M., Manera, M., Grotta, L., Abete, M. C., Tarasco, R., & Amorena, M. (2011). Heavy metal (Hg, Cr, Cd, and Pb) contamination in urban areas and wildlife reserves: Honeybees as bioindicators. *Biological Trace Element Research, 140*, 170–176.

Pickett, S. T., Cadenasso, M. L., Grove, J. M., Nilon, C. H., Pouyat, R. V., Zipperer, W. C., & Costanza, R. (2001). Urban ecological systems: Linking terrestrial ecological, physical, and socioeconomic components of metropolitan areas. *Annual Review of Ecology and Systematics, 32*(1), 127–157.

Russo, D., & Ancillotto, L. (2015). Sensitivity of bats to urbanization: A review. *Mammalian Biology, 80*(3), 205–212.

Sachanowicz, K., Ciechanowski, M., & Piksa, K. (2006). Distribution patterns, species richness and status of bats in Poland. *Vespertilio, 9–10*, 151–173.

Scolozzi, R., & Geneletti, D. (2012). A multi-scale qualitative approach to assess the impact of urbanization on natural habitats and their connectivity. *Environmental Impact Assessment Review, 36*, 9–22.

Starik, N., Gygax, L., & Göttert, T. (2024). Unexpected bat community changes along an urban–rural gradient in the Berlin–Brandenburg metropolitan area. *Scientific Reports, 14*(1), 10552.

Stone, E. L., Jones, G., & Harris, S. (2009). Street lighting disturbs commuting bats. *Current Biology, 19*, 1123–1127.

Website

Inaturalist. (2024). Bats of Poland. Assessed on 22.04.2025, https://inaturalist.ca/check_lists/4460178-Bats-of-Poland?view=taxonomic

Repair. (2012). Pull Łódź (PL). Assessed on 22.04.2025, https://h2020repair.eu/case-studies/pull-lodz-pl/

Sas, Adriana. (2025). Largest cities in Poland in 2023, by number of population. Assessed on 22.04.2025, https://www.statista.com/statistics/1455315/poland-largest-cities-by-population/

7. Table with budget of the project.

	Amount in PLN
Direct costs, including	726 695,34
- personnel costs and scholarships	192 000,00
- research equipment/device/software cost	238 885,78
- other direct costs	295 809,56
Indirect costs, including:	159 873,01
- indirect costs of OA	14 533,91
- other indirect costs	145 339,10
Total costs	**886 568,35**

8. Breakdown of project costs including justification and relevance for the tasks in the project.

a. **Direct costs- personal salaries**

Position/Contributor	**Salary per month (PLN)**	**Salary collection period [in months]**	**Total (PLN)**
PI 1	4000	24	96 0000,00
Contribution to the project	Research planning,coordination, field work, participation in data collection, data analyses and writing manuscripts		
PI 2	4000	24	96 0000,00
Contribution to the project	Research planning,coordination, field work, participation in data collection, data analyses and writing manuscripts		
Total			**192 000,00**

b. **Direct costs- list of equipment to be purchased**

Name of apparatus	**Year of purchase**	**Unit Cost (PLN)**	**Amount**	**Cost (PLN)**
Ultrasound Detector	2026	7 585,26	18	75 852,60
External Microphone	2026	1 803,45	18	18 034,50
Justification of purchase	The use of an ultrasonic detector with a microphone made it possible to record the echolocation voices of bats in the field.			
Weather Station data logger	2026	7 154, 96	18	71 549,60
Light sensor	2026	1 179,56	18	11 795,60

Name of apparatus	Year of purchase	Unit Cost (PLN)	Amount	Cost (PLN)
Justification of purchase	Essential for measuring temperature, humidity and light intensity			
Sound level meter	2026	2 324,00	18	23 240,00
Justification of purchase	Necessary for measuring noise level			
Laptop with programme licence Office and EndNote	2026	5000,00	4	10 000,00
Justification of purchase	Computers for everyday work, planning the studies, data analyses, writing manuscripts for PI			
Portable drive	2026	500,00	4	1 500,00
Justification of purchase	Disc used for backups and data archivization for PI			
Avisoft-SASLab software	2026,2027	18 347,76	1	18 347,76
BatIdent software	2026,2027	8 565,72	1	8 565,72
Justification of purchase	Professional analysis of bat echolocation signals			
Total				**238 885,78**

c. **Other direct costs justification**

Materials

Costs: **11 200,00 PLN**

Cost of materials used to install survey equipment in the field:

Protective and mounting components (weatherproof enclosures, brackets, poles, tripods, vibration-damping elements): **3 200, 00 PLN**

Power supply infrastructure (connectors, waterproof cables): **2 100,00 PLN**

Installation and assembly materials (screws, clamps, fasteners, cable ties, basic tools): **2 000,00 PLN**

Transport and contingency (field cases, spare mounting parts): **2 700,00 PLN**

Office materials including printer cartridge: 600 per year. Cost for 2 years: **1 200,00 PLN**

Conferences

(by members of the research team)

Cost: **64 200, 00 PLN**

Costs of attending conferences of the two PI e.g. International Bat Research Conference in 2026 (USA), European Bat Research Symposium in 2027 (German), International Bioacoustics Congress in 2027 (France)

Average costs of return flight (2 people): 10 000 PLN, average early fee (2 people): 4 000,00 PLN average accommodation for a week (2 people): 4 000, 00 PLN; daily allowance for a week (2 people): 3 400,00 PLN; in total for one conference: 21 400,00 PLN.

Costs of 3 conferences: 64 200,00 PLN

Visits and consultations

(travel expenses / travel expenses by external collaborators and/or consultants and costs of meetings)

Cost: **10 409, 56 PLN**

Visit of a bat echolocation sound specialist at the University of Munich for both PI: return flight Munich- Kraków: 2 569,56 PLN, accomodation in the University Guest house: 360,00 PLN /day; daily allowance: 200,00 PLN /day, in total for two weeks 2 569,56 + 7 840,00 = 10 409,56 PLN

Collective investigators

Cost: **210 000,00 PLN**

The number of collective investigators will be 15 and their task will be to assist with field research; The salary of each will be 1000,00 PLN/month and the pay period will be 14 months

d. **Indirect costs**

(such as do not fit into other categories, including the cost of disseminating the results)

Cost: **159 873,01 PLN**

Costs of publishing (this includes figure artwork and publication fees, such as extra-page charges): 14 533,91 PLN

Other indirect costs: 145 339,10 PLN

Authors' Declaration

We declare that AI has been used in the preparation of this proposal for translation purposes and sentences improvement.

2. Terraforming Mars with Fungi: Exploring the radiotrophic and radioprotection properties of Cladosporium sphaerospermum in Martian simulated conditions

Team: Aya Dridi, Patrycja Wąchała

Project (initial proposal)

Title: Terraforming Mars with Fungi: Exploring the radiotrophic and radioprotection properties of *Cladosporium sphaerospermum* in Martian simulated conditions

Authors: Aya Dridi, Patrycja Wąchała

Summary:

People have dreamed of going into space for many years, and now that we have done that and landed on the Moon, the frontier is moving further - we want to land on another planet. Mars stands out for its potential, but it poses a greater risk because the levels of UV radiation are higher on it compared to Earth. Consequently, scientists are looking for new materials to protect astronauts from this danger. And now scientists are turning their attention from artificial materials to fungi from Chernobyl. *Cladosporium sphaerospermum* is a worldwide species that grew near the Chernobyl power plant after the disaster. It has also visited the International Space Station, where it has not only survived but grown. It is thought that this is due to a high concentration of melanin - a pigment that protects against UV radiation - and a presumed ability to convert ionising radiation into an energy source. In our project, we want to focus on discovering the mechanisms behind this ability to survive in space conditions. Using a vacuum chamber and high UV radiation, we will simulate the Martian atmosphere and test whether melanin is only responsible for protection from UV radiation, or whether it is essential for further growth by using UV radiation as an energy source. We will also investigate whether *Cladosporium sphaerospermum* can be used as a bio-shield to protect human cells. If the results are promising, it will unlock the potential for further work on UV protection in space and further space exploration.

1) Scientific goal of the project

While radiation studies on Earth have focused mainly on gamma and X-rays (Shahbazi-Gahrouei et al., 2013), the Martian surface presents a different challenge. The absence of a global magnetic field and a thin atmosphere mean that Mars is exposed to intense Galactic Cosmic Rays (GCRs), Solar Energetic Particles (SEPs), and unfiltered UV radiation (Hassler et al., 2013; Chen et al., 2022). These conditions result in a major increase in the radiation dose — it is expected that astronauts on Mars are exposed to ~400 mSv in one year, compared to ~2 mSv on Earth (ANS, 2020).

Recent discoveries showcase that *Cladosporium sphaerospermum*, a melanized fungus found in high-radiation zones like Chernobyl, not only survives but may even thrive under

radiation, potentially using it as an energy source ("radiotrophy") (Averesch et al., 2022). Its unique feature offers a promising opportunity to explore biological strategies for space radiation protection.

This project is an attempt to simulate Martian conditions (UV exposure, atmosphere, temperature) to investigate the biological mechanisms behind *C. sphaerospermum's* resilience and radiotrophic potential. Specifically, we will explore the role of melanin and ergosterol in protecting cellular structures, the fungus's capacity for DNA repair and regrowth after UV exposure, and its potential application as a living radiation shield for human cells.

In our project, we want to answer two main questions:

Q1: Is melanin essential for protecting fungi from UV-ionization by preserving ergosterol integrity and/or protecting DNA from damage?

Q2: Can *C. sphaerospermum* be explored as a self-replicating biological shield to protect humans in space, such as on the Martian surface?

Based on the previously announced questions, we hypothesize that:

H1a: Melanin protects ergosterol from UV-induced degradation, maintaining membrane stability and fungal viability.

H1b: Knock-out or suppression of melanin results in decreased growth and membrane damage.

H1c: Synthetic shielding materials (e.g. UV-absorbing polymers) only partially replicate melanin's protective role.

H2: *C. sphaerospermum* can act as an effective biological radiation shield for human cells, reducing radiation-induced cytotoxicity.

2) Significance of the project

One of the core questions of modern science is whether life can exist — and be sustained — beyond Earth. Mars is considered a prime candidate for human exploration due to its relative proximity and geological features. However, its lack of a magnetic field and thin atmosphere lead to intense exposure to cosmic and UV radiation, posing serious threats to both biological organisms and mission infrastructure (Checinska Sielaff & Smith, 2019; Montmessin & Lefèvre, 2013).

Radiation damages DNA, induces oxidative stress, and compromises material integrity — challenges that must be tackled for potential long-term missions (Guo et al., 2021). UV radiation, in particular, can disrupt essential cellular processes in humans and erode key materials employed in space missions (Abd El-Hameed, 2022).

In this context, fungi — especially melanized species like *Cladosporium sphaerospermum* — present an innovative opportunity. Not only has this species survived in high-radiation environments on Earth and in space, but it may also use radiation as a metabolic driver (Zhdanova et al., 2000; Averesch et al., 2022). Its capacity to function as a biological

radiation shield could offer sustainable, self-repairing protection for both living systems and sensitive electronics during extraterrestrial missions.

By investigating the underlying processes allowing for this organism's resilience, this project could contribute to the development of bio-based shielding strategies and expand our understanding of life's adaptability in extreme environments — critical insights for both astrobiology and human spaceflight.

3) Concept and work plan

Table 1. Experimental Design for Investigating the Role of Melanin and Ergosterol in Fungal Viability, Radiotrophic, and Radioprotection Behavior under UV Radiation.

Research question	H	Experiment	Purpose	Methods	Expected result
Q1	H 1a	Melanin's role in UV-induced damage preventing ergosterol degradation	Er gosterol levels, melanin content, Vitamin D2 pr oduction	HPLC for e rgosterol and vitamin D2 quant ification	Me lanin-deficient strain shows decreased viability and higher Vitamin D2 conversion under UV exposure; a decrease in ergosterol levels
	H 1b	Effect of CRISPR/Cas9 knockouts on DNA oxidative damage and fungal growth after UV exposure	DNA o xidative damage marker, m easuring DNA i ntegrity to d etermine survival rate, Fungal growth conf irmation	8-OHdG ELISA, PCR and s equencing for mutation conf irmation, CFU counting	Me lanin-deficient strains exhibit significantly increased DNA oxidative damage (higher 8-OHdG levels) and reduced survival as well as growth (lower CFUs)
	H 1c	Effect of Non-Ionizing Radiation on Fungal Growth and Survival (With and Without Melanin):	Testing the sy nergetic n ecessity of both melanin and er gosterol for the enh	Growth rate (measuring biomass), ATP p roduction using ATP lum inescence	Wild-type strains show increased growth and energy production under non-ionizing radiation; me lanin-deficient strains show diminished

		radiotrophic abilities	ancement of fungal growth under non-ionizing r adiation by co nverting it into energy	assay, ROS p roduction (ROS detection assays)	response; Ergosterol is essential for fungal survival under electromagnetic radiation
Q2	H2	Testing the rad ioprotective effects of *Cladosporium sph aerospermum* on human cells lines (HaCat-human k eratinocytes and HepG2 cells-human liver cells)	Evaluate if *Clad osporium sphaero spermum* and its b ioactive c ompounds (melanin and erg osterol) can mitigate r adiation - induced damage to human cells	Radiop rotection effects	Potential Radioprotective Effect: The pre-treatment of human cells with *C. sphaerospermum* extracts or purified melanin and/or ergosterol will show significantly reduced DNA damage and higher cell viability compared to untreated, irradiated controls

Work plan

The duration of the work is scheduled to be two years, with the cultivation of *C. sphaerosporum* being carried out predominantly on the designated substrate during the majority of this period. The initial year will be dedicated to the identification of answers to Q1, and each hypothesis will be tested with a different experiment. The subsequent year will be dedicated to addressing Q2. The project will be distributed between two Principal Investigators (PIs). The construction of the Martian chamber will be undertaken by external physicists. The project is scheduled to culminate in two publications, each addressing a distinct research question, and a conference to disseminate the findings. Throughout the duration of the project, efforts will be made to popularise it and disseminate the knowledge gained.

Table 2. Gantt chart for the workplan. Legend: blue – both PIs (PI1, PI2); green – PI1; orange – PI2; violet – external; red – milestones.

Tasks	Year 1				Year 2			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Cultivation of fungi	PIs	PIs	PIs	PIs	PIs	PIs	PIs	

Construction of Martian chamber								
Creating knock–out fungi	PI1							
Ergosterol testing in chambers		PI1						
Ergosterol testing – normal cond.		PI2						
DNA damage test - chambers			PI1					
DNA damage test – normal			PI2					
Biomass - chambers				PI2				
Biomass - normal				PI1				
Cultivation of human cell lines					PIs			
Shielding potential testing -						PIs		
Statistical analysis			PI2	PI2	PI2		PI1	PI1
Paper writing				PIs	PIs			PIs
Dissemination		PIs	PIs	PIs	PIs	PIs	PIs	PIs
MILESTONES								
First publication								
Second publication								
Conference								

Risk assessment

In order to ensure that there will be no prior discrepancies between *C. sphaerospermum* individuals, it is imperative that specimens are not taken from the environment; rather, known laboratory strains will be purchased and cultivated in the same conditions prior to the experiment. To enhance the chances of achieving correct Martian conditions, the model of chamber will be based on Skubała et al. (2025). The construction of this chamber will be undertaken by physicists. To ensure the accuracy of ergosterol level measurements, samples will be sent to specialists for HPLC analysis.

For participants' safety, special personal protective equipment will be used (glasses, gloves, remote control of UV light).

Collaborations:

We will collaborate with Faculty of Biochemistry, Biophysics and Biotechnology (Jagiellonian University), Faculty of Chemistry (Jagiellonian University) and Faculty of Physics, Astronomy and Applied Computer Science (Jagiellonian University).

4) Research Methodology

Simulation of Martian conditions

In order to simulate Martian conditions, a vacuum chamber equipped with a source of UV radiation and a CO_2 supply will be utilised. A cooling table will be connected to the cooling unit and installed inside the chamber. The temperature and humidity will be regulated by sensors installed within the chamber and connected to a computerised control system. The UV radiation will be regulated by external controllers. The pressure within the chamber will be regulated by a vacuum pump. Fungi samples will be attached to the metal grid, with the UV radiation source positioned at a distance of 35 cm perpendicular to this.

The pressure will be maintained within the range of 5 – 7 mbar, reflecting the atmospheric conditions of Mars, with a gas composition of 95% CO_2 and 5% air. The temperature will vary between 15°C during the day simulation and –30°C during the night simulation, while the relative humidity (right next to the fungi) will be 8 – 32%. The model is based on Skubała et al. (2025).

Importance of UV protection

In order to determine whether melanin is essential for survival and/or growth per se (possibly through radiotrophy), or whether it is important for its UV-protective properties, we will use melanin knock-out samples. However, it is acknowledged that UV radiation is detrimental to cells, and it is hypothesised that at these levels of UV radiation, which are significantly higher than those typically encountered on Earth, fungi will be protected by materials that block UV (including UVB and UVC). In the subsequent text, these two types are referred to as melanin (regular fungi) and non-melanin (knockout of melanin, use of protective material). For each group, 20 specimens will be used. The model underpinning this study is derived from Skubała et al. (2025).

Fungal strain cultivation for comparative assays

At a first step, we propose to generate knockout mutants of *C. sphaerospermum* (ATCC 11288). We will use the CRISPR/Cas technique, which uses the Cas protein to modify targeted sequences using artificially generated sgRNA (guide RNA), which guides the Cas9 protease to effectively cut the double strands of DNA and form double-strand breaks. The dihydroxynaphthalene (DHN)-melanin gene biosynthesis in *C. sphaerospermum* will be disrupted by targeting the gene encoding polyketide synthase (PKS) using BLAST, a key enzyme in the pathway homologous to the gene that has been previously genomically encoded in annotated strain UM843 and is conserved across other *C. sphaerospermum* strains (Yew et *al*., 2016). sgRNAs will be designed to target conserved early exons in the PSK gene. A fungal-compatible CRISPR/Cas9 expression plasmid will be constructed, incorporating Cas9, the sgRNA, and a selectable marker (hygromycin resistance [hph]). Fungal transformation will be carried out using PEG-mediated protoplast transformation, followed by selection on hygromycin-containing media. Mutants will be screened by PCR and confirmed via Sanger sequencing. Phenotypic validation will be depicted by the loss of pigmentation on PDA plates using spectrophotometric quantification of melanin. We will then cultivate around 20 biological replicates of each strain, the original wild-type strain, and the CRISPR/Cas9-generated knockout strains separately under identical conditions for the following analysis.

Impact on ergosterol

Ergosterol is a vital component of fungi cell walls, as it serves to stabilise its structure (a function analogous to that of cholesterol in animal cells). However, upon exposure to UV radiation, ergosterol is converted into vitamin D2, a process that is not essential for fungal growth. Consequently, this results in a decrease in the amount of ergosterol and a subsequent destabilisation of the cell structure.

The present study aims to ascertain whether melanin can protect fungi by stabilising the cell structure via the protection of ergosterol. To this aim, we will compare melanin and non-melanin samples both in the Mars chamber and under normal conditions (climate chamber 20°C, 101 kPa, UV lamp emitting standard UV light). Following a five-hour acclimatisation period, the samples will be preserved and sent to specialists for HPLC analysis of ergosterol levels.

DNA damage test

In order to evaluate the capacity of melanin to shield DNA from harm in Martian conditions, we will perform a test similar to the ergosterol level test, but after acclimatisation we will conduct 8-OHdG detection tests (as a marker of DNA oxidative damage).

Growth

In order to check whether *C. sphaerosporum* is able not only to survive but also to grow under Martian conditions (which would support the hypothesis that it converts UV radiation into energy for growth), we will dry the melanin and non-melanin samples and then weight the biomass.

Radioprotection in Fungi

In order to evaluate the radioprotective potential effects of *Cladosporium sphaerospermum,* the use of biological cell lines is being considered, with a particular focus on human cell lines, such as HaCaT-human keratinocytes, which are derived from skin and are highly relevant for studying skin responses, particularly in cases of UV exposure, which is predicted to primarily affect skin cells (Khalil et al., 2017). A secondary cell line that is to be studied in this section is HepG2 cells, which are human liver cells. It is imperative to comprehend the potential of radioprotective compounds derived from fungi in mitigating these effects, particularly in the context of prolonged space missions where radiation exposure is a concern. The use of these cells will help to determine the efficacy of different derived bioactive compounds in protecting HepG2 from radiation-mediated DNA damage. Such evaluations have the potential to pave the way for subsequent in vivo studies or clinical trials.

The methodological demarche for this part will be as follows:

1) Co-culture assays in vitro simulated Martial conditions, using both fungi batchs (wild-type, CRISPR/Cas9 melanin knockouts, purified melanin extracts) 2) Assess cell viability and apoptosis post-UV exposure using MTT technique for viability assessment and trypan blue technique, LDH assay for membrane integrity and necrosis, and flow cytometry for apoptosis quantification 3) Determine if fungal melanin is responsible for the reduction of reactive oxygen species (ROS), which were produced due to oxidative stress. We will use fluorescent probes to measure ROS levels post-irradiation 4) Detect and quantify UV-induced DNA damage and the potential protective effect of the fungi. We can use 8-OHdG

ELISA to determine DNA oxidative damage increase or reduction 5) Assess long-term radioprotection: using transcriptomic (RNA-sequencing) and proteomic profiling (using western blot) would be conducted on both HaCat and HepG2 cell lines following UV exposure. We would check which tested batch will induce a sustained upregulation of antioxidant response genes (HMOX1), DNA repair mediators and survival regulators. Such testing will provide mechanistic insight into how *C. sphaerosporum* mediates its protective effects and if melanin alone is the bioproduct responsible for radioprotective effect observed in cells or are additional fungal components required for a significant effect?

Statistical analysis

Statistical analysis will be carried out with the use of free software RStudio.

5) Composition and qualifications of the research team

Principal Investigator 1: Aya Dridi – PhD student who holds a biochemistry master's diploma, with hands-on experience with chromatography methods, including gas chromatography–mass spectrometry (GC-MS) and Thin-Layer chromatography technique (TLC), which makes her well-suited for the biochemical profiling components of the following project.

Principal Investigator 2: Patrycja Wąchała - PhD student in the Biology Programme, Master of Science (biology); experience in statistics (SPSS, Statistica, R) and coding (R); experience in writing manuscripts (*Przegląd Przyrodniczy*; one paper currently under revision in another journal) and presenting at conferences (Studencka Konferencja Teriologiczna); experience in designing and directing student scientific projects as a President of Students Naturalists Association for 2 years.

6) Project literature

- Abd El-Hameed, A. M. (2022). Radiation effects on composite materials used in space systems: A review. *NRIAG Journal of Astronomy and Geophysics*, *11*(1), 313-324.
- ANS (2020). *Radiation Dose Calculator. American Nuclear Society*. Available online at: https://ans.org/pi/resources/dosechart/msv.php (accessed April 12, 2025).
- Averesch, N. J., Shunk, G. K., & Kern, C. (2022). Cultivation of the dematiaceous fungus Cladosporium sphaerospermum aboard the International Space Station and effects of ionizing radiation. *Frontiers in microbiology*, *13*, 877625.
- Bhardwaj, A., Sam, L., Buchroithner, M. F., & Galofre, A. G. (2022). Advances in Mars research and exploration. *Frontiers in Astronomy and Space Sciences*, *9*, 971104.
- Chen, J.-L., Yun, S.-J., Dong, T.-K., Ren, Z.-Z., & Zhang, X.-P. (2022). Studies of the radiation environment on the Mars surface using the Geant4 toolkit. *Nuclear Science and Techniques*, *33*(1),
- Checinska Sielaff, A., & Smith, S. A. (2019). Habitability of mars: How welcoming are the surface and subsurface to life on the red planet?. *Geosciences*, *9*(9), 361.

- Chowaniec, K., Latkowska, E., & Skubała, K. (2023). Effect of thallus melanisation on the sensitivity of lichens to heat stress. *Scientific Reports*, *13*(1), 5083.
- Cortesão, M., Schütze, T., Marx, R., Moeller, R., & Meyer, V. (2020). Fungal biotechnology in space: why and how?. *Grand challenges in fungal biotechnology*, 501-535.
- Guo, J., Zeitlin, C., Wimmer-Schweingruber, R. F., Hassler, D. M., Ehresmann, B., Rafkin, S., ... & Wang, Y. (2021). Radiation environment for future human exploration on the surface of Mars: The current understanding based on MSL/RAD dose measurements. *The Astronomy and Astrophysics Review*, *29*, 1-81.
- Hassler, D. M., Zeitlin, C., Wimmer-Schweingruber, R. F., Ehresmann, B., Rafkin, S., Eigenbrode, J. L., … Bohm, E. (2013). *Mars' Surface Radiation Environment Measured with the Mars Science Laboratory's Curiosity Rover. Science, 343(6169), 1244797–1244797.*
- Khalil, C., & Shebaby, W. (2017). UVB damage onset and progression 24 h post exposure in human-derived skin cells. *Toxicology reports*, *4*, 441-449.
- Li, Y., Wang, Y., Wang, H., Shi, T., & Wang, B. (2024). The genus *Cladosporium*: A prospective producer of natural products. *International Journal of Molecular Sciences, 25*(3), 1652.
- Montmessin, F., & Lefèvre, F. (2013). Transport-driven formation of a polar ozone layer on Mars. *Nature Geoscience*, *6*(11), 930-933.
- Shahbazi-Gahrouei, D., Gholami, M., & Setayandeh, S. (2013). A review on natural background radiation. *Advanced Biomedical Research*, *2*(1), 65.
- Shunk, G. K., Gomez, X. R., Kern, C., & Averesch, N. J. (2020). A self-replicating radiation-shield for human deep-space exploration: Radiotrophic Fungi can attenuate ionizing radiation aboard the international space station. *BioRxiv*, 2020-07.
- Sinha, R. P., & Häder, D. P. (2002). UV-induced DNA damage and repair: a review. *Photochemical & Photobiological Sciences*, *1*(4), 225-236.
- Skubała, K., Chowaniec, K., Kowaliński, M., Mrozek, T., Bąkała, J., Latkowska, E., & Myśliwa-Kurdziel, B. (2025). Ionizing radiation resilience: how metabolically active lichens endure exposure to the simulated Mars atmosphere. *IMA fungus*, *16*, e145477.
- Yew, S. M., Chan, C. L., Ngeow, Y. F., Toh, Y. F., Na, S. L., Lee, K. W., Hoh, C.-C., Yee, W.-Y., Ng, K. P., & Kuan, C. S. (2016). Insight into different environmental niches adaptation and allergenicity from the Cladosporium sphaerospermum genome, a common human allergy-eliciting Dothideomycetes. *Scientific Reports*, *6*(1), 27008.
- Zhdanova, N. N., Zakharchenko, V. A., Vember, V. V., & Nakonechnaya, L. T. (2000). Fungi from Chernobyl: mycobiota of the inner regions of the containment structures of the damaged nuclear reactor. *Mycological Research*, *104*(12), 1421-1426.

7) Budget

Type of costs	Amount in PLN
Direct costs, including	**600,500**
- personnel costs and scholarship	36,000
- research equipment/device/software cost	444,000
- other direct costs	120,500
Indirect costs, including:	**132,110**
- indirect costs of OA	12,010
- other indirect costs	120,100
Total costs	**732 610**

8) Breakdown of projects costs

Category	**Description**	**Amount (PLN)**
Direct costs		
Salaries / Scholarships	2 PhD Students x 750 PLN x 24 months	36,000 PLN
CRISPR/Cas9 Gene Editing	Cas9, sgRNA design and cloning, electroporation supplies, selection reagents	25,000 PLN
Cell Culture	HaCaT and HepG2 cell lines (2 types x 1500 PLN) Media, FBS, antibiotics, flasks Substrate: 2 x 1500 PLN	6,000 PLN
Melanin Purification	Solvent extraction, dialysis membranes, consumables	6,000 PLN
Cell Viability Assays	MTT, LDH, Trypan Blue kits, flow cytometry reagents	18,000 PLN
ROS Measurement	Fluorescent dyes (e.g., DCFDA, MitoSOX), microplate reader filters	7,500 PLN
Transcriptomics / Proteomics	RNA extraction, RNA-Seq/qPCR, protein blots (HSP70, p21, Nrf2)	35,000 PLN
DNA Oxidative Damage	8-OHdG ELISA kits (x2), DNA extraction kit, consumables	5,800 PLN
Fungal Strain and Culture Substrates	*Cladosporium sphaerospermum* active cultures (x5), growth substrates	5,500 PLN
HPLC Analysis	80 melanin or ergosterol samples x 150 PLN/sample	12,000 PLN
Special Equipment for	Vacuum chamber – 20,000 PLN	65,000

Simulated Space Conditions	UV lamp – 20,000 PLN Gas supply + pressure regulation – 15,000 PLN Cooling table – 5,000 PLN Control computer – 5,000 PLN	PLN
Other Devices	Climate chamber – 25,000 PLN Solar lamp – 4,000 PLN Flow cytometry – 350,000 PLN	3 79,000 PLN
Others	Conference costs IBCF: International Conference on Fungal Biology (Location varies, commonly in Europe) The conference focuses on the survival of life in extreme environments, e.g. Greece 2025 (2 people x 3 days, 50 EURO/day diet, 160 EURO transport, fee 350 EURO)	5700 PLN
	Miscellaneous	5000 PLN
	Consumables: Eppendorf tubes, pipette tips, PCR tubes, Petri dishes, gloves	5000 PLN
	Biohazard Waste Disposal	3000 PLN
	Shipping costs: reagents, cultures	6000 PLN
	Safety and personal protective equipment	
Indirect costs		
Publication of results (open access journals)		12,010
Other indirect costs		12,010

Declaration

During the preparation of this work the authors used ChatGPT in order to estimate the budget and DeepL in order to improve the grammar. Perplexity was used for literature research. After using this tools,, the authors reviewed and edited the content as needed and take full responsibility for the content of the submitted proposal.

Reviews

Alice Ballabio

Simplified form for grant proposal review

Title of the project: Terraforming Mars with Fungi: Exploring the radiotrophic and radioprotection properties of *Cladosporium sphaerospermum* in Martian simulated conditions

1. **Assessment of scientific quality of the research project**

- Overall good, relevant and original: studies about UV and radiation protection are important for many fields, like medicine or energy research. Moreover, given the problematic with air pollution, UV protection is getting more and more popularity. Studies on organisms that resist to high energy radiation (which are detrimental to biological tissues and therefore to humans) need to gain more popularity and have more space in the scientific community, so I believe this project is very relevant in this sense.

- It is too ambitious, I would have focused mostly on one of the parts of the project, the exploration of the protective mechanism of melanin in the fungus, with more experiments in that sense.

- The layout is very clear and nice, readable and logical.

2. **Assessment of potential impact of the research project**

- The questions assessing the exploration of the mechanisms of radioprotection is interesting for the scientific community and it has the potential to set collaborations with other universities outside Poland and also the involvement of spatial agencies.

- The publications have the potential to be of great impact in the scientific community, especially in the not well-known field of astrobiology.

3. **Assessment of feasibility of the research project**

- The space suits used from NASA and ESA are well tested against UV radiation and the major concerns about human Mars exploration are against cosmic radiations. It is given that Mars have a higher amount of UV radiation, but since the suits are 'good enough', the project needs to highlight an effective and reasonable improvement of said suits to justify the change.
- The timing of the project does not take into account some difficulties that can arise from using some of the sequencing and gene editing techniques used: CRISPR-Cas9 unfortunately has a low rate of time-wise success in editing genes (it takes long time to actually edit the gene!) So, I think more time is needed for the project or a different gene editing technique is required. I wouldn't go with Sanger sequencing method since is not as efficient as NGS in exploring potential pathways, but it may be a personal preference.

- In the means of the Martian chamber, it seems overall well thought, I fear it would not be the strongest part of the project, given what I previously affirmed.

- I would have gone more into details into radiotrophy, since to me is the most innovative and important part of the project, which is unfortunately just mentioned. This could be exploited in the means of spatial exploration as potential fuel for rovers (they rely mostly on solar energy, which sometimes is inefficient, like in the Rosetta mission in which the rover got stuck in a dark cave and it couldn't be operational anymore).

- I would have explored more the viability of the fungus in extreme environments with actual methods to verify how long a single fungus can resist against UV in Martial environment before dying. Moreover, I would have tested if and how the life cycle and if the emergence of the next generation is affected by UV if the parental one is exposed, especially as potential part of biomaterial (if their ability to replicate changes in martial environment, how would you cope?)

- There is lack of PhDs, Postdocs and technicians in the projects, which are very useful resources.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

- The budget seems to little in my opinion. Sequencing is generally very expensive and hiring people to take care of the Martian chamber as well.

5. **Strengths of the proposal**

- Original and well written, the experiments are stated clearly, and it follows a good logic.

- I strongly believe in the potential of the work in the means of exploration the mechanisms of *Cladosporium sphaerospermum,* the genome editing and sequencing can give a good idea on what pathway to explore. I think it is a good pilot project.

6. **Weaknesses of the proposal**

- Too short timing

- Not enough scientific justification to go into human shield protection against UV

- Not enough exploration of radiotrophy

- Too little budget

Kinga Szafrańska

Title of the project: Terraforming Mars with Fungi: Exploring the radiotrophic and radioprotection properties of *Cladosporium sphaerospermum* in Martian simulated conditions

1. **Assessment of scientific quality of the research project**

The authors of this study propose to examine the impact of simulated Martian conditions in order to investigate the biological mechanisms behind the resistance and radiotrophic potential of *C. sphaerospermum*. This approach is innovative and pioneering due to the remarkable property of this fungal species to convert ionising radiation into a usable

energy source. This species has been identified in the Chernobyl nuclear disaster site. The authors have demonstrated the disparities between Earth and Mars, which is a significant strength of the study. However, it would have been beneficial to include a discussion of the conditions at the Chernobyl site. Furthermore, the incorporation of an example elucidating the geomorphological features that render Mars a favourable candidate for human exploration (line 56) would have been advantageous. However, I think that the authors have described the problem very well and that the research project is of high scientific quality.

2. **Assessment of potential impact of the research project**

Given the intensive development of research into the survival of organisms in space, this project will undoubtedly contribute to the development of this field of research. The project has identified the potential of using this fungus species as a living radiation shield for human cells. The proposal to use this fungus as a biological shield against radiation seems bold and needs further substantiation. It would be worthwhile for the authors to refer to existing studies that confirm or at least support this possibility. I consider it a very great asset for the authors to have planned two scientific publications and to have participated in a conference on this topic. Furthermore, it is to be commended that the authors also want to popularise the results of their research on a regular basis.

3. **Assessment of feasibility of the research project**

There are a lot of test methods planned in the project. The authors mention (lines 95 and 96) that in order to ensure the accuracy of the ergosterol measurement they send samples to external specialists from where the question arises what about the other analyses and their accuracy. This may be a poorly phrased sentence as the authors' planned collaboration with specialists from different fields suggests that they will be done accurately. I would also suggest a more extensive description of the measurement of fungal biomass growth. As for the description of the facility simulating Martian conditions, it is very detailed but it would be useful to include a diagram as well, which would make it easier to imagine. The risk management assessment takes many variables into account. However, the question arises whether researchers if they encounter a problem are able to modify the analysis plan. The description of “Fungal strain cultivation for comparative assays” is very detailed, which indicates the authors’ knowledge of the topic, but I wonder whether it could be simplified.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The budget has been planned in a clear and logical manner, but it would have been useful to add a short justification for the purchase of individual items of equipment. Such a procedure could further strengthen the legitimacy of these expenditures and facilitate their acceptance.

5. **Strengths of the proposal**

The project is well thought of and has an application-level approach to it. The authors provided very good justification for undertaking a given research topic, which, despite the short time for preparing the project, proves a good review of the literature. Thanks to the

graphical representation of experimental design, it is clear what a given analysis will be used for.

6. Weaknesses of the proposal

In the project I would add or change a few things that I mentioned above. I note that Table 2 does not indicate who will perform the chamber and when it will take place. I would also avoid stating that ergosterol is essential for fungal survival under electromagnetic radiation.

Rama S. Krovi

Simplified form for grant proposal review

Title of the project:

1. **Assessment of scientific quality of the research project** (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)

   This project is **highly relevant and important** in the context of space exploration and astrobiology. The challenges posed by Martian radiation are significant hurdles for future missions, and this research directly addresses a critical aspect of astronaut safety and the potential for establishing a sustained presence on Mars. Although other fungi were tested for a long time, this species is new. The fungus's unique history in a high-radiation environment on Earth and its survival in space make it a compelling subject for this research. As mentioned it could potentially be a new avenue for research. However, the project needs to be carefully assessing the potential after-effects when sending anything to space as it might pose a problem later. The authors haven't given details of how they expect the three factors are going to interact with each other.

2. **Assessment of potential impact of the research project** (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)

The potential for **substantial international impact** in several research fields is high. The findings could significantly advance our understanding of life's adaptability to extreme extraterrestrial environments and inform the search for life beyond Earth and so it would be a leap into astrobiology and related fields. Successful demonstration of *C. sphaerospermum*'s radioprotective capabilities could revolutionize astronaut safety protocols and enable longer and more ambitious deep-space missions, including to Mars. As mentioned in the references, the Polish scientists have already demonstrated in other species that this is possible for five hours which looks promising so aiming for a longer time could be more impactful? The project could yield novel insights into the mechanisms of radiation resistance and potential radioprotective compounds, which might even have applications on Earth. Discovering the genetic and biochemical pathways involved in and radioprotection in this fungus could lead to the development of new biomaterials or

biotechnological solutions.
The project clearly aims for **high-quality research publications**, with a plan for two distinct papers.

3. **Assessment of feasibility of the research project** (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)

The proposal demonstrates a reasonably good understanding of the project's feasibility, though some aspects need closer scrutiny and more detailed explanations given the project is planned for 2 people for two years. I consider it to be highly ambitious. International cooperation is not mentioned but would prove to be invaluable in this project. The proposed methods appear generally well-suited to address the research questions and test the hypotheses although I am a little skeptical given the times for knockout strains, building the apparatus etc. The use of a simulated Martian chamber, genetic manipulation of the fungus, and various analytical techniques for assessing ergosterol, DNA damage, growth, and radioprotection of human cells is logical. However, in assessing the ergosterol and also the growth, no explanation was given as to why they consider a five-hour acclimatization period. Incase of the growth experiment as well, do they expect the fungi to grow continuously for five hours?If yes, a possible explanation with a reference would be useful.

Risk management: The risks, such as ensuring consistent fungal strains and achieving accurate Martian conditions, are difficult to maintain. The proposed mitigation strategies, like using lab strains and basing the chamber design on existing research, are reasonable first steps in this case. However, a more detailed contingency plan for potential technical challenges with the chamber construction or unexpected experimental outcomes could be useful.
Research facilities and equipment: The proposal lists necessary equipment, including a vacuum chamber, UV lamp, and cell culture facilities. The reliance on external physicists for chamber construction and specialists for HPLC analysis will be extremely useful.
International cooperation: While collaborations with different faculties within Jagiellonian University are mentioned, there is no explicit mention of international cooperation. Depending on the specific expertise required, exploring international collaborations could further enhance the project.
Other factors affecting the feasibility of the project: The two-year timeframe seems ambitious given the scope of the proposed experiments, including the generation of knockout strains and the complex cell-based assays. A more detailed timeline within each year, outlining specific milestones for each experiment, would provide a clearer picture of the project's feasibility within the proposed duration

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

Overall, the costs seem generally justified :)

5. **Strengths of the proposal**

- addressing a critical challenge in space exploration with an innovative biological approach.
- It has a strong scientific rationale which is well-supported by existing literature on radiation in space and the unique properties of *C. sphaerospermum*. Successful preliminary work has already been published by their collaborators.
- clearly defined research questions and testable hypotheses.
- utilizing a range of appropriate methodologies to address the research questions.
- Potential for high impact: significant implications for astrobiology, space exploration, and potentially other fields.

6. **Weaknesses of the proposal**

- Feasibility within the two-year timeframe might be challenging given the number and complexity of experiments. A more granular timeline would be beneficial.
- Under the subheading, "Radioprotection in Fungi", it was not clearly mentioned on how you plan to use the human cell lines and test the use of beneficial effects of these fungi. Co-culturing assay is possibly how it is done but a mention about the details is missing.
- Simulating the exact conditions of Martian radiation (galactic cosmic rays, solar particle events), atmospheric pressure, temperature swings, and the thin, CO2-rich atmosphere in a lab setting is tough. How well will lab results truly translate to years on the Martian surface?
- A more detailed overview of the statistical analysis is needed.
- Risk management could be more detailed, including contingency plans for potential setbacks but the potential collaborators (Skubała et al., 2025) seem promising.
- International collaboration could aid in such a project.
- Budgets are not calculated according to the proposal requirements.

Dr hab. Joanna Sudyka

Grant proposal review

Title of the project: Terraforming Mars with Fungi: Exploring the radiotrophic and radioprotection properties of *Cladosporium sphaerospermum* in Martian simulated conditions

1. **Assessment of scientific quality of the research project** (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)

This project aims to explore the biological mechanisms behind *C. sphaerospermum's* resilience and radiotrophic potential in extraterrestrial (Martian) conditions. While relevant and important, I have some specific concerns regarding the scientific quality:

-My basic doubt is how any fungi can protect astronauts, as stated in the summary? How will it protect human cells? Will they have to wear the fungus? At this moment, I cannot imagine how this will practically happen, and this undermines the logic and rationale of the project. Perhaps the focus could be shifted from protecting astronauts, as this is too far-fetched, in particular in such a short project, into terraforming alone or simply the impact of the UV on the physiology of the fungus. If humanity thinks of colonizing Mars, introducing pioneering species such as these fungi could be the means to start. What are the basic metabolites of "radiothrophy"? Are these metabolites a step to forming an atmosphere where other species could develop? In this context, the project title does not describe the scientific goal well.

-How will the effects of UV exposure be disentangled from temperature and atmosphere on the fungus growth when the "simulation of Martian conditions" is applied? It is mentioned that "we will compare melanin and non-melanin samples both in the Mars chamber and under normal conditions", but this needs further explanations and careful experimental design, perhaps analysing the impacts on the fungus factor-by-factor (UV, pressure, temperature). Will there be a replicate for each treatment? It would serve the project well to be simpler yet more precise.

-Ergosterol is introduced next to melanin only in the study goals, without any rationale behind this being explained. The same is true for the role of Vitamin D2. It is only mentioned late in the methods. These biological pathways and the potential impacts on their functioning by the UV have to be first laid out, before introducing them in the project aims.

-The focus of the project, as I understood from the summary, is to explore the impact of UV-radiation, but in the H1c (Table 1) there is a switch to non-ionizing radiation in general. This includes visible, infrared, microwaves etc, except for the UV band. How does this fit in the study goals? In general, ionising radiation is the bigger problem for humans, while in space, so it has to be explained well why focus on the UV here?

-The hypothesis H1c mentions "Synthetic shielding materials (e.g. UV-absorbing polymers)", whereas the experimental design in Table 1 H1c pertains to something different. This is pretty confusing.

2. **Assessment of potential impact of the research project** (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)

If the rationale and the subject of the hypotheses to be tested are adequately stated and reasonably reduced to a clear experimental schedule, the project is potentially very impactful. It addresses a fundamental issue for human endeavors to explore space. This obviously would have a large international reception and publication potential.

3. **Assessment of feasibility of the research project** (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)

It is hard to assess the feasibility of the project because the timelines are not adequately described. The Gantt chart is massive yet not very informative, takes too much space that could be better used to substantiate the rationale and better explain the exact focus of the project (which type of radiation is to be studied, and what are the exact biological pathways to be explored). Instead, a simplified Gantt chart could be used, which primarily presents whether the tasks go in parallel or stem form one another (are there any key intermediate goals?) and are altogether feasible within the planned 2 yrs. For example, how long does the cultivation of *C. sphaerosporum* take? Is it days/months/weeks, how much does it take to get the amount needed for the experiment and how much is needed for each of the experiments?

Risk assessment is very brief. What will happen if the fungi don't want to grow at all in the chamber, or develop an infection and die out?

Within the project methodology, experimental schedules require more explanation. Perhaps using figurative schemes would make it easier and more readable. There is no mention of treatment replicates except for "20 biological replicates of each strain" in one of the assays, which remains unclear.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The costs are mostly justified, except for personnel costs. **"**The construction of the Martian chamber will be undertaken by external physicists" and there will be "specialists for HPLC analysis". I imagine these people should be well-paid, even more so if commercial services are used. Even if that is an agreed collaboration within the University, the costs of the respective equipment use/maintenance should be included. No remuneration was planned here though. Also, the scholarships of the PhD students are really low, especially if this is their only source of income (if not, that was not specified).

5. **Strengths of the proposal**

The project tackles important and timely research questions. The planned assays are coherent and build well on one another. It contains novel and ambitious aspects like CRISPR gene editing, which would help to disentangle the causality behind observed relationships.

6. **Weaknesses of the proposal**

The primary research goal is not well defined. It is not certain what remains in focus: the impact of UV, non-ionizing radiation in general or Martian conditions (atmosphere, temperature, pressure etc.). The experimental design requires more attention. The title is detached from the contents, neglecting the terraforming aspect. The timeline remains unclear, and therefore, it is not possible to evaluate the feasibility of the project. Risk assessment is also too brief, and no contingency plans are put in place.

Final version

Title: Terraforming Mars with fungi: exploring *Cladosporium sphaerospermum* as a pioneer species in Martian-simulated conditions

Authors: Aya Dridi, Patrycja Wąchała

Summary:

For decades, humans have dreamed of traveling beyond Earth. After landing on the Moon, the next logical step is Mars. However, Mars lacks a protective magnetic field, making its surface highly exposed to harmful cosmic and solar radiation. Data from the Mars Odyssey probe show radiation levels up to 2.5 times higher than on the International Space Station, with peaks during solar events reaching up to 2,000 millirads in a single day.

To protect future astronauts, scientists are now looking beyond synthetic shielding materials—and toward biology. One surprising candidate is a fungus: *Cladosporium sphaerospermum*, known for growing in high-radiation environments like the Chernobyl Exclusion Zone. Remarkably, it also survived and grew aboard the International Space Station, possibly thanks to its high melanin content and its potential to use radiation as an energy source (a process called radiotrophy). In our project, we will simulate Martian conditions—including UV radiation, ionic radiation, low pressure, and Martian soil—to test how this fungus survives and whether melanin plays a key role. We will also explore whether it can act as a **pioneer species**, modifying Mars-like soil to support the growth of other microbes like cyanobacteria.

Our findings could inform the development of biological shielding systems or early life support tools for Mars missions—and even help explain whether life could exist on the Red Planet under such extreme conditions.

1) Scientific goal of the project

While radiation studies on Earth have focused mainly on gamma and X-rays (Shahbazi-Gahrouei et al., 2013), the Martian surface presents a different challenge. The absence of a global magnetic field and a thin atmosphere mean that Mars is exposed to intense Galactic Cosmic Rays (GCRs), Solar Energetic Particles (SEPs), and unfiltered UV radiation (Hassler et al., 2013; Chen et al., 2022). These conditions result in a major increase in the radiation dose — it is expected that astronauts on Mars are exposed to ~400 mSv in one year, compared to ~2 mSv on Earth (ANS, 2020).

Recent discoveries showcase that *Cladosporium sphaerospermum*, a melanized fungus found in high-radiation zones like Chernobyl, not only survives but may even thrive under radiation, potentially using it as an energy source ("radiotrophy") (Averesch et al., 2022). Its unique feature offers a promising opportunity to explore biological strategies for space radiation protection.

This project is an attempt to simulate Martian conditions (UV and cosmic exposures, atmosphere, temperature) to investigate the biological mechanisms behind *C. sphaerospermum's* resilience and radiotrophic potential. Specifically, we will explore the role of melanin in protecting cellular structures, the fungus's impact on regolith properties

in Martian-like conditions and explore whether its colonization facilitates subsequent growth of cyanobacteria as secondary colonizers.

In our project, we want to answer a main question:

Q1: Can *C. sphaerospermum* act as a pioneer species on Mars-like regolith, initiating biological changes that support further microbial colonization?

Based on the previously announced question, we hypothesize that:

H1: *C. sphaerospermum* can survive and grow in Martian-simulated conditions (I)

H2: *C. sphaerospermum* alters the physicochemical properties of the regolith, creating a more habitable substrate (II)

H3: Substrates colonized by *C. sphaerospermum* are more supportive of secondary microbial colonizers, such as cyanobacteria (III)

2) Significance of the project

The human race is on the cusp of becoming a multi-planetary species. The planet Mars is considered the most suitable candidate for this expansion due to its geological features, past evidence of water, and relatively accessible surface conditions in comparison to other planets or moons. The colonisation of Mars is a concept that has been demonstrated to offer a solution to the long-term survival of the human species. Furthermore, it has been shown to present opportunities for the acquisition of new scientific knowledge and technological innovation. Nevertheless, the most significant impediments to the process of colonization are radiation, low temperatures, unbreathable atmospheres, and a scarcity of fertile soil. The majority of proposed life-support systems for Mars are dependent on substantial infrastructure and imported resources, both of which are costly and logistically challenging.

The success of this fungal strain could lead to its integration as a critical component of early-stage bioregenerative life support systems on Mars. The potential applications of this material include bioremediation of Martian regolith, pre-conditioning the soil for other life forms (e.g. photosynthetic cyanobacteria), and even serving as a bioshield for critical systems and habitats. It is posited that, in the longer term, an understanding of its adaptation strategies may result in a range of biotechnological applications, including the development of radiation shielding materials and new forms of bioenergy. The project's emphasis on an organism that has already demonstrated its capacity to withstand extreme radiation and spaceflight provides a pragmatic, biology-based approach to addressing the fundamental challenges of Martian exploration.

The aspiration to inhabit extraterrestrial environments is driven by a combination of curiosity and necessity. The Earth, while exhibiting a unique capacity for habitation, is confronted with mounting challenges, including climate change, biodiversity loss, and geopolitical instability. The expansion of human civilisation to other planets is a long-term strategy which may ensure the survival of the human race. In consideration of the factors relevant to this study, it is the contention of the present study that Mars is the most realistic candidate for such expansion. This is due to the fact that it is relatively close, has seasons, polar ice caps, and signs of past water flow, and may even harbor subsurface

water at the present time (Bhardwaj et al., 2022). The colonisation of Mars is not merely a matter of survival; it is also a quest for progress. It is hypothesised that this will drive innovations in biotechnology, sustainability, and closed-loop life support systems. Ultimately, it may assist in the understanding of the origins of life on Earth and elsewhere in the universe.

3) Concept and work plan

We based our work on several preliminary findings as Dadachova et al. (2007) demonstrated that *C. sphaerospermum* is a resisting species to radiation due to its melanin content. Zhdanova et al. (2004) demonstrated that melanized fungi exhibit directional growth in the direction of radiation resource, suggesting the radiotrophic role of this fungal species. More recent research reported that *C. sphaerospermum* measurable colonization of Mars-simulant soils under earth atmospheric conditions (Shunk et al., 2021).

First, our plan is to start with a survival assessment (I) during which we will test *C. sphaerospermum* survival across varied simulated Martian conditions in a Mars simulation chamber which we will be building ourselves in collaboration with Faculty of Physics, Astronomy and Applied Computer Science.

Second, we will be assessing the regolith potential modifications (II) via analyzing the physicochemical changes in colonized regolith samples under various Martian conditions using a variety of measuring techniques such as pH measurement, organic carbon accumulation, and nutrient availability among others that will be more explained in the following part –research methodology. We are also considering testing purified melanin to determine its direct effects without the living organisms.

Third, following the potential results of both the first and second phases of this project, we will be testing the effect of *C. sphaerospermum* on a secondary colonizer (III). We will evaluate the potential of fungal-modified regolith in supporting secondary colonization by cyanobacteria, in a first step. Three different cyanobacterial species with pre-known extremophile properties will be cultivated on regolith samples previously colonized by *C. sphaerospermum*. In a second step, we will compare both direct co-culture by inoculating both of the fungi and cyanobacteria and sequential colonization methods by introducing the cyanobacteria after establishing fungal growth in order to determine optimal establishment conditions. In this part of the research, we will analyze and determine both the potential symbiotic relationships between the fungus and cyanobacteria and synergistic effects that might enhance the resilience of the bacteria in Martian environment.

In the last part of the following project, we will integrate all our findings to develop optimal Martian conditions for fungal-cyanobacterial consortium in order to refine environmental parameters and inoculation protocols to maximize biomass production and regolith modification rates. Furthermore, we will create a mathematical model predicting terraformation timeline and resource requirements accounting for various Martian regional conditions in collaboration with the computational Biophysics and Bioinformatics department, and finally we will submit the final report with recommendations for Mars potential mission applications that highlights *C. sphaerospermum* as a biological catalyst for establishing initial life support systems on the Martian surface.

Division of responsibilities:

PIs: project coordination and design, oversight of experiments and hypothesis refinement, data analysis and interpretation. **Lab technician:** preparing regolith simulant and media, maintaining chamber operations, daily growth checks, assisting in measurements and sampling. **External partners:** supporting with Martian simulation chamber and chemical analysis (Faculty of Physics, Astronomy and Applied Computer Science), developing mathematical model (Department of Computational Biophysics and Bioinformatics).

Table 1. Gantt chart for the timeline of the project.

Task	Year 1				Year 2				Year 3			
Pilot setup, fungal adaptation and baseline												
Full Martian simulation and substrate impact												
Microbial succession experiments												
Analysis												
Mathematical model												
Writing papers												
Conferences												
Popularisation, dissemination												

Obraz zawierający tekst, zrzut ekranu, linia, kwadrat Zawartość wygenerowana przez sztuczną inteligencję może być niepoprawna.

Risk assessment:

Our risk analysis has identified several potential challenges that require mitigation strategies. One of the most recurrent problems that we might experience with the simulation chamber is the appearance of contaminants, for which we will work to maintain the decontamination of the experimental chamber by applying rigorous sterilisation protocols. Another challenge could be that the fungi may not show sufficient growth or survival under simulated Mars conditions. In this case, the project will shift to evaluating the functional properties of fungal-derived materials, such as melanin-rich extracts or inactivated biomass (Plan B). These extracts can still be tested and used to answer our hypotheses for the second and third parts of this project (H2 and H3). This Plan B still answers key questions regarding the biotechnological potential of fungal components in space environments and can provide publishable data even in the absence of viable growth. The limited colonisation and modification of regolith is also a major concern. This can be countered by modifying the water content, adding minimal nutrients or pre-conditioning the regolith, with the ability to test and select different types if required. To mitigate the risk of cyanobacteria failing to grow on colonised substrate, we will use three different strains to maximise the chances of success. If there is no measurable physicochemical change in the substrate, we can test its interaction with more reactive regolith simulant types. There is also the possibility of equipment failure in the Mars simulation chamber, although this is very unlikely to occur but would have a major impact. Such issues will be addressed by scheduled regular calibration and access to back-up equipment to ensure continuity.

4) Research methodology

Materials

We will be utilizing in our study various biological and regolith materials. The biological materials included both melanized wild-type and non-melanized albino mutant strains of *Cladosporium sphaerospermum*, with the latter produced through gene knockout sing the CRISPR/cas technique. For secondary colonization, we selected three cyanobacterial species: desert-adapted *Chroococcidiopsis sp*. CCMEE 029, nitrogen-fixing *Anabaena cylindrica* UTEX B629, and halotolerant *Synechococcus sp.* PCC 7002. We also employed purified fungal melanin extracted using acid hydrolysis. Our regolith materials consisted primarily of JSC Mars-1A Mars regolith simulant, complemented by MMS-2 (Mojave Mars Simulant) for comparative analyses.

Setting of the Mars environment simulation chamber (MESC)

In order to simulate Martian conditions, a vacuum chamber equipped with a radiation source and a CO_2 supply will be utilised. A cooling table will be connected to the cooling unit and installed inside the chamber. The temperature and humidity will be regulated by sensors installed within the chamber and connected to a computerised control system. The radiation will be regulated by external controllers. The pressure within the chamber will be regulated by a vacuum pump. Fungi samples will be attached to the metal grid, with the radiation source positioned at a distance of 35 cm perpendicular to this.

Different conditions will be tested (check table 2 for more details).

Table 2. Different conditions tested in the Martian simulation chamber.

Parameter	Mars simulation condition
Atmospheric Pressure	6-10 mbar (0.6-1.0 kPa)
Gas Composition	95% CO_2, 2.7% N_2, 1.6% Argon, traces of O_2 and H_2O
Temperature	-80°C to +20°C
UV Radiation	High UV flux (280-400 nm) with minimal atmospheric filtering
Solar/Cosmic Radiation	~0.2–0.7 mSv/day (much higher than Earth)
Desiccation	Very low humidity (~0-10% RH): extreme dry conditions
Freeze-Thaw Cycles	Rapid temperature fluctuations
Gravity	0.38g (38% of Earth's gravity)

We will compare the results from both wild-type (melanized) and knock-out mutant strains to determine melanin's protective role of the fungus and its effect on its growth. As a substrate, we will use Certified Martian Regolith Simulant (MMS-2).

H1: To test whether *C. sphaerospermum* can survive and grow on regolith with minimal organic nutrients, we will inoculate sterilised Martian regolith simulant with fungal spores and then incubate under simulated Martian environmental conditions for 7, 14 and 21 days. Then, we will monitor growth via biomass yield, analyse visual colony coverage using image analysis and conduct a survival assessment.

H2: To assess changes in physicochemical properties of regolith after fungal colonization we will measure regolith pH, electrical conductivity (EC) and soluble minerals (e.g. phosphorus, magnesium) and compare inoculated and uninoculated controls.

H3: To evaluate whether fungal-treated regolith supports colonization by cyanobacteria we will use common cyanobacteria strains. We will inoculate them onto: a. untreated regolith, b. fungal-colonised regolith and c. extract-colonised regolith (plan B) for 7, 14 and 21 days. Then we will assess photosynthetic activity via chlorophyll *a* extraction and fluorescence. We will monitor biomass growth via dry weight and microscopy.

Plan B: If fungal growth is insufficient, we will prepare extract from *C. sphaerospermum* biomass and apply extract to regolith simulant as a "treatment layer". Then we will act similarly as in H2 and H3.

Fungal and co-culture preparation

The preparation of fungal cultures involved maintaining *C. sphaerospermum* stock cultures on Potato Dextrose Agar (PDA) at 25°C. Each suspension will be prepared in triplicate to ensure consistency and verified using fluorescein diacetate (FDA) staining, with 200 spores counted per sample across five replicates to establish baseline viability percentages. The inoculum density was standardized spectrophotometrically at OD_{600} = 0.5, with measurements taken from ten independent cultures to account for potential variability (Averesch et al., 2022).

Cyanobacterial cultures will be harvested during the exponential growth phase and resuspended in modified BG-11 medium and standardised by chlorophyll a concentration (10 µg Chl-a/mL) to acclimate to Martian conditions, with osmolarity adjusted to match the fungal suspension (Macário et al. 2022).

Fungal strain cultivation for comparative assays

We propose to generate knockout mutants of *C. sphaerospermum* (ATCC 11288). We will use the CRISPR/Cas technique, which uses the Cas protein to modify targeted sequences using artificially generated sgRNA (guide RNA), which guides the Cas9 protease to effectively cut the double strands of DNA and form double-strand breaks. The dihydroxynaphthalene (DHN)-melanin gene biosynthesis in *C. sphaerospermum* will be disrupted by targeting the gene encoding polyketide synthase (PKS) using BLAST, a key enzyme in the pathway homologous to the gene that has been previously genomically encoded in annotated strain UM843 and is conserved across other *C. sphaerospermum* strains (Yew et *al*., 2016). sgRNAs will be designed to target conserved early exons in the PSK gene. A fungal-compatible CRISPR/Cas9 expression plasmid will be constructed, incorporating Cas9, the sgRNA, and a selectable marker (hygromycin resistance [hph]). Fungal transformation will be carried out using PEG-mediated protoplast transformation, followed by selection on hygromycin-containing media. Mutants will be screened by PCR and confirmed via Sanger sequencing. Phenotypic validation will be depicted by the loss of pigmentation on PDA plates using spectrophotometric quantification of melanin. We will then cultivate around 20 biological replicates of each strain, the original wild-type strain, and the CRISPR/Cas9-generated knockout strains separately under identical conditions for the following analysis.

Mars Simulation Chamber Experiments (MSCE)

For the simulation experiments, we will prepare 5 cm × 5 cm regolith beds (2 cm depth) in sterile Petri dishes, with a total of 120 individual samples to accommodate all experimental conditions and replicates. Each regolith sample will be inoculated with 2 mL of

standardized spore suspension. The samples will be introduced into the MESC under various conditions including baseline conditions (-60°C/+15°C diurnal cycle, 7 mbar pressure, 95% CO_2 atmosphere, 8 hours daily UV exposure at 10 W/m^2), temperature variants, pressure variants (3 mbar, 7 mbar, and Earth atmospheric pressure as control), radiation variants (UV-shielded, UV-exposed at 10 W/m^2, cosmic radiation), and moisture variants (dry at <1% w/w, low moisture at 5% w/w, moderate moisture at 10% w/w). For each condition, we plan to prepare six replicates, to maintain the experiments for 90-day cycles. Parallel experiments with 30 samples each of wild-type and knockout strains would be conducted for comparison across all environmental parameters.

Survival assessment methods

Survival assessment will be performed using colony-forming unit (CFU) enumeration every 15 days by removing small samples (0.5 g) from the MESC, with three subsamples taken from each of the six replicates per condition to assess the strains viability. Metabolic activity assessment included FDA hydrolysis assay for esterase activity, and ATP quantification using luciferin-luciferase bioluminescence assay, with each assay performed in quadruplicate on all samples in order to measure cellular activity in stressed cells. Genomic integrity was assessed through DNA analysis to assess genetic damage and repair mechanisms. These methods will collectively determine how the fungus, focusing mainly on its byproduct: melanin responds to Martian stressors including radiation, extreme temperatures, and desiccation.

Growth assessment

In order to check whether *C. sphaerosporum* is able not only to survive but also to grow under Martian conditions (which would support the hypothesis that it converts radiation into energy for growth), we will dry the melanin and non-melanin samples and then weight the biomass.

Physicochemical analysis

For physical properties, we will measure particle aggregation using laser diffraction and wet sieving tests to detect soil structure changes. Porosity and water retention capacity measurements will reveal changes in regolith's ability to support water movement and retention. As for the chemical modification assessment, we will track the changes in colonized regolith through pH and electrical conductivity monitoring. Nutrients alteration will be assessed via ion chromatography and inductively coupled plasma mass spectrometry (ICP-MS).

Growth and interaction assessment

We will investigate interactions between fungal primary colonizers and cyanobacterial secondary colonizers in Mars regolith simulants. Four treatment groups will be established: non-inoculated controls, plus regolith with recent (2 weeks), moderate (2 months), and extensive (4 months) fungal colonization. All groups will receive standardized cyanobacterial inoculations and undergo different Mars simulation gradient with progressively intense conditions.

Growth assessment will include first chlorophyll-a quantification by extracting this photosynthetic pigment from cyanobacterial cells using methanol or acetone, then

measuring concentration spectrophotometrically. This test will provide quantification of the biomass. Second, we will apply oxygen evolution monitoring in order to track oxygen production during light periods and consumption during dark periods, and nitrogen fixation measurement via acetylene reduction assay. Furthermore, metabolic interactions will be investigated. We will employ isotope labeling (^{15}N and ^{13}C) to track nutrient exchange, perform targeted metabolomic analysis to identify shared compounds and signalling molecules using chromatography-mass spectrometry, and use RT-qPCR to assess stress response and symbiosis-related gene expression. These approaches will demonstrate whether fungal colonists create protected microenvironments that enable cyanobacterial survival and growth, supporting our main question that fungi could serve as pioneer species for Mars terraforming.

Statistical analysis

H1: two-way ANOVA to assess the effects of substrate nutrient level and time on growth, post-hoc Tukey's HSD for pairwise comparisons, regression analysis to model growth trends over time.

H2: one-way ANOVA for comparing inoculated vs. uninoculated regolith, multivariate analysis (MANOVA) to analyse multiple dependent variables (pH, EC, minerals), PCA for visualising how samples cluster based on physicochemical properties.

H3: T-test for comparing cyanobacterial growth on treated vs. control substrates.

If normality and homogeneity of variance will not be met, non-parametric tests will be used (Kruskall-Wallis, Mann-Whitney U).

Mathematical model

To estimate the long-term terraforming potential of *C. sphaerospermum*, we will collaborate with a Department of Computational Biophysics and Bioinformatics. Using our experimental data (e.g., fungal growth rates, substrate modification effects, and compatibility with cyanobacteria), we will parameterize a simplified mathematical model predicting colonization dynamics under varying Martian environmental conditions. The model will simulate regolith bio-conditioning timelines, resource requirements (moisture, nutrient input), and potential scalability. This step will be completed in the final project year and supported by environmental datasets from Mars missions (e.g., NASA, ESA). Outcomes will inform final recommendations for potential applications in mission planning.

Dissemination and popularisation

To ensure the broad impact of the project, we will implement a multi-tiered dissemination plan including publication in peer-reviewed journals (examples: *Life Sciences in Space Research*, *International Journal of Astrobiology*) presentation at international astrobiology and microbiology conferences (examples: *AbGradCon (Astrobiology Graduate Conference)*, *Astrobiology Science Conference (AbSciCon)*), and open-access sharing of results. For science popularisation, we will engage with the public through social media, university science communication outlets, and participation in outreach events like Researchers' Night. Educational materials and workshops for secondary school students will also be developed, fostering early interest in microbiology and space sciences.

5) Composition and qualifications of the research team

Principal Investigator 1: Aya Dridi – PhD student who holds a biochemistry master's diploma, with hands-on experience with chromatography methods, including gas chromatography–mass spectrometry (GC-MS) and Thin-Layer chromatography technique (TLC), which makes her well-suited for the biochemical profiling components of the following project.

Principal Investigator 2: Patrycja Wąchała - PhD student in the Biology Programme, Master of Science (biology); experience in statistics (SPSS, Statistica, R) and coding (R); experience in writing manuscript and presenting at conferences (Studencka Konferencja Teriologiczna); experience in designing and directing student scientific projects as a President of Students Naturalists Association for 2 years.

6) Project literature

- ANS (2020). *Radiation Dose Calculator. American Nuclear Society*. Available online at: https://ans.org/pi/resources/dosechart/msv.php (accessed April 12, 2025).
- Averesch, N. J., Shunk, G. K., & Kern, C. (2022). Cultivation of the dematiaceous fungus Cladosporium sphaerospermum aboard the International Space Station and effects of ionizing radiation. *Frontiers in microbiology*, *13*, 877625.
- Bhardwaj, A., Sam, L., Buchroithner, M. F., & Galofre, A. G. (2022). Advances in Mars research and exploration. *Frontiers in Astronomy and Space Sciences*, *9*, 971104.
- Chen, J.-L., Yun, S.-J., Dong, T.-K., Ren, Z.-Z., & Zhang, X.-P. (2022). Studies of the radiation environment on the Mars surface using the Geant4 toolkit. *Nuclear Science and Techniques*, *33*(1),
- Hassler, D. M., Zeitlin, C., Wimmer-Schweingruber, R. F., Ehresmann, B., Rafkin, S., Eigenbrode, J. L., … Bohm, E. (2013). *Mars' Surface Radiation Environment Measured with the Mars Science Laboratory's Curiosity Rover. Science, 343(6169), 1244797–1244797.*
- Shahbazi-Gahrouei, D., Gholami, M., & Setayandeh, S. (2013). A review on natural background radiation. *Advanced Biomedical Research*, *2*(1), 65.
- Yew, S. M., Chan, C. L., Ngeow, Y. F., Toh, Y. F., Na, S. L., Lee, K. W., Hoh, C.-C., Yee, W.-Y., Ng, K. P., & Kuan, C. S. (2016). Insight into different environmental niches adaptation and allergenicity from the Cladosporium sphaerospermum genome, a common human allergy-eliciting Dothideomycetes. *Scientific Reports*, *6*(1), 27008.
- Zhdanova, N. N., Zakharchenko, V. A., Vember, V. V., & Nakonechnaya, L. T. (2004). Fungi from Chernobyl: mycobiota of the inner regions of the containment structures of the damaged nuclear reactor. *Mycological Research*, *104*(12), 1421-1426.
- Dadachova, E., Bryan, R. A., Huang, X., Moadel, T., Schweitzer, A. D., Aisen, P., Nosanchuk, J. D., & Casadevall, A. (2007). Ionizing radiation changes the electronic properties of melanin and enhances the growth of melanized fungi. PLoS ONE, 2(5), e457.

- Shunk, G. K., Gomez, X. R., Averesch, N. J., Oakley, C. A., Montgomerie, R., Delaney, N. F., Iwanicki, N. S., & Venditti, J. L. (2020). Growth of Melanized Fungi on the International Space Station and Implications for Astronaut Safety. bioRxiv 2020.07.16.205534
- Macário, I. P. E., Veloso, T., Frankenbach, S., Serôdio, J., Passos, H., Sousa, C., Gonçalves, F. J. M., Ventura, S. P. M., & Pereira, J. L. (2022). Cyanobacteria as candidates to support Mars colonization: Growth and biofertilization potential using Mars regolith as a resource. Frontiers in Microbiology, 13, 840098

7) Budget of the project

Type of cost	**Amount in PLN**
Direct costs, including:	**589 520 PLN**
- personnel costs and scholarships	330 000 PLN
- research equipment/device/software cost	225 000 PLN
- other direct costs	34 520 PLN
Indirect costs, including:	**129 694,4 PLN**
- indirect costs of OA	11 790,40 PLN
- other indirect costs	117 904 PLN
Total costs	**719 214,4 PLN**

8) Breakdown of projects costs

Category	Description	Amount (PLN)
Direct costs		
Scholarships	36 months * 2 PIs * 1500 PLN	108000 PLN
Salary	For lab technician (36 months * 4500 PLN)	162000 PLN
Salary for collaborators	Physicists and bioinformatics (2*30000 PLN)	60000 PLN
CRISPR/Cas9 Gene Editing	Cas9, sgRNA design and cloning, electroporation supplies, selection reagents	25000 PLN
Microbial material	Cladosporium sphaerospermum wild-type; Cyanobacteria (Chroococcidiopsis sp. CCMEE 029, Anabaena cylindrica UTEX B629, Synechococcus sp. PCC 7002); PDA media, BG-11, mutagenesis reagents	8000 PLN
Melanin purification materials	Solvent extraction, dialysis membranes, consumables	6000 PLN
Cell Viability Assays materials	FDA, TTC, ATP kits, culture media	12000 PLN

Category	Description	Amount (PLN)
Special Equipment for Simulated Space Conditions	Vacuum chamber – 20,000 PLN Radiation lamp – 20,000 PLN Gas supply + pressure regulation – 15,000 PLN Cooling table – 5,000 PLN Control computer – 5,000 PLN	65000 PLN
Regolith simulants & modification supplements	JSC Mars-1A, MMS-2, Fe-rich, MgSO4-supplemented, Mg(ClO4)2-supplemented variants	15000 PLN
Physicochemical analysis	Particle aggregation (laser diffraction), porosity, BET, SEM imaging, ICP-MS, TOC analyzer, XRD, FTIR, contact angle meters	40000 PLN
Secondary colonization studies	Chlorophyll quantification, acetylene reduction assay, isotope labeling (15N, 13C), metabolomics, RT-qPCR kit	20000 PLN
Statistical and imaging software	Important for the analysis of survival	5000 PLN
Other Devices	Climate chamber – 25,000 PLN Solar lamp – 4,000 PLN	29000 PLN
Others	Conferences (based at the example of AbGradCon): (accomodation 750 PLN * 4 days + diets 220 PLN * 4 days) * 2 people * 2 conferences	15520 PLN
	Miscellaneous	5000 PLN
	Consumables: Eppendorf tubes, pipette tips, PCR tubes, Petri dishes, gloves	5000 PLN
	Biohazard Waste Disposal	3000 PLN
	Shipping costs: reagents, cultures	6000 PLN
	Safety and personal protective equipment	
Indirect costs		
Publication of results (open access journals)		11 790,40 PLN

Category	Description	Amount (PLN)
Other indirect costs		117 904 PLN

Declaration

During the preparation of this work the authors used ChatGPT in order to estimate the budget and DeepL in order to improve the grammar. Perplexity was used for literature research. After using this tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the submitted proposal.

3. How to communicate with the dangerous neighbours: Testing the adaptive meaning of wing coloration patterns in Atlas moths

Team: Alice Ballabio, Adam Solecki, Antonii Bakai

Project (initial proposal)

How to communicate with the dangerous neighbours

Testing the adaptive meaning of wing coloration patterns in Atlas moths.

Authors: Alice Ballabio, Adam Solecki, Antonii Bakai

Summary

Lepidopterans show intricate and colorful patterns on their wings. This fascinating phenomenon has always tackled the curiosity of scientists and amateurs, leading to development of the idea of mimicry. We address this phenomenon by studying Attacus atlas, a giant moth from Southeast Asia, which appears to have a snake represented on the tip of its wings.

Artificial Intelligence is gaining increasing popularity in many fields, proving to be of great help especially in the scientific world, dealing with modelling and huge data manipulation. We jump on this opportunity by improving an existing modelling tool and implementing it to analyze and differentiate wing patterns.

Therefore, our project aims to explore whether the wing pattern of this moth is an important adaptation that enables predator detering, confusion or signaling to conspecifics? If so, does it work similarly for different kinds of predators or is it aimed at a selected group?

1) Scientific goal of the project

The “arms race” between prey and predators was one of the evolutionary fenomena puzzling the minds of researchers since the times Van Vallen presented his Red Queen Hypothesis (VanValen 1973). Mimicry is one of the ways how prey can outsmart the predator (Stevens, 2005). So how do you speak with your dangerous neighbour? Well if you ask a seemingly defenceless organism, like a large and nutritious moth the answer might be - you look like its worst nightmare and try not to talk at all.

Intricate and colorful patterns on lepidopteran wings are often associated with mimicry (Stevens, 2005), both Batesian - resembling other, more dangerous species (Carpenter & Ford 1933) and satiric - confusing the predator and employing it for the delay or misdirection of the attack (Howse 2013). There are also reports on the mimics that combine visual and acoustic signals for a better effect (Barber & Conner 2007; Moore & Hassall 2016).

Although this phenomenon received a lot of attention from the researchers across the years, we rarely see the study that would combine the field observation with an experimental approach. We tend to take a supposed mimicry for granted, without entertaining the other possible explanations (possibly no less exciting), and sometimes interpret the observed features as a mimicry of a given model species or group of species, without proper empirical evidence.

In this regard we would like to propose a study that combines the field observations with behavioral experiments and machine learning to investigate the functions of the shape and pattern of the *Attacus atlas* moth.

The first hypothesis is based around the assumption that the pattern displayed on the wings of *Attacus atlas* is an important adaptation and its **key features are conservative:**

1). The pattern and shape of the *Attacus atlas* wings is conservative across the range of the species with a slight, regional variance

Our second hypothesis would approach the current, observation-based consensus - **Bathesian mimicry**:

2). The pattern and the shape of the *Attacus atlas* wings is a mimicry of a snake model and it can deter predators.

The third would be constructed around the assumption that it might achieve a similar goal by different means so rather confuse and slow, than deter - **satirical mimicry:**

3). The pattern and the shape of the *Attacus atlas* wings is an example of satirical mimicry, used to confuse predators and compel them to attack non vital parts of the moth

With the fourth one we want to look at the problem from a **perspective of the moth and communication with conspecifics:**

4). The pattern and the shape of the *Attacus atlas* wings is a signal and a tool for communication, enabling recognition of conspecifics.

Finally we are going to assess the effect of the pattern on the atack **preferences of different predators in the field:**

5). Predators from different taxa and representing different feeding strategies will react differently to different kinds of wing pattern alterations or lack thereof.

2) Significance of the project

The Atlas moth *Attacus atlas* L. is a charismatic insect that attracts people's attention due to its size and peculiar wing pattern. Inhabiting the mature growth () it can serve as an umbrella species for nature conservation. Despite the attention it gets and the potential for economic significance (Kumara & Kumar 2022), its biology is not sufficiently studied. For instance, this moth is speculated to use its wing pattern resembling venomous snake as a defense mechanism against predators (Howse 2013; Kumara& Kumar, 2022), but no experimental evidence has been provided so far. It is also unclear whether possible

defensive mimicry works on Batesian or satyric basis. With our noninvasive approach, based on the archival and commercially available specimens, we are introducing new ways to conduct studies of mimicry, and behavioral biology while reducing our disturbance of the studied ecosystem. The only interference we will introduce to the environment will be the presence of our artificial moth models.

Despite such limitations this study will enhance our understanding of the evolution of complex visual patterns, mimicry and their behavioral and ecological significance, as well as for the development of accurate tools that would enable fine scale analysis of visual patterns in nature.

Artificial intelligence can play a crucial role in the exploration of the complexity of the wing pattern of Attacus atlas. Convolution neural networks (CNNs) have gained much popularity nowadays because of their ability to scan through many images and extract the main features to further classify said images according to a general trend (Kavi et al, 2020). CNNs have been used in the medical field to recognize cancerous cells or to classify different cell types with accurate precision and a relatively easy workflow (Schouten et al, 2021; Tsuneki, 2022). Our model aims to adapt the CNN model -used by Schouten et al- to perform pattern recognition and analysis of Attacus atlas wings. It will be able to extract the main features of the complex multicolored wing pattern and establish whether a common fashion among different populations is present.

The potential of the CNN model is not limited to our project but can be extended to other lepidopterans or even other taxa for species detection and visual pattern analyses. Also, with our usage of museum collections and growing digitalisation of those resources we are creating the tool that will increase its value and potential overtime, with each pattern added to the database.

3) Concept and work plan

To test our hypothesis, we developed a range of subsequent experiments, with the crucial assumption of the conservativity of the key components of the pattern. The research will be conducted as follows:

1) Assessment of the variability of the wing pattern in the Atlas moth photos originated from museum collections and the natural environment using machine learning tools, and compare them to moths obtained from breeders. If the breed individuals have the main pattern preserved and are not significantly different from the wild individuals, they will be used for laboratory experiments. Otherwise we will have to catch the wild moth and breed them to have sufficient test subjects or replace the living moths with dummies in our laboratory experiments (3) - hypotheses I.

2) Field experiment with usage of 3D-printed edible moth dummies and camera traps directed to test native predator behaviour toward our prey models (hypotheses II, III and V). The tropical habitat of the Atlas moth does not limit us with the seasonality, so the field work can have a plastic schedule. Given that it is a field observation, we may also observe conspecific recognition, that we can use as additional data in testing the IV hypothesis.

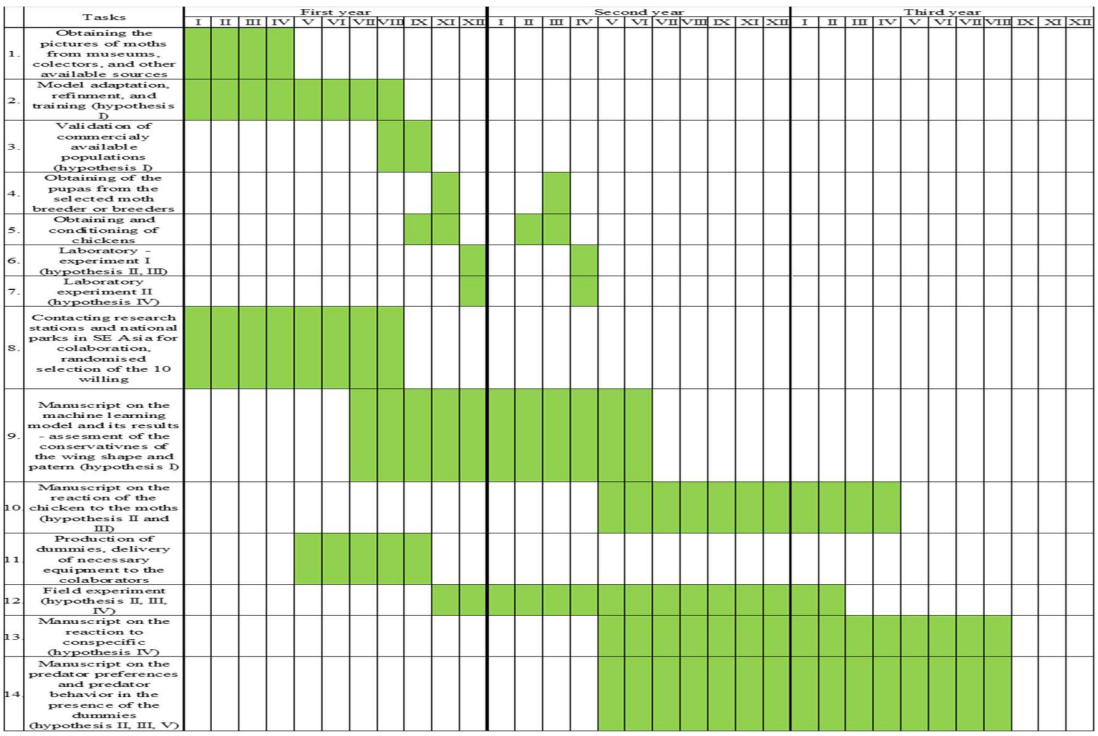
3) Laboratory experiment in which the chickens will be used as the predators. The moth (originated from a farm or the 3D-printed model) will be used to test whether wing pattern and will influence a) a predator behavior (scared/discouraged/alarmed/neutral/curious) -

hypothesis II; b) time of naive - hypothesis III and conditioned predator attack - hypothesis II; c) direction of the attack (wing margins/wing center/thorax and abdomen) - hypothesis III. For this experiment the moth-chicken pair will be divided into four groups: moth with integral wing pattern +conditioned chicken; moth with integral wing pattern + naive chicken; moth with altered wing pattern (painting in neutral color) + conditioned chicken;moth with altered wing pattern + naive chicken.

4) Laboratory experiment in which wing shape and pattern recognition will be tested by exposure of the male moths to the dummies with integral or disrupted pattern or shape of the wings and evaluation of their interactions (interested/ignorant/interaction) - hypothesis IV . .

During the first year of the project our team will focus on testing the variation of the wing pattern and the first field experiment (collaboration with national parks//research station in SE Asia). We predict that the survey of potentially available collections and the photos database arrangement will take around two to four months. Simultaneously we will be working on the machine learning model and artificial mimic design. The model development and testing will take around eight months. In the meantime we will contact the national parks and research stations to establish collaborations for the ongoing field experiment. The ideal goal is to have 10 locations in SE Asia. We are planning to distribute the two question surveys in all possible research sites (national parks and research stations), asking if they know that *Attacus atlas* is present in their area and if they are willing to participate in the experiments (for a fee or participation in field based papers). From those willing to collaborate we would randomly choose 10 locations (with the limit of 3/country). As soon as we have our wing pattern results we will use them to complete artificial mimics designs with apropriate patterns (based on the local variants if we found them during picture analysis or generalised if we found the pattern to be very conservative) . The mimics and other necessary tools will be passed to the local personnel to set the experiment, as we will rely on the local experts (field guides and entomologist) for the determination of the best time and locations. The experiment will last for 14 days: 2 days to organize and set the experiment, 10 days of observation, as this is the average lifespan of the imagos, and an additional two days to fold the field experiment and it'll be repeated in the next month or later if local collaborators decide that the conditions are not favorable we expected that both replicates are going to be done in the span of 16 months across all experimental locations. After the field experiment (or during, in case it will have to be delayed) we will start the laboratory experiment, again with two replicates to test **II, III and IV hypotheses**. Preparations such as setting the equipment, conditioning the chickens and obtaining imagos will approximately take us two months, and the experiment itself will be conducted during the following month. We will also be working on the manuscript about machine learning for publication.

At the middle of the second year we're expecting our manuscript about machine learning to be ready for publication. The next eight months of the project's second year we will focus on the manuscripts based on data from our predation lab experiment (II and III hypotheses), conspecific recognition lab experiment (IV hypothesis) and the predator preference and behaviour in the presence of mimics field experiment (II, III and V hypotheses).T



According to the scheduling of the first experiment, we will replicate the same one a year after.

The third year of the project will be used to polish the manuscripts. Project schedule is presented in the Gant chart below:

During the project we expect a range of possible risks and complications connected with the data obtained:

1. Too high variability of the wing pattern. If this occurs, either it will require more time to train the model (the amount of pictures to train it could be insufficient) or it would be harder to assess the proper mimic pattern for experiments, in that case we will develop the regional variants of the model based on the selection of experimental sites which will be reduced to eight or five depending on the feasibility.

2. Shortage of museal samples. In that case we will compensate for the lack of data with photos from other sources such as wild individuals and private collections. If the captivity bred moth is not representative comparingly to museal records, we will be forced to use dummies intended for the field experiment.

3. Unexpected weather conditions might pose a challenge to conduct the field experiment, but as the experiment is short and not limited by strict seasonality, - we can delay it until suitable weather occurs or with the observations of imagos in each experimental location.

4) Research methodology

To test the first hypothesis, we will adapt a pre-existing convolution neural network (CNN) used in leucocyte recognition to wing pattern recognition, modifying it to be more efficient

in pattern, color and contrast discrimination. The model will be trained with images provided by museums and private collectors from different countries (India, Brunei, Malaisia, Thailand, Indonesia, Great Britain, Germany, USA, the Netherlands) and assess the pattern similarity among different populations of the same species. Then, the model will be used to define the similarity of the pattern in the wings of the commercially available moth to those originated from the natural environment and define whether they are applicable for laboratory experiments.

The second hypothesis is going to be tested in a laboratory setup. The experiment involves conditioning chickens to avoid snakes and subsequently verify if they avoid the moth once exposed to it. The chickens will be aversive conditioned by exposing them for 5 minutes to air puffs and presenting at the same time pictures of different SE Asian snakes. The conditioning occurs for 23 training sessions one hour apart from each other, as described in du Plessis et al, 2021. After the training, the moth is positioned in the chicken cage, and we check and record the reaction of the chicken. For the experiment, we will alter the wing pattern in 24 of the moths, by spraying uniform paint on its entire upper surface. That way we will have two types of moth imagos, - one with normal wing pattern and the second with the altered. Each moth type will be exposed to conditioned chickens and unconditioned ones, making it four treatments, 12 pairs (moth+chicken) each, having in the end 48 observations, we will perform 6 simultaneous trials. We are going to replicate the procedure after approximately a month with different animals. At the same time, we test the third hypothesis - the eventual presence of satirical mimicry, by measuring the time response of the chicken to the moth: if it has a statistically significant delayed response, the chicken is startled. The chicken is also allowed to attack the moth for the period of 30 s. from the first contact.feast on the moth, and The place of the attack will be analyzed to confirm the type of mimicry: if it occurs on the pattern, it may mean that the chicken is startled.

Exploration of the third hypothesis, the determination of whether the terminal part of *Attacus atlas* a distraction for predators from the vital part of the moth is, is also donedone also in the field. A survey is prepared to find collaborators in national parks and research stations with a stable atlas moth population in their areas. Moreover, we prepare four different types of edible dummies, which will show the preferred bite spot of predators. After assessing the areas for the experiment, we place 48 edible dummies in 10 field locations to check for bite marks. The dummies are produced by a 3D printer from edible polymers by our team.

We planned four types of dummies (12 per type):

- Normal pattern (as the model assessed) and normal shape
- Normal pattern and disrupted shape (without the wing appendages that supposedly resembles the snakes head)
- Disrupted pattern (neutral coloration) and normal shape
- Disrupted pattern and disrupted shape

The experiment will be repeated twice. The placement of dummies in the field is semi-random, dummies are going to be distributed on four most common host plants in each

area. We also provide 9 camera traps that are going to record the predators approaching the dummies and natural behavior of *Attacus atlas* in its native environment (2 camera traps/treatment + additional one for the eventuality in which we are able to find a female *Attacus atlas* and set the camera trap so it can observe it for reference).

The fourth hypothesis revolves around the idea that patterns on the wings of our moth is a signal for conspecifics. To tackle this, we prepare a laboratory experiment in which we expose male moths to dummies in four different conditions as in the field experiment. We plan to present each variation of the dummy to 12 male moths and replicate the experiment after a month with different animals (the moths are going to be used with the chicken experiment later on), we are planning for six simultaneous trials.

The final hypothesis is set around the assumption that the pattern or shape of *Attacus atlas* wings will affect different taxa of predators differently. For that we are going to analyze the bite marks on the dummies from the field experiments and support our findings with the camera traps materials (the amount of camera traps was reduced for feasibility of analyses)

5) composition and qualifications of the research team,

Alice Ballabio- PIs: expertise in neural network modelling, trained models of insect learning behavior (trace conditioning modelling) and large data manipulation.

Adam Solecki - PIs: 10 years of experience with field, animal related studies (mostly fauna diversity, distribution and monitoring), including an experiment with a moth larvae model used for predation assessment in relation to hiding/feeding strategy, experience with field studies in the tropics (Danum Valey in Sabah State, Malaysia).

Antonii Bakai - PIs: experience in field, animal related studies, GIS tools, qualified for work with laboratory animals

6) Project literature

Schouten, J.P.E., Matek, C., Jacobs, L.F.P. *et al.* Tens of images can suffice to train neural networks for malignant leukocyte detection. *Sci Rep* 11, 7995 (2021). https://doi.org/10.1038/s41598-021-86995-5,

source code: https://github.com/marrlab/ClassificationALL

Asayuki Tsuneki, Deep learning models in medical image analysis,Journal of Oral Biosciences, Volume 64, Issue 3,2022, Pages 312-320, ISSN 1349-0079, https://doi.org/10.1016/j.job.2022.03.003.

Kavi B. Obaid, Subhi R. M. Zeebaree & Omar M. Ahmed (2020). Deep Learning Models Based on Image Classification: A Review. International Journal of Science and Business, 4(11), 75-81. doi: https://doi.org/10.5281/zenodo.4108433

Yadav, S.S., Jadhav, S.M. Deep convolutional neural network based medical image classification for disease diagnosis. *J Big Data* 6, 113 (2019). https://doi.org/10.1186/s40537-019-0276-2

du Plessis EW, Beausoleil NJ, Bolwell CF, Stafford KJ (2021) Validation of a combined approach-avoidance and conditioned stimulus aversion paradigm for evaluating aversion in chickens. PLoS ONE 16(2): e0247674. https://doi.org/10.1371/journal.pone.0247674

Barber J.R., Conner W.E. 2007. Acoustic Mimicry in a Predator–Prey Interaction. *Biological Sciences,* 104: 9331–9334. https://doi.org/10.1073/pnas.0703627104

Carpenter G.D.H., Ford E.B. Mimicry. London: Methuen, 1933.

Howse P.E. 2013. Lepidopteran Wing Patterns and the Evolution of Satyric Mimicry. *Biological Journal of the Linnean Society,* 109: 203–214. https://doi.org/10.1111/bij.12027

Moore C.D., Hassall C. 2016. A Bee or not a Bee: an Experimental Test of Acoustic Mimicry by Hoverflies. *Behavioral Ecology,* 27: 1767–1774. https://doi.org/10.1093/beheco/arw107

Van Valen L. 1973. New Evolutionary Law. *Evolutionary Theory*. 1: 1–30.

Ravi Kumara, R., & Kumar, H. (2022). The Fagara Silkworm (Attacus atlas L.): An underutilized Vanya Silkworm of India. *Journal of Biodiversity*, *8*(5), 1-11.)

Bhawane, Ganesh & Mamlayya, Amol & Koli, Yogesh & Phonde, Y & Aland, Santosh & Gaikwad, Sunil. (2011). LIFE HISTORY OF ATTACUS ATLAS LINN. (SATURNIIDAE: LEPIDOPTERA) ON SAPIUM INSEGNEBENTH. FROM WESTERN GHATS, MAHARASHTRA. The Bioscan. 6. 497-500.

Stevens M. The role of eyespots as anti-predator mechanisms, principally demonstrated in the Lepidoptera. Biol Rev Camb Philos Soc. 2005 Nov;80(4):573-88. doi: 10.1017/S1464793105006810. PMID: 16221330.

7. Budget for the project

	Amount in PLN
Direct costs, including	1628232
-personnel costs and scholarships	10000x3x36 (1080000) 330000x1 - Senior data scientist
-research eq uipment/devise/software cost	• 9000 3d printer • 50x1000 mimics (50000) • camera traps 90x500. (45000) • chicken 30x120 + food 3600 (7200) • Moth 100x150 + containers 150x3. • 13000 computer setup , • average of 9000 for gathering 200 images from museums and national parks.

	Amount in PLN
	• Conditioning setup: 90 x 6 chamber with separation + 70 x6 air puff canister (1050)
-other direct costs	delegations abroad 23000x3 (69000)
Indirect costs, including	79850
-Indirect costs of OA	2000
- other indirect costs	publications fee 7550+6600+10700+8000; conference cost host 21000, visit 8000x3; deliver tools 200x10
Total costs	1708082

Reviews

Aya Dridi

Grant proposal review

Title of the project: How to communicate with the dangerous neighbours: Testing the adaptive meaning of wing coloration patterns in Atlas moths.

1. **Assessment of scientific quality of the research project** (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)

This project is innovative and has international importance as it addresses several evolutionary questions in regards to the adaptive significance of wing coloration in *Attacus atlas*, particularly the concept of satirical mimicry, which has not been widely studied in moths. The conception of this project is original as it combines AI based concepts such as the convolutional neural networks (CNNs) to model the wings perception of patterns to predators with the 3D technology for the conception of dummies (a non-invasive methodology) to mimic real moths in field experiments (for response measurement of the predators). It tackles also the behavioral experimental aspect. The integration of such multi-faceted aspects (AI with traditional experimental biology) is what makes this project promising.

2. **Assessment of potential impact of the research project** (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)

This project presents the potential of making a substantial international impact in multidisciplinary fields. First by understanding the mimicry and coloration patters of *Attacus atlas* wings, such findings can be highly relevant in the ecology and evolutionary biological fields, helping researchers understand animals' adaptation to their environment and predation avoidance. Furthermore, combining behavioral experimentation and field investigative studies can reveal valid information about how visual cues influence

predation risk and help in understanding the natural selection behind coloration in certain species. The integration of CNNs and machine learning tools in the study of pattern recognition open huge doors for AI use in analyzing complex biological data. Lastly, the invention of edible moth dummies could be a very promising application for future research.

The interdisciplinary nature of this project would allow it for the generation of high-quality publications in well-known journals with high impact factors and these publication would likely attract significant interest from both academia as well as industry. Added to that, the potential findings and approaches of this project could be applied globally to other insects' species.

3. **Assessment of feasibility of the research project** (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)

The experimental design demonstrates a well-structured planning. The use of both laboratory experimental aspect (test with conditioned chickens) and field experimentation (with natural predators) will allow for cross validation of the results and concrete validations of the hypotheses. It also includes a logical progression from computational analysis to behavioral validation making the research methodology ambitious but achievable and each technique is reinforcing the other. The use of edible material in 3D printing is a creative solution which could be further investigated in similar research questions.

The project depicts three potential risks: variability of wing patterns, museum's samples shortage and weather conditions and they thought of strategies to address these risks which strengthens the project's feasibility. However, other potential challenges were not thoroughly addressed. For example, for the dummy predation trial, there was no mention of how environmental and seasonal variabilities will be accounted for or mitigated.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The total budget seems reasonable enough for a three-year research project, especially due to the fact that it involves field work across multiple sites in Asia, experimental approaches testing in both laboratory and field, as well as advanced computational analysis. The requirement of a specialist seems appropriate for a research-intensive project. However, some of the budget areas lack specific clarifications such as the 3D printer, it needs to include specificities about the type, the used material (even though it was mentioned in the research methodology, yet it needs to be specified in the budget) with explanation of the cost of each mimic production. For the predator experiments, ethics board fees need to be included. The museum image acquisition costs is unclear whether it includes travel costs, digitization fees, or just licensing costs. There are no allocations for compensating local collaborators at field sites (who were mentioned in the methodology). Furthermore, there were no provisions made for potential equipment failure and replacement needs.

5. **Strengths of the proposal**

- This project's idea is original and novel and based on a strong theoretical grounding as it combines machine learning, behavioral ecology as well as field work for an innovative approach to understand mimicry and predator deterrence.
- The project makes efficient use of resources as it is leveraging existing museum collections rather than just extensive new specimen collection and it intends to develop innovative complex tools like the CNN model with potential application beyond the immediate project.
- The research is clearly structured with testable hypotheses and strengthened with controlled laboratory settings and a clear experimental framework with a previously thought of alternative solutions for the discussed risks in the proposal.

6. **Weaknesses of the proposal**

- For other direct costs, conferences costs are listed without any explanation of which conferences are targeted and the objectives behind it.
- Even though the idea of using 3D-printed edible dummies is very innovative however it raises few concerns regarding the ecological realism of this model as dummies may not fully replicate the subtle differences in movement or chemical cues that real moths exhibit. Such differences might influence predator responses.
- Sample size might be considered small for drawing generalizable conclusions about behavioral responses.

Napat Emdee

Title of the project:

How to communicate with the dangerous neighbours: Testing the adaptive meaning of wing coloration patterns in Atlas moths

1. **Assessment of scientific quality of the research project**

The research questions of this project are undoubtedly original, as the evolutionary biology of the Atlas moth (*Attacus atlas*) remains largely unexplored. Although the hypotheses proposed for the function of wing coloration patterns in the moth are not entirely novel within lepidopteran studies, they are nonetheless valid, diverse, and scientifically testable. Thus, the scientific quality is quite strong and relevant in an international context.

2. **Assessment of potential impact of the research project**

As aforementioned, the research fields of mimicry and predator-prey interaction is already well-established, especially in lepidopterans. Nevertheless, the Atlas moth is an alluring and novel model, and the convolution neural networks (CNNs) the authors plan to combine with 3D-printing is a cutting-edge machine learning approach that could pave the way for the future of 'imagenomics' in evolutionary biology. Thus, the expected finding (probably, at least two research articles) has potential to be published in high-quality international and/or interdisciplinary journals. In addition, the simplicity and accessibility of the research topic might attract interest of general public in terms of popular science.

3. **Assessment of feasibility of the research project**

The overall research methodologies the authors employ to test the hypotheses are appropriate and feasible. It is reasonable and commendable that they plan to make a collaboration with national parks/research stations in Southeast Asia, which could give valuable insights into the moth and save their effort and time. The potential risks are well-mentioned. The experimental design using chickens as a model predator and modification of edible 3D-model is brilliant. However, there is still a big room for improvement, as listed below in detail.

a) Due to difficulty in finding the Atlas moth in their native environment and scarcity of museum specimens, the author should consider expanding their focus from the sole species to other *Attacus* moths and related saturniid genera. This will also help improve the impact of their finding, especially in the terms of using CNN to describing uniqueness and variation in evolution of wing pattern. CNN has not only been used in leucocyte recognition but also employed in the related topic of insect identification (see https://doi.org/10.1016/j.aspen.2020.11.015)

b) In the experiment of predator (chicken) behaviour toward the wing pattern, the authors need the proper-defined ethograms to describe the words scared/discouraged/alarmed/neutral/curious. For example, alarmed can be describe by specific sound frequency which may be acquire from some previous literature on chicken behaviour. If I understood correctly, the author plan to use time of naïve to address the hypothesis III (compelling predators to attack non-vital parts). But time of naïve can also imply the hypothesis II (mimicking snake), thus it's pretty ambiguous (see https://doi.org/10.1093/czoolo/61.4.749 and https://doi.org/10.1093/beheco/aru154). Painting the model is more accurate than spraying the model. And Chicken rearing should be mentioned and included in the schedule of the work plan.

c) The edible 3D-model may not display all aspects of moth's wing coloration pattern in terms of texture or UV-reflectance, given that avian eye sight are delicate in discriminating color.

d) The hypothesis IV addresses the function of wing patter in conspecific recognition (or to be specific, intersexual recognition within the same species) is interesting. Besides observing behaviour of male moth toward the model, the authors may consider more pairing condition between live moth and the model (male-female, female-male male-male, female-female). There could also be sexual dimorphism in wing coloration pattern, in which can also be analysed by CNN.

e) The specific statistical analyses may be addressed.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The budget is generally reasonable but could be optimized. It is acceptable for the principal investigators (PIs) to receive compensation; however, their salaries appear relatively high compared to their expected workload. Some of these costs could be reduced or redistributed to other budget categories. The cost per 3D

model and camera trap seems appropriate, but the number of each could be reduced, especially if they are reusable and not all of them will get damaged. Additionally, the project outcomes are likely to be published in no more than three separate papers, so the allocated publication fees could be adjusted.

5. **Strengths of the proposal**

All the hypotheses and research methodology are clearly delivered by the authors. The interdisciplinary approach using machine learning and a collaboration with international personnels are the main strength in this grant proposal. The workplan and table of schedule are described in very fine detail, allowing me to understand the whole process (it would be even better if the authors can increase the resolution of the schedule table).

6. **Weaknesses of the proposal**

The project focus on single moth species. With all the resources available, the author could expand their project to include more diversity of wing coloration pattern.

Dr hab. Aneta Arct

Title of the project: How to communicate with the dangerous neighbours. Testing the adaptive meaning of wing coloration patterns in Atlas moths.

1. **Assessment of scientific quality of the research project**

I find the topic of the proposed research visual mimicry in Atlas moths to be scientifically relevant, as it touches on a classic question in evolutionary biology: how organisms adaptively respond to predation pressure through morphological traits. I appreciate the project's ambition to integrate AI, behavioural ecology, and field experimentation, which could indeed offer a valuable contribution to the study of mimicry. However, in my view, the scientific quality is weakened by conceptual and structural issues. The proposal puts forward too many hypotheses without clear prioritization or rationale for their selection. Some of these hypotheses seem to overlap or lack distinctiveness, and there is no explanation of how findings from one set of experiments might influence or constrain the others. I also feel that the theoretical framework is underdeveloped, and the novelty is questionable predator mimicry has already been widely studied. Most importantly, I have serious doubts about the central assumption of the project: that the wing pattern of *Attacus atlas* represents a specific case of snake mimicry. The proposal hinges on a visual resemblance, yet fails to provide even a single photo to support this claim — something that naturally invites visual verification. Moreover, it is plausible that the wing pattern does not mimic a specific species at all, but rather reflects a more general aposematic motif a combination of shape and contrast shared across multiple taxa, including snakes, that serves as a broad warning signal to predators. This alternative, grounded in sensory ecology and predator psychology, is not considered in the proposal and should be acknowledged as a plausible competing explanation.

2. **Assessment of potential impact of the research project**

The potential for methodological novelty exists, particularly through the use of AI in analysing wing patterns, and such tools could be applied to other taxonomic groups. However, similar mimicry studies both conceptual and empirical already exist. The

proposal does not sufficiently engage with the existing body of international literature, making it difficult to understand how the project advances current knowledge. Furthermore, the proposal does not convincingly show how its findings would advance the field beyond descriptive observations. A more mechanistic perspective through genetic or physiological approaches would significantly enhance the depth of the study's conclusions.

3. **Assessment of feasibility of the research project**

The feasibility of the project is questionable. The timeline is unrealistic given the number of hypotheses, the variety of experimental setups, and the lack of detail about core logistics. No clear museum list is provided, nor is there a realistic plan for acquiring or accessing specimens. Field sites are described vaguely and seem to rely heavily on potential, yet unconfirmed, international collaborations. Additionally, while the authors intend to collect data in 10 tropical field sites, no information is provided on permissions, collaborations, or the availability of the moth in these locations. Field experiments using dummy moths, camera traps, and live chickens combined with AI model development would require a level of coordination, time, and staffing that is not reflected in the work plan. Risk analysis exists but fails to account for the true bottlenecks in specimen access and international coordination.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The budget covers a wide range of expenses related to the multidisciplinary nature of the project. The inclusion of items such as a 3D printer, camera traps, artificial moth models, and conditioning setups is consistent with the experimental design. However, a separate "Senior Data Scientist" salary of 330,000 PLN is listed, yet the specific responsibilities and time commitment of this position are not described. The cost of obtaining museum images (9,000 PLN on average for 200 images) is not clearly broken down. Does it include licenses, travel, or digitization fees? Similarly, the logistics surrounding international collaboration (3 delegations at 23,000 PLN each) are vague and could be better justified with destination details, purpose of travel, and expected outcomes. The only omission is the cost of purchasing a qualified electronic signature.

5. **Strengths of the proposal**

Application of AI to biological pattern analysis is an emerging and valuable field. The species under study is relatively under explored experimentally.

6. **Weaknesses of the proposal**

I must admit that when I first saw the full stop at the end of the project title, a part of me wondered whether it was an intentional suggestion to stop reading altogether and I briefly felt relieved at the thought of having one less proposal to review. The title, "How to communicate with the dangerous neighbours. Testing the adaptive meaning of wing coloration patterns in Atlas moths", is not particularly inviting. Its tone oscillates between a popular-science headline and a formal research framing, which creates confusion rather than intrigue. A title is often the first indication of the project's clarity and focus, and in this case, it suggests a conceptual looseness that unfortunately continues throughout the proposal. As I mention before the main weaknesses of the proposal is excessive number of hypotheses with no prioritization or realistic plan for addressing them all and unclear

experimental and logistical details, including museum/sample access and field locations. Another important weakness of the proposal is the lack of collaboration with experts who specialize in mimicry, lepidopteran biology, or visual ecology. This is particularly surprising given the complex and interdisciplinary nature of the research question.

Final version

WING (Wing-pattern **I**dentification using **N**eural **G**eneration**) testing the adaptive meaning of wing coloration patterns in Atlas moths**

Summary

Artificial Intelligence is gaining increasing popularity in many fields, due to its ability to deal efficiently with modelling and huge data manipulation. In this project we intend to exploit AI by using WING (Wing Pattern Identification using Neural Generation), a perfectioned AI-based modelling tool to study the mimicry in *Attacus atlas* moth - a gentle giant from Southeast Asia with a snake-like pattern on its wings. Despite popular belief, the ground under this peculiar pattern is unclear. Therefore, we are planning a set of laboratory and field experiments to study the interplay between this moth and its predators, focusing on the role of the wing pattern.

1) Scientific goal of the project

The "arms race" between prey and predators was one of the evolutionary phenomena puzzling the minds of researchers since the times Van Vallen presented his Red Queen Hypothesis (VanValen 1973). Mimicry is one of the ways in which prey can outsmart the predator (Stevens, 2005). If you are a seemingly defenceless organism, like a large and nutritious moth the best defence might be to look like the predator's worst nightmare.

Intricate and colourful patterns on lepidopteran wings are often associated with mimicry (Stevens, 2005), both Batesian - resembling other, more dangerous species (Carpenter & Ford 1933) and satiric - confusing the predator and employing it for the delay or misdirection of the attack (Howse 2013). There are also reports on the mimics that combine visual and acoustic signals for a better effect (Barber & Conner 2007; Moore & Hassall 2016).

Although this phenomenon received a lot of attention from the researchers across the years, we rarely see the study that would combine the field observation with an experimental approach. We tend to take a supposed mimicry for granted, often interpreting the observed features as a mimicry of a given model species or group of species, without entertaining other possible explanations (perhaps no less exciting).

In this regard we would like to propose a study that combines the field observations with behavioural experiments and machine learning to investigate the functions of the shape and pattern of the *Attacus atlas* moth.

The first hypothesis is based around the assumption that the pattern displayed on the wings of *Attacus atlas* is an important adaptation and its **key features are conservative:**

1). The pattern and shape of the *Attacus atlas* wings is conservative across the range of the species with a slight, regional variance

Our second hypothesis would approach the current, observation-based consensus - **Batesian mimicry**:

2). The pattern and the shape of the *Attacus atlas* wings is a mimicry of a snake model and it can deter predators.

The third would be constructed around the assumption that it might achieve a similar goal by different means so rather confuse and slow, than deter - **satirical mimicry:**

3). The pattern and the shape of the *Attacus atlas* wings is an example of satirical mimicry, used to confuse predators and compel them to attack non vital parts of the moth

With the fourth one we want to look at the problem from a **perspective of the moth and communication with conspecifics:**

4). The pattern and the shape of the *Attacus atlas* wings is a signal and a tool for communication, enabling recognition of conspecifics.

Finally, we are going to assess the effect of the pattern on the attack **preferences of different predators in the field:**

5). Predators from different taxa and representing different feeding strategies will react differently to different kinds of wing pattern alterations or lack thereof.

2) Significance of the project

The Atlas moth *Attacus atlas* L. is a charismatic insect that attracts people's attention due to its size and peculiar wing pattern. Despite the attention it gets and the potential for economic significance (Kumara & Kumar 2022), its biology is not sufficiently studied. For instance, this moth is speculated to use its wing pattern resembling venomous snake as a defence mechanism against predators (Howse 2013; Kumara& Kumar, 2022), but no experimental evidence has been provided so far. It is also unclear whether possible defensive mimicry works on Batesian or satiric basis. With our non-invasive approach, based on the archival and commercially available specimens, we are introducing new ways to conduct studies of mimicry, and behavioural biology while reducing our disturbance of the studied ecosystem. The only interference we will introduce to the environment will be the presence of our artificial moth models.

Artificial intelligence can play a crucial role in the exploration of the complexity of the wing pattern of Attacus atlas. Convolution neural networks (CNNs) have gained much popularity nowadays because of their ability to scan through many images and extract the main features to further classify said images according to a general trend (Kavi et al, 2020). CNNs have been used in the medical field to recognize cancerous cells or to classify different cell types with accurate precision and a relatively easy workflow (Schouten et al, 2021; Tsuneki, 2022). WING aims to adapt the CNN model -used by Schouten et al- to perform pattern recognition and analysis of Attacus atlas wings by extracting the main features of the complex multi-coloured wing pattern and establishing whether a common fashion among different populations is present.

Overall, this study will enhance our understanding of the evolution of complex visual patterns, mimicry and their behavioural and ecological significance, as well as develop the accurate tools for fine scale analysis of visual patterns in living organisms.

3) Concept and work plan

To test our hypothesis, we developed a range of subsequent experiments, with the crucial assumption of the conservativity of the key components of the pattern. The research will be conducted as follows:

1) Assessment of the variability in Atlas moth wing pattern on the photos originated from museum collections and the natural environment using WING and later comparison of these patterns with moths obtained from breeders, testing our first hypothesis. If the breed individuals are not critically different from the wild individuals in terms of pattern, they will be used for laboratory experiments. Otherwise, we will use moth models.

2) Field experiment with usage of 3D-printed edible moth dummies and camera traps directed to test native predator behaviour toward our prey models (hypotheses 2, 3 and 5). The tropical habitat of the Atlas moth does not limit us with the seasonality, so the field work can have a plastic schedule. Given that it is a field observation, we may also observe conspecific recognition, that we can use as additional data in testing the 4th hypothesis.

3) Laboratory experiment in which we will test predator behaviour using chickens as models. The moth (originated from a farm or the 3D-printed model, according to the results of point 1) will be used to test whether wing pattern has an influence on the predator behaviour (H2), predator reaction time (H2a) and the attack direction (H3).

4) Laboratory experiment to test male moth reaction on female dummies with normal and altered wings (H4).

During the first year of the project our team will focus on testing the variation of the wing pattern and the first field experiment (collaboration with national parks/research stations in SE Asia). We predict that the survey of potentially available collections and the photos database arrangement will take around two to four months. Simultaneously we will be working on the machine learning model and artificial mimic design. The model development and testing will take around eight months. In the meantime, we will contact the national parks and research stations that have Atlas moth present in their territory to confirm the participation in the ongoing field experiment. From those willing to collaborate we would randomly choose 10 locations (with the limit of 3/country). As soon as we have our wing pattern results, we will use them to complete artificial mimics designs with appropriate patterns (based on the local variants if we found them during picture analysis or generalised if we found the pattern to be very conservative). The mimics and other necessary tools will be passed to the local personnel via postal services. We will travel to one of the chosen sites to set the experiments with the local personnel and prepare visualised field protocol for the rest of collaborators. In the remaining 9 sites, local experts (field guides and entomologist) will set the experiments accordingly. The experiment will last for 14 days: 2 days to organize and set the experiment, 10 days of observation, as this is the average lifespan of the imagos, and an additional two days to fold the field experiment and it'll be repeated in the next month or later if local collaborators decide that the conditions are not favourable we expected that both replicates are going to be done in

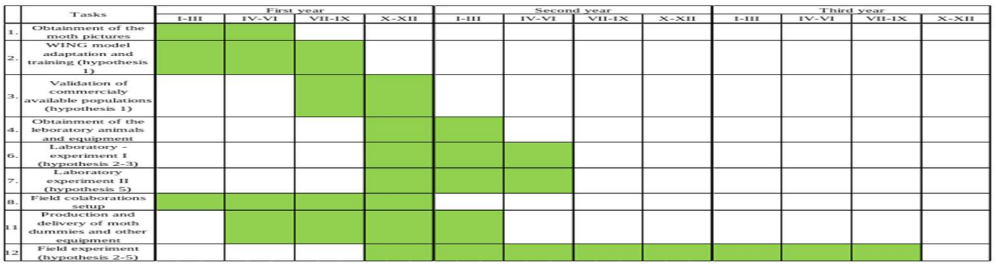
the span of 16 months across all experimental locations . After the field experiment (or during, in case it will have to be delayed) we will start the laboratory experiment, again with two replicates to test second, third and fourth hypotheses. Preparations such as setting the equipment, conditioning the chickens and obtaining imagos will approximately take us two months, and the experiment itself will be conducted during the following month. We will also be working on the manuscript about machine learning for publication.

At the middle of the second year, we're expecting our manuscript about machine learning to be ready for publication. The next eight months of the project's second year we will focus on the manuscripts based on data from our predation lab experiment (H2 and H3), conspecific recognition lab experiment (H4) and the predator preference and behaviour in the presence of mimics' field experiment (H2, H3 and H5).

According to the scheduling of the first experiment, we will replicate the same one a year after.

The third year of the project will be used to polish the manuscripts and if needed to repeat field or laboratory experiments if we would encounter problems such as extreme weather in some locations.

Project schedule is presented in the Gant chart below:



During the project we expect a range of possible risks and complications connected with the data obtained:

1. Too high variability of the wing pattern. If this occurs, either it will require more time to train the model (the amount of pictures to train it could be insufficient) or it would be harder to assess the proper mimic pattern for experiments, in that case we will develop the regional variants of the model based on the selection of experimental sites which will be reduced to eight or five depending on the feasibility.

2. Shortage of museal samples. In that case we will compensate for the lack of data with images from other sources such as wild individuals and private collections. If the captivity bred moth is not representative comparingly to museal records, we will be forced to use dummies intended for the field experiment.

3. Unexpected weather conditions might pose a challenge to conduct the field experiment, but as the experiment is short and not limited by strict seasonality, - we can delay it until suitable weather occurs or with the observations of imagos in each experimental location.

4. Chickens do not get conditioned or do not react properly after exposure to the moth. If the chickens do not get conditioned or the chickens do not react as expected to the moth exposure, we will focus more on the natural behaviour of the moths in the field, while considering that the pattern of the moth would appear different according to bird vision (tetrachromatic vision with vision field in the near-UV spectrum).

4) Research methodology

To test the first hypothesis, we will adapt a pre-existing convolution neural network (CNN) used in leucocyte recognition to wing pattern recognition, modifying it to be more efficient in pattern, colour and contrast discrimination. The model, which is called WING- (Wing-pattern Identification using Neural Generation), will be trained with images provided by museums and private collectors from different countries (India, Brunei, Malaisia, Thailand, Indonesia, Great Britain, Germany, USA, the Netherlands) and assess the pattern similarity among different populations of the same species. Then, the model will be used to define the similarity of the pattern in the wings of the commercially available moth to those originated from the natural environment and define whether they are applicable for laboratory experiments.

The second hypothesis is going to be tested in a laboratory setup. The experiment involves conditioning chickens to avoid snakes and subsequently verify if they avoid the moth once exposed to it. The chickens will be aversive conditioned by exposing them for 5 minutes to air puffs and presenting at the same time pictures of different SE Asian snakes. The conditioning occurs for 23 training sessions one hour apart from each other, as described in du Plessis et al, 2021. After the training, the moth is positioned in the chicken cage, and we check and record the reaction of the chicken. For the experiment, we will alter the wing pattern in 24 of the moths, by spraying uniform paint on its entire upper surface. That way we will have two types of moths imagos, - one with normal wing pattern and the second with the altered. Each moth type will be exposed to conditioned chickens and unconditioned ones, making it 2x2 treatments, 12 pairs (moth + chicken) each, having in the end 48 observations, we will perform 6 simultaneous trials. We are going to replicate the procedure after approximately a month with different animals. At the same time, we test the third hypothesis - the eventual presence of satirical mimicry, by measuring the time response of the chicken to the moth: if it has a statistically significant delayed response, the chicken is startled. The chicken is also allowed to attack the moth for the period of 30 s. from the first contact. The place of the attack will be analysed to confirm the type of mimicry: if it occurs on the pattern, it may mean that the chicken is startled.
For the experiment on vertebrates we are planning to obtain permission from the National Ethical Commission for Animal Experiments. After the experiment the naive chicken will stay in the facility, and the conditioned chicken will be disposed of.

Exploration of the third hypothesis, the determination of whether the terminal part of Attacus atlas a distraction for predators from the vital part of the moth, is also done in the field. A survey is prepared to find collaborators in national parks and research stations with a stable atlas moth population in their areas. Moreover, we prepare four different types of edible dummies, which will show the preferred bite spot of predators. After assessing the areas for the experiment, we place 48 edible dummies in 10 field locations to check for bite marks. The dummies are produced by a 3D printer from ecofriendly polymers (PHA) by a third party from our blueprint.

We planned four types of dummies (12 per type):

- Normal pattern (as the model assessed) and normal shape
- Normal pattern and disrupted shape (without the wing appendages that supposedly resembles the snakes head)
- Disrupted pattern (neutral coloration) and normal shape
- Disrupted pattern and disrupted shape

The experiment will be repeated twice. The placement of dummies in the field is semi-random, dummies are going to be distributed on four most common host plants in each area. We also provide 9 camera traps that are going to record the predators approaching the dummies and natural behaviour of Attacus atlas in its native environment (2 camera traps/treatment + additional one for the eventuality in which we can find a female Attacus atlas and set the camera trap so it can observe it for reference).

The fourth hypothesis revolves around the idea that patterns on the wings of our moth is a signal for conspecifics. To tackle this, we prepare a laboratory experiment in which we expose male moths to dummies in four different conditions as in the field experiment. We plan to present each variation of the dummy to 12 male moths and replicate the experiment after a month with different animals (the moths are going to be used with the chicken experiment later on), we are planning for six simultaneous trials.

The final hypothesis is set around the assumption that the pattern or shape of Attacus atlas wings will affect different taxa of predators differently. For that we are going to analyse the bite marks on the dummies from the field experiments and support our findings with the camera traps materials (the number of camera traps was reduced for feasibility of analyses)

5) Composition and qualifications of the research team,

Alice Ballabio- PIs: expertise in neural network modelling, trained models of insect learning behaviour (trace conditioning modelling) and large data manipulation.

Adam Solecki - PIs: 10 years of experience with field, animal related studies (mostly fauna diversity, distribution and monitoring), including an experiment with a moth larvae model used for predation assessment in relation to hiding/feed strategy, experience with field studies in the tropics (Danum Valey in Sabah State, Malaysia).

Antonii Bakai - PIs: five-year experience in field, animal related studies, GIS tools, qualified for work with laboratory animals (including vertebrates).

6) Project literature

Asayuki Tsuneki, Deep learning models in medical image analysis,Journal of Oral Biosciences, Volume 64, Issue 3,2022, Pages 312-320, ISSN 1349-0079, https://doi.org/10.1016/j.job.2022.03.003.

Barber J.R., Conner W.E. 2007. Acoustic Mimicry in a Predator–Prey Interaction. *Biological Sciences,* 104: 9331–9334. https://doi.org/10.1073/pnas.0703627104

Bhawane, Ganesh & Mamlayya, Amol & Koli, Yogesh & Phonde, Y & Aland, Santosh & Gaikwad, Sunil. (2011). LIFE HISTORY OF ATTACUS ATLAS LINN. (SATURNIIDAE: LEPIDOPTERA) ON SAPIUM INSEGNEBENTH. FROM WESTERN GHATS, MAHARASHTRA. The Bioscan. 6. 497-500.

Carpenter G.D.H., Ford E.B. Mimicry. London: Methuen, 1933.

du Plessis EW, Beausoleil NJ, Bolwell CF, Stafford KJ (2021) Validation of a combined approach-avoidance and conditioned stimulus aversion paradigm for evaluating aversion in chickens. PLoS ONE 16(2): e0247674. https://doi.org/10.1371/journal.pone.0247674

Howse P.E. 2013. Lepidopteran Wing Patterns and the Evolution of Satyric Mimicry. *Biological Journal of the Linnean Society,* 109: 203–214. https://doi.org/10.1111/bij.12027

Kavi B. Obaid, Subhi R. M. Zeebaree & Omar M. Ahmed (2020). Deep Learning Models Based on Image Classification: A Review. International Journal of Science and Business, 4(11), 75-81. doi: https://doi.org/10.5281/zenodo.4108433

Moore C.D., Hassall C. 2016. A Bee or not a Bee: an Experimental Test of Acoustic Mimicry by Hoverflies. *Behavioral Ecology,* 27: 1767–1774. https://doi.org/10.1093/beheco/arw107

Ravi Kumara, R., & Kumar, H. (2022). The Fagara Silkworm (Attacus atlas L.): An underutilized Vanya Silkworm of India. *Journal of Biodiversity*, *8*(5), 1-11.)

Schouten, J.P.E., Matek, C., Jacobs, L.F.P. *et al.* Tens of images can suffice to train neural networks for malignant leukocyte detection. *Sci Rep* 11, 7995 (2021). https://doi.org/10.1038/s41598-021-86995-5,

Stevens M. The role of eyespots as anti-predator mechanisms, principally demonstrated in the Lepidoptera. Biol Rev Camb Philos Soc. 2005 Nov;80(4):573-88. doi: 10.1017/S1464793105006810. PMID: 16221330.

Van Valen L. 1973. New Evolutionary Law. *Evolutionary Theory*. 1: 1–30.

Source code: https://github.com/marrlab/ClassificationALL

7. Budget for the project

	Amount in PLN
Direct costs, including	**1,585,827**
-personnel costs and scholarships	1,392,000
-research equipment/devise/software	99,958

	Amount in PLN
cost	
-other direct costs	93,869
Indirect costs, including	**43,630**
-Indirect costs of OA	2,000
- other indirect costs	41,630
Total costs	**1,629,457**

Budget explanation:

All the amounts are in PLN.

- IP fee - average month fee for PHD researcher in Poland for the duration of project 9000x3x36 (972000).
- Senior data scientist services will be crucial for proper model development 330000 for one year. This figure will help with the implementation and maintenance of WING along with the PIs.
- production of 800 mimics (approximately 6000 according to market prices)
- camera traps KeepGuard KG790 90x529 + batteries (10 packs) 72x9
- chicken 30x120 + food for 3 month (3600)
- Living Moth 100x150 + containers 150x3 pln. (15450)
- 13000 computer setup
- average of 9000 for gathering 200 images from museums and national parks.
- Conditioning setup: 90 x 6 chamber with separation + 70 x6 air puff canister (1050)
- delegations abroad with 1-2 IP for participation and guidance in field research in SE Asia. Costs including flight tickets (approximately 2x2x4000), vaccination (approximately 2x2000), local transportation (approximately 1000), booking in field stations (2x14x600) and diets (2x14x230) in total 44240.
- Fee for collaborators in national parks and research stations (11 days)- 3 sites, 5 collaborators per site: 6000 x15 – 90,000
- The conference will be hosted in the Jagiellonian University. Costs of booking for the 20 participants (Hotel + diet 400 - fee 200 x 3 days). Budget planned for core speakers (experts in the field of our research) - flight tickets 2500x2x2, hotel 330x3x2, diet 80x3x2. The main auditory will be the local students, and the main goal is topic promotion and science popularization.
- The budget planned for participation in conferences connected to the topic is planned for 1 conference per IP. We consider the international level of engagement,

and will aim for SE Asia conferences, thus the budget mostly covers the flight (4000+), and the rest is attendance fee (750), hotel (6040x3) and diet (230x3).

- delivering tools to (moth dummies, camera traps) 200x10 - the average cost of international delivery service for packages sent to 10 national parks in SE Asia
- publications fee 10637 (Frontiers in Zoology) +3381 (AI Open) +17318 (Current Opinion in Insect Science) +10294 (Animal Behaviour);
- Qualified electronic signature for 3 years: 429.

All animal-related procedures were conducted in accordance with ethical standards and approved by the appropriate institutional animal care and use committee.

We acknowledge the use of ChatGPT to check for English grammar and spelling.

4. Deciphering biological functions of astaxanthin in Atlantic salmon (Salmo salar)

Team: Napat Emdee, Rama S. Krovi, Jakub P. Płachta

Project (initial proposal)

Title: Deciphering biological functions of astaxanthin in Atlantic salmon (*Salmo salar*)

Authors:

Napat Emdee, Rama S. Krovi, Jakub P. Płachta

Summary

Ever wonder how Atlantic salmon pull off the epic upstream journeys? It's not just willpower that drives them. These species have been studied on the genetic and physiological factors that aid them and we want to dig deeper into a secret or not so secret weapon: the astaxanthin (Ax), the vibrant orange-pink pigment which gives them their beautiful hue based on which you try to choose for dinner. Think of it as more than just the salmon's food colouring. We suspect that it plays a vital role in their incredible migrations as we already know from previous studies that they accumulate the Ax in the muscles. We hypothesize that this aids in the protection against the stress of their powerful swims. We also know that the salmon's muscles naturally break down during their journey releasing some of the Ax into their bloodstream acting like an antioxidant. This could also help boost their immune system, making them tougher against the bacteria and other parasites they encounter during their journey. To investigate this, we will raise two groups of Atlantic salmon with and without Ax in their diet, simulate the migration to see how the Ax levels affect their swimming stamina, oxidative stress and their immune response. This study will solve the mystery about Ax's precise role in these long-distance migrations. It would lead us to better ways of raising salmon in fish farms making them healthier and more resilient and resistant to infections.

SHORT DESCRIPTION OF THE RESEARCH PROJECT

1) Scientific goal of the project

Many members of the Salmonidae family are capable of traveling astounding distances (up to 3000 km) in order to reproduce in freshwater, a behaviour known as anadromy. This journey constitutes a major strain on their physiology, resulting in degradation of muscle and mobilization of lipids and proteins (Bowerman et al. 2017; Johnson et al., 1997). This may result in increased oxidative stress, and in turn increase susceptibility to pathogen infection by impairing the immune system (Wilson et al., 2014). Crucially to this work, muscle tissue is also the main storage of astaxanthin (Ax) (Rajasingh et al., 2007), a carotenoid attached to α-actinin in myofilament (Matthews et al., 2006). It gives salmon flesh its signature colouration, so sought after that it constitutes a 60 billion USD market worldwide. Exercise-induced muscle degradation results in a release of Ax into the bloodstream. Ax possesses potent antioxidative properties, which may then be used to scavenge reactive oxygen species (ROS) released during the migration effort. While the role

of carotenoids in the skin for mate selection is well-described (Lehnert et al., 2016), the biological role of Ax storage in muscle and its subsequent presence in blood remain poorly understood. Two questions crucial for filling this gap are (1) the effect of Ax on the muscle tissue, which we hypothesize to has a beneficial influence on endurance during migration by reducing exercise-induced oxidative stress (H1); (2) the utilization of Ax in the bloodstream - here we propose that Ax helps prevent oxidative stress by scavenging harmful ROS (H2a). Alternatively, Ax enhances the immune response of the fish, improving its resistance to pathogen infection (H2b).

2) Significance of the project

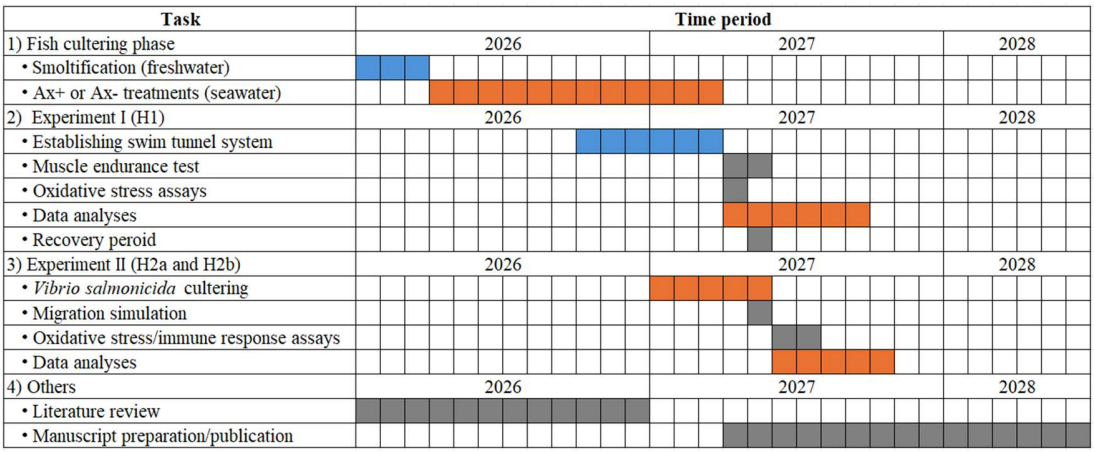
Salmonid fish hold a significant ecological and economic value. Their Ax-based vibrant pigmentation is an important trait whose evolutionary and physiological drivers remain sparsely understood. Studies in humans and rodents demonstrate the beneficial effects of Ax on muscle endurance (Polorov et al., 2014; Liu et al., 2018). These effects are attributed to Ax's ability to prevent redox imbalance (Polorov et al., 2014), alongside its role in improving insulin sensitivity and glucose metabolism (Ishiki et al., 2013). However, the functional significance of the substantial Ax accumulation in the muscle tissue of salmonids remains poorly understood, especially in the context of their energetically demanding migratory behaviour, which leads to muscle degradation and a redistribution of energy reserves in the forms of lipid and protein (Jonsson et al., 1997; Bowerman et al., 2017). Only a few studies have addressed the potential association of Ax on salmonid muscle tissue, for example, by showing its role in upregulating the glycolytic gene *fbp1* (Schmeisser et al., 2021) and its binding capacity to α-actinin in myofilament (Matthews et al., 2006). Our project will advance this area of research using state-of-the-art swim tunnel experiments (Prescott et al., 2023) to test the effect of Ax on muscle endurance (H1).

Beyond its role in muscle physiology, Ax exhibits antioxidant effects in systemic circulation and may enhance immune responses (Park et al., 2010; Does et al., 2016). These properties not only contribute to disease prevention and delayed aging in humans (Ekpe et al., 2018), but also promote growth and health status in aquatic species (Lim et al., 2022). Evidence suggests that oxidative stress (Wilson et al., 2014) and pathogen burden (Dolan et al., 2016; Chapman et al., 2020) increase significantly during the spawning migration of salmonid fish. Our project will therefore explore Ax's protective role in reducing systemic oxidative stress (H2a) and enhancing immune function (H2b) under simulated migration conditions. This approach integrates various research fields, including behavioural evolution, life-history theory, physiological ecology, and immunology. Our findings could have implications for aquaculture practices potentially informing dietary supplementation strategies to enhance fish health, resilience and overall production efficiency.

3) Work plan

The project spans three years. The first phase will focus on culturing Atlantic salmon (*Salmo salar)* with or without astaxanthin (Ax) supplementation. After approximately one year of rearing, the hypothesis-testing phase will begin using salmon at the smolt stage, when they are physiologically prepared for migration. The same groups of fish will be used to test both H1 and H2a/H2b in chronological order. H1 aims to investigate the specific role of Ax within muscle tissue. Fish will be subjected to forced exercise in a swim tunnel to assess muscle endurance, and blood samples will be collected before and after the exercise

to evaluate oxidative stress levels associated with short-term muscle activity. H2a and H2b will explore the systemic role of Ax outside the muscle. Migration will be simulated by generating a constant water flow in tanks over the course of one month, inducing muscle degradation and promoting the release of Ax into the bloodstream. Blood samples will then be analyzed to assess oxidative stress markers and immune responses. Tasks are allocated as follows (see the Gantt chart): blue indicates responsibilities of the lab technician, gray represents tasks assigned to the principal investigator (PI), and orange denotes tasks shared between the PI and the lab technician.



Risk analysis: The list of possible risks and mitigation strategies is addressed in the below table.

Risk and description	**Mitigation strategy**
Biological variability Variation in individual fish response to dietary treatments and experimental conditions is a potential risk.	Mitigation strategies include using a robust statistical analysis.
Bacteria maintenance Maintaining a viable culture of *Vibrio salmonicida*	Establish a culturing protocol or plan for sourcing parasites from external laboratories
Technical issues Equipment malfunctions (e.g., swim tunnel, respirometer, ELISA reader) could delay experiments.	Regular maintenance and having backup equipment, great collaborators and access to alternative facilities will mitigate this risk.
Data loss or mismanagement	We will implement a robust data management plan, including secure storage, regular backups, and standardized data entry procedures.
Fish health and welfare The experiments may cause stress or harm	We will adhere to strict animal care protocols, including anesthesia and biopsy procedures, and monitor fish health

to the fish.	closely. We will also optimize experimental conditions to minimize stress.
Dietary effects The experimental diets, while designed to isolate the effects of Ax, could have unintended consequences on fish physiology.	We will carefully formulate and analyze the diets, and include control groups to account for any non-carotenoid effects.
Sampling stress Repeated sampling (e.g., blood draws, muscle biopsies) could stress the fish and affect the results.	We will optimize sampling procedures to minimize invasiveness and frequency, and allow for adequate recovery periods.
Data loss or mismanagement There is a risk of losing or mismanaging the data collected during the experiments.	We will implement a robust data management plan, including secure storage, regular backups, and standardized data entry procedures.

4) Research methodology

(underlying scientific methodology, methods, techniques and research tools, methods of results analysis, equipment and devices to be used in research);

Culturing: The study will be conducted on Atlantic salmon (*Salmo salar*). The fish will be reared from the egg stage in four tanks with: constant temperature (15 °C); filtered, recirculated water; 24:0 L:D photoperiod until smolt stage (1 year). During the first 3 months, the fish will be fed a standardised crushed cod pellet diet, twice per day, to satiation. After 3 months of growth, we will replace the water in the tank with seawater (35 ppt). Fish in two tanks will continue to be fed with a standardised diet (Ax- group), while the other two tanks will be supplemented with astaxanthin (Ax+ group, 100 mg/kg). After 1 year, we will tag the dorsal fin of each individual for identification and the fish will be ready for experimental procedures. We specifically chose non-lethal methods so **no fish will be euthanized** for this project. All assays will be done using commercially available kits.

Experiment I (H1): 50 Ax+ fish and 50 Ax- fish will be used in this experiment. 10 individuals will be placed in a swim tunnel (Figure 1) left to acclimate overnight. Critical speed (*Ucrit*; Bret, 1964) and time-to-fatigue (Nikora et al., 2002) as well as maximum metabolic rate (MMR; Prescott et al., 2023) will be measured. Before and after exercise, blood samples will be taken (non-lethal sampling) for oxidative stress analysis: oxidative damage (dROMs), antioxidant capacity (OXY-adsorbent) and lipid peroxidation (TBARS).

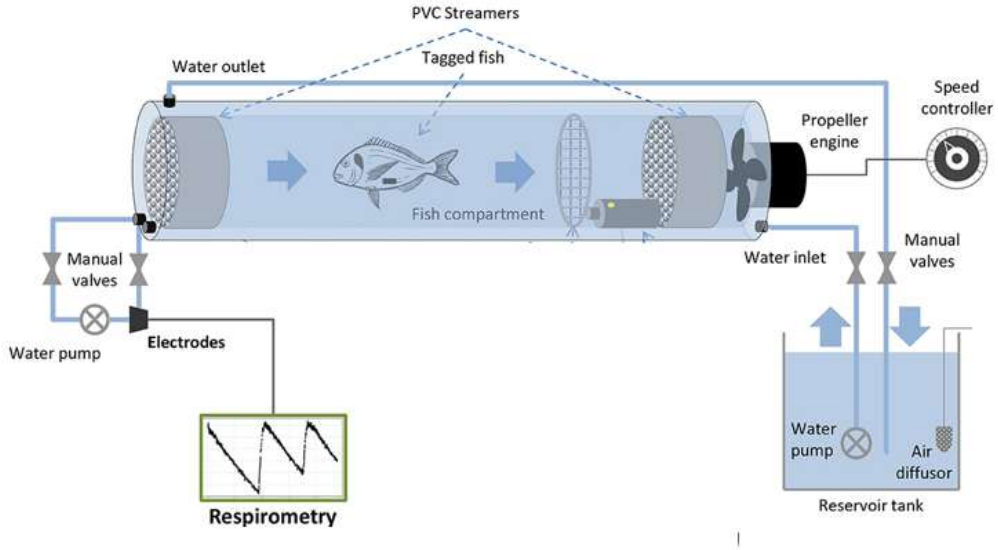


FIGURE 1 Schematics of the swim tunnel equipped for respirometry (Modified from Van Den Thillart et al., 2004)

Experiment II (H2a): All fish used in Task 1 will be anaesthetised and blood samples will be collected from the caudal vein. A muscle biopsy will be performed (dorsal muscle, non-lethal; Henderson et al., 2016) and the fish will be left to recover for 7 days. Then, all smolts will be placed into a common tank with a constant generated current (1.2 m/s) and fasted there for 2 weeks. These conditions mimic the effort required for long-period oceanic migration. After 2 weeks, blood and muscle sampling procedures will be repeated.

Ax levels in muscle and blood will be assessed with the use of HPLC. This analysis will be outsourced to a commercial analytics lab, since equipment necessary for this analysis is prohibitively costly. Oxidative stress will be assessed in the same way as in Task 1.

Experiment II (H2b): For this analysis, we will use blood obtained from Task 2 (before and after experimental procedure). Activity of the immune system will be assessed for: (i) phagocytes (peak respiratory burst) using luminol-enhanced chemiluminescence; (ii) cytokine expression using ELISA; bacterial killing assay (BKA) using *Vibrio salmonicida*, a common salmon pathogen.

Statistical analyses:

Measured variables will be used as dependent variables in linear mixed models (MLM) with diet as a grouping factor (experiment I) or astaxanthin levels as a covariate (experiment II). Fish ID will be used as a random factor to account for repeated measurements. These models will then be analysed using ANOVA with Satterwaite's correction for degrees of freedom.

5) Composition and qualifications of the research team,

Napat Emdee: 1st year PhD student with a background in stress responses in laboratory rats and tardigrades

Rama Sarvani Krovi: 3rd year PhD student with experience in molecular techniques, lab management, data analysis and paper writing. Following is the publication list.
https://doi.org/10.1111/imb.12973
https://doi.org/10.1007/s10531-024-02940-8
https://doi.org/10.1038/s41598-018-20946-5

Jakub Płachta: 2nd year PhD student, experience in oxidative stress research in birds including oxidative stress assays and data analysis.

6) Project literature

Bowerman TE, Pinson-Dumm A, Peery CA & Caudill CC (2017) Reproductive energy expenditure and changes in body morphology for a population of Chinook salmon *Oncorhynchus tshawytscha* with a long distance migration. J*ournal of Fish Biology 90:* 1960–1979. https://doi.org/10.1111/jfb.13274

Chapman JM, Teffer AK, Bass AL, Hinch SG, Patterson DA, Mikker Km & Cooke SJ (2020) Handling, infectious agents and physiological condition influence survival and post-release behaviour in migratory adult coho salmon after experimental displacement. *Conservation Physiology 8(1)*: coaa033. https://doi.org/10.1093/conphys/coaa033

Dolan BO, Fisher KM,Colvin ME (2016) Innate and adaptive immune responses in migrating spring-run adult chinook salmon, *Oncorhynchus tshawytscha*. *Fish & Shellfish Immunology 48*: 136–144. https://doi.org/10.1016/j.fsi.2015.11.015

Dose J, Matsugo S, Yokokawa H, Koshida Y, Okazaki S, Seidel Y, Eggersdorfer M, Rimbach G & Esatbeyoglu T (2016) Free radical scavenging and cellular antioxidant of astaxanthin. *International Journal of Molecular Sciences 17*:103. https://doi.org/10.3390/ijms17010103

Epke L, Inaku K & Ekpe V (2018) Antioxidant effects of astaxanthin in various diseases—a review. *Journal of Molecular Pathophysiology 7*:
http://doi.org/10.5455/oams.20180315075538

Henderson CJ, Stevens TF & Lee SY (2016) Assessing the suitability of a non-lethal biopsy punch for sampling fish muscle tissue. *Fish Physiology and Biochemistry 42*: 1521–1526. https://doi.org/10.1007/s10695-016-0237-z

Ishiki M, Nishida Y, Ishibashi H, Wada T, Fujisaka S, Taikawa A, Urakaze M, Sasaoka T, Usui I & Tobe K. (2013) Impact of divergent effects of astaxanthin on insulin signaling in L6 cells. *Endocrinology 154*: 2600 –2612. https://doi.org/10.1210/en.2012-2198

Jonsson N, Jonsson B & Hansen LP (1997) Changes in proximate composition and estimates of energetic costs during upstream migration and spawning in Atlantic salmon *Salmo salar*. *Journal of Animal Ecology 66(3)*: 425–436. http://doi.org/10.2307/5987

Lehnert S, Pitcher TE, Devlin RH & Heath DD (2016) Red and white Chinook salmon: genetic divergence and mate choice. *Molecular Ecology 25*: 1259–1274. https://doi.org/10.1111/mec.13560

Lim KC, Yusoff FM, Karim M & Natrah FMI (2022) Carotenoids modulate stress tolerance and immune responses in aquatic animals. *Reviews in Aquaculture 15(2)*: 872–894. https://doi.org/10.1111/raq.12767

Liu SZ, Ali AS, Campbell MD, Kilroy K, Shankland EG, Roshanravan B, Marcinek DJ & Conley KE (2018) Building strength, endurance, and mobility using an astaxanthin formulation with functional training in elderly. *Journal of Cachexia, Sarcopenia and Muscle 9(5):* 826–833. https://doi.org/10.1002/jcsm.12318

Matthews SJ, Ross NW, Lall SP & Gill TA (2006) Astaxanthin binding protein in Atlantic salmon. *Comparative Biochemistry and Physiology, Part B 144*: 206–214. https://doi.org/10.1016/j.cbpb.2006.02.007

Nikora VI, Aberle J, Biggs BJF, Jowett IG & Sykes JRE (2003) Effects of fish size, time-to-fatigue and turbulence on swimming performance: A case study of *Galaxias maculatus*. *Journal of Fish Biology 63*: 1365–1382. https://doi.org/10.1111/j.1095-8649.2003.00241.x

Park JS, Chyun JH, Kim YK, Line LL & Chew BP (2010) Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. *Nutrition & Metabolism 7:* 18*.* https://doi.org/10.1186/1743-7075-7-18

Polotov TG, Vardaris CV, Mihaliuc AR, Gonçalves MS, Pereira B, Ganini D & Barros MP (2014) Astaxanthin supplementation delays physical exhaustion and prevents redox imbalances in plasma and soleus muscles of Wistar rats. *Nutrients 6*: 5819–5838. https://doi.org/10.3390/nu6125819

Prescott LA, Symonds JE, Walker SP, Miller MR, Semmens JM & Carter CG (2023), Long-term sustained swimming improves swimming performance in Chinook salmon, *Oncorhynchus tshawytscha*, with and without spinal scoliosis. *Aquaculture 574*: 739629. https://doi.org/10.1016/j.aquaculture.2023.739629

Rajasingh H, Våge DI, Pavey SA & Omholt SW (2007) Why are salmonids pink?. *Canadian Journal of Fisheries and Aquatic Sciences 64(11)*:1614–1627. https://doi.org/10.1139/f07-119

Schmeisser J, Verlhac-Trichet V, Madaro A, Lall SP, Torrissen O & Olsen RE (2021) Molecular mechanism involved in carotenoid metabolism in post-smolt Atlantic Salmon: Astaxanthin metabolism during flesh pigmentation and its antioxidant properties. *Marine Biotechnology 23*: 652–670. https://doi.org/10.1007/s10126-021-10055-2

Van Den Thillart, G. E. E. J., Van Ginneken, V., Körner, F., Heijmans, R., Van derLinden, R., and Gluvers, A. (2004) Endurance swimming of European eel. *J.Fish Biol.* 65, 312–318. https://doi.org/10.1111/j.0022-1112.2004.00477.x

Wilson SM, Taylor JJ, Mackie TA, Patterson DA, Cooke SJ & Willmore WG (2014) Oxidative stress in Pacific salmon (*Oncorhynchus* spp.) during spawning migration. *Physiological and Biochemical Zoology 87(2)*: 346–352. https://doi.org/10.1086/674798

6) Table with budget of the project.

Item	**Funds for the each budget year (zł)**			
	2026	**2027**	**2028**	**Total**
1.Direct costs	363,177	181,588.50	181,588.50	726,354

1/Salaries and benefits	51,000	51,000	51,000	153,000
2/Equipment	123,000	0	0	123,000
3/Other direct costs	183,452	103,452	163,450	450,354
2.Indirect costs	72,634	36,318	36,318	145,270
Total costs (1+2)	435,811	217,906.50	277,906.50	931,624

7) Breakdown of project costs including justification and relevance for the tasks in the project

Salaries and benefits:

1. Salaries for three PIs: 36 months x 3 people x 1250 zł (Responsibilities: Constructing swim tunnel, performing experiments and biochemical assays, data analysis, manuscript writing, conference attendance)
   Total: **135 000 zł**

2. Technical assistant:

   Animal care: 36 months x 500 zł (Responsibilities: maintaining fish colony, including experimental diet administration)
   Total: **18 000 zł**

Equipment:

1. Swim tunnel construction (respirometer, temperature, oxygen and flow rate control etc.): **43 000 zł**
   Will allow for measuring endurance in salmon smout in Experiment I

2. Fish tanks (four grow tanks and one common tank with flow rate control): **80 000 zł**
   Necessary for housing the animals and performing exercise in Experiment II

Other Direct Costs:

1. Summary cost of oxidative stress analysis (dROMs, OXY-adsorbent, TBARS combined):

   a. Experiment I - 200 samples x 2 replications x 37 zł per sample = **14 800 zł**

   b. Experiment II - 200 samples x 2 replications x 37 zł per sample = **14 800 zł**

2. Analysis of astaxanthin concentration (HPLC, outsourced to a commercial analytics lab):

   a. Experiment I - 200 samples x 440 zł per sample = **88 000 zł**

   b. Experiment II - 200 samples x 440 zł per sample = **88 000 zł**

3. Summary cost of immunological assays (peak respiratory burst, cytokine expression, BKA):
    a. Experiment II - 100 samples x 2 replications x 120.5 zł per sample = **24 100 zł**
4. Animal colony maintenance for 3 years (feed, electricity, repairs, cleaning materials, Ax supplement): **160 554 zł**
5. PIT tags for fish identification: **1000 zł**
6. 2 International conferences (attendance for all 3 PIs): **60 000 zł**

Reviews

Antonii Bakai

Simplified form for grant proposal review

Title of the project: Deciphering biological functions of astaxanthin in Atlantic salmon (*Salmo salar*)

1. **Assessment of scientific quality of the research project** (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context):

The research is applicable for many different scientific fields, such as food science, biomedicine and biology. It also present the novel approach to given scientific problem. The project relevance is justified, as its main topic, – the oxidative stress in living organisms, is currently under active studying. The research is also indirectly connected with the problem of food safety and nutrition value, – one of the main global challenges for humanity nowadays.

2. **Assessment of potential impact of the research project** (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context):

The research project not only demonstrates the new organism for oxidative stress studies, but it also might give us a new insight on the astaxanthin role and possible applications. The study does not undermines the previous findings in the field, but filling the existing knowledge gape.

3. **Assessment of feasibility of the research project** (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project):

I failed to see any issues with the number of fish in the experiment. The sample size is minimally sufficient for obtaining significant results, which is good from perspective of labour, costs and bioethics. The authors managed to address to many crucial risks

connected to experiment and came up with effective ways to mitigate it. Although, they does not mentioned the risks connected with the fish colony. It would be wise to came up with backup solutions in case problems arise with the main source of experimental data. I might assume that authors took it in to account, as they decided to using two tanks for each treatment, but in that case it should be mentioned in the risks chapter.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The costs in research project are mostly justified. The equipment prises are adequate and the decision to use third party for expertise services is logical. However, the authors does not provide the information about fish fate after project's end. In case they have the way to dispose of the fish after the end of experiment, there is little need to maintain the aquaculture for the third year of the project, that was declared for manuscript preparation and publication. It is also unclear how the colony rearing costs were accessed, as I failed to find the information about number of individuals in the culture. It is indeed hard to estimate how many individuals we get rearing the fish from the egg, but approximate amount should be given. Another note is concerning salary justification. I am aware about the limits PRELUDIUM impose on PI remuneration, but I found that 500 zł for the technical assistant remuneration is to low, considering the load of work and level of project engagement.

5. **Strengths of the proposal:**

The project is relatively cheap and methodology is not unnecessary complicated. It has little of risks, most of which could be effectively mitigated. The subject animals will be treated with an accordance to 3R rule. The results of the research are relevant regarding to actual scientific problems and applicable at both local and international level.

6. **Weaknesses of the proposal:**

The main weaknesses of the proposal are the unclear fish colony mitigation and lack of educational component. The contribution in scientific progress and possible economic applications are valuable elements of the research, but one of the main goals of science is to present the knowledge to the public, and in my opinion publications in specialized scientific journals are not enough. Thus I recommend authors to consider cooperation with universities or colleges, by taking a bachelor or master student as an assistant. This way they'll be able to obtain technical support and, at the same time, – promote the research in the academic circles, and maybe even general public.

Failasuf Nugroho

Simplified form for grant proposal review

Title of the project: Deciphering biological functions of astaxanthin in Atlantic salmon *(Salmo salar)*

1. **Assessment of scientific quality of the research project** (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)

The idea provided regarding coloration is very interesting. We often prefer to eat salmon with red color, but we don't know whether salmon benefits from the presence of this color. The project investigating the biological function of astaxanthin in Salmon has good scientific quality. Overall, the research is written well with good flow and construction that builds logically. The hypotheses are well-stated by connecting muscle physiology, oxidative stress, and immune function. However, I missed **results expectations from the hypotheses**. The originality of the project comes when it integrates behavioral ecology, physiology, and immunology. However, some parts would benefit if stated in more detail. For example **regarding the tasks**, it would be helpful if you also mention in a table about commercial analytical labs that will be involved in the project, and at which times they will be involved. Additionally, it would be **important to state the preparation time** to build your equipment; in this case, I would assume that you will build the equipment yourself. I wonder that the project would generate a lot of data requiring many statistical analysis processes, so it would be clearer if you state briefly on the statistical analysis connecting to the hypotheses.

2. **Assessment of potential impact of the research project** (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)

Since, the project integrates many domain of field biology, the findings are potentially useful both fundamental physiology research and applied aquaculture practices. Publications resulting from this work would likely appear in a good journals in disciplines evolutionary biology to aquaculture. In the global perspective, the findings may implied on the methods for evaluating fish welfare and health. It may also increases people awareness on this function and consequences of coloration in Salmon. However, I would mentioned a minor things that you stated that the findings may help farmers on diet supplementation strategies. In which way findings you would convince the farmers? (*minor*).

3. **Assessment of feasibility of the research project** (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)

The project is feasible, with a well-designed experimental approach and well-managed risk mitigation strategies. However, I am not certain how you will manage the equipment for the experiments, as well as the fish-rearing process. You stated that you will grow the fish from eggs to 1 year old, but is that sufficiently mature for your sample needs? Additionally, how many eggs will you start with, and how many individual fish do you expect to obtain from them? Regarding hypothesis 1, if you place 10 samples in a swim tunnel, what will be the dimensions of the tunnel? I can imagine that if 10 fish swim together in a tunnel against the current, some fish might position themselves behind others, or one fish might lead while others follow behind to reduce drag. Would this positioning affect your measurements of metabolic rate?

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The presented budget justification is quite comprehensive and well-detailed. There are a couple of parts where additional information might further strengthen your justifications. First, the equipment section could benefit from some clarification regarding the custom-built devices. You presented a good picture but it would be helpful to understand if you put also size of the equipment, because so far would be hard to imagine it. Second, it might be worthwhile to expand the indirect cost section with a bit more justification for these expenses.

5. **Strengths of the proposal**

- The project is well-written in terms of structure, background, and hypotheses
- Hypotheses are clearly defined
- It has an integrative approach in the field
- Good identification and assessment of risk management
- Methodology is well-written

6. **Weaknesses of the proposal**

- Minimal information on the facilities and dimensions of the equipment for experiments
- Only one species involved in the experiment
- Limited international collaborators
- Statistical analysis is unclear

Patrycja Wąchała

Simplified form for grant proposal review

Title of the project: **Deciphering biological functions of astaxanthin in Atlantic salmon (*Salmo salar*)**

1. Assessment of scientific quality of the research project

The research plan uses previously used and described experimental set-ups, but with modifications and to explore new things. It addresses the scientific gap by showing that similar things have been researched in other taxa but not in fish, and that the role of astaxanthin has been previously researched in fish but in a different aspect.

It also proposes two alternative explanations for the second question, which are not necessarily mutually exclusive - which is good, because if one hypothesis is disproved, there is still an alternative reason for research.

2. Assessment of potential impact of the research project

In my opinion, the part about the significance of the project is well written as it addresses the existing knowledge gap. However, most of this part focuses only on filling this gap, while there is only one sentence (the last one) talking about the impact on the wider society (and one sentence about the salmon market in the scientific aim part). I suggest adding a few more sentences saying why this research might be important and/or useful to people outside science, e.g. how popular salmon is as food, maybe there is research on assessing the health of fish in aquaculture (this proposal suggests that the results can improve fish health, so does this mean that fish are currently in poor health?)

3. Assessment of feasibility of the research project

The whole experiment seems complicated and difficult, but it is described in such a clear and understandable way that the authors convinced me that they could do it well. I am only surprised that they want to build the experimental set on their own, without the involvement of experts in building such things. Perhaps it would be beneficial to at least add consultations with people experienced in building such experimental sets? To make it more reliable.

Methods are well-matched to the goals.

The "risk analysis" section shows that the authors have given a lot of thought to the plan, as it addresses various possible problems and proposes relevant solutions to avoid them.

I feel confused about the research team - in the "work plan" and the Gantt chart description, there are only the Principal Investigator (PI) and the lab technician. But then in the research team there are 3 PhD students and in the budget we can see a salary for 3 students and a lab technician. So there are 3 PIs, not one? Does this mean that the work in the Gantt chart, which is done by the PI, is divided between 3 people? Or is only one person responsible for it? I think that if there are 3 PIs, the Gantt chart should include this information, because someone might nitpick about the impression that only one PI is working and the other two are getting salaries without doing any work.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?

The costs are well described, broken down into specific experiments and well justified, even the responsibilities of the research team.

I would just add some more information about conferences to make it more reliable - maybe one or two examples of conferences, where they were held, what the fee was, and estimates of travel and accommodation costs, because now it is just the total cost based on unknown assumptions.

5. Strengths of the proposal

In my opinion, the proposal benefits from a really well-written scientific aim of the project. The structure of the description makes it easy to read and understand the idea of the experiment. It is really important that the authors included the graphical representation of the experimental set, as it allows reviewers to visualise the whole concept.

And in my opinion, it was a good move to devote more than half a page to risk analysis and mitigation, as it shows that the authors have thought deeply about the experimental design

and are aware of potential problems and are trying to solve or avoid them at the planning stage.

6. Weaknesses of the proposal

For me, the weakness is that there is no information about obtaining permission to test on live animals. Maybe it is not necessary in the short version, but I think adding a short information about it would show the reviewer that the authors are aware of the need for such permission.

Also, in the Methods section, the authors have bolded the sentence "no fish will be euthanised". This is good information, but... One day the project will end. And then what? Will they just keep these salmon until they die? If so, they will have to maintain the colony longer than the duration of the project. Will you release fish into the wild? This is illegal. Will the fish be killed after the project? The authors should think about this and include this information in the proposal, because now it looks like they have not even thought about it.

Dr hab. Aneta Arct

Title of the project: Deciphering biological functions of astaxanthin in Atlantic salmon (*Salmo salar*)

1. **Assessment of scientific quality of the research project**

I find this to be a scientifically sound and well-structured project that addresses a clearly defined research gap. The role of astaxanthin (Ax) in Atlantic salmon physiology particularly during their demanding migratory journeys has not yet been comprehensively explored. The hypotheses are well-grounded in relevant literature and build upon previous findings from both aquatic and mammalian models. The methodology effectively integrates experimental physiology, oxidative stress biology, and immunology in a coherent and realistic manner.

2. **Assessment of potential impact of the research project**

In my view, this topic holds broad ecological, physiological, and applied significance, with direct implications for aquaculture practices. By integrating life-history theory with physiology and immune function, the project extends its relevance beyond fish biology alone. If successful, I believe the results could lead to high-impact publications in journals focused on animal physiology, aquaculture, and ecological immunology. The interdisciplinary approach combining behavior, physiology, and immune response further enhances its international appeal.

3. **Assessment of feasibility of the research**

I consider the project design ambitious yet realistic, supported by a detailed methodology and a clear timeline for task allocation. The risks have been thoughtfully identified, and the proposed mitigation strategies appear plausible. The with analytical labs for HPLC, and a strong emphasis on fish welfare all contribute to the project's high feasibility. The inclusion of swim tunnel assays and standardized protocols for immune and oxidative stress markers demonstrates the team's technical competence.

4. **Are the costs to be incurred well justified with regards to the subject and scope of the research?**

The cost structure is highly appropriate for the scope and ambition of the project. The only omission is the cost of purchasing a qualified electronic signature.

5. **Strengths of the proposal**

I find several key strengths in this proposal. The project addresses an understudied yet ecologically significant physiological trait, which I believe provides a strong foundation for meaningful scientific contributions. In my assessment, the use of well-established methodologies ensures reliability and reproducibility, strengthening the validity of the expected results. I appreciate the thorough literature review and integration of existing knowledge, which solidifies the project's conceptual framework. The emphasis on ethical research practices aligns with modern standards in animal research. The proposal presents a realistic and well-planned approach, with clear contingency measures, which I consider crucial for successful execution. I see high value in the project's ability to advance fundamental science while also offering practical applications in aquaculture.

6. **Weaknesses of the proposal**

The immune function analysis, while relevant, relies on a limited set of markers (phagocyte activity, cytokine expression, and BKA). Inclusion of additional immune parameters e.g., gene expression profiling could enhance mechanistic insights. The project could also benefit from the inclusion of additional molecular tools such as RNA-seq, which would allow for a comprehensive analysis of gene expression changes associated with astaxanthin supplementation. This would provide broader insight into the systemic physiological responses beyond the selected oxidative stress and immune markers revealing specific molecular pathways involved in stress resistance, metabolic regulation, and immune modulation. Such data would considerably deepen the mechanistic interpretation of the findings and increase the publication potential of the project. The statistical approach described linear mixed models followed by ANOVA with Satterthwaite's correction may not fully utilize the complexity of repeated-measures data. More advanced techniques (e.g., generalized linear mixed models, multivariate analyses) and explicit testing of interaction effects would strengthen the analysis. Overall, this is a strong, promising project with high potential for international impact. The weaknesses noted are minor and do not substantially affect the feasibility or scientific relevance of the proposal.

Final version

Title: Deciphering biological functions of astaxanthin in Atlantic salmon (*Salmo salar*)

Authors:

Napat Emdee, Rama S. Krovi, Jakub P. Płachta

Summary

Ever wonder how Atlantic salmon pull off the epic upstream journeys? It's not just willpower that drives them. These species have been studied on the genetic and physiological factors that aid them and we want to dig deeper into a secret or not so secret weapon: the astaxanthin (Ax), the vibrant orange-pink pigment which gives them their

beautiful hue based on which you try to choose for dinner. Think of it as more than just the salmon's food colouring. We suspect that it plays a vital role in their incredible migrations as we already know from previous studies that they accumulate the Ax in the muscles. We hypothesize that this aids in the protection against the stress of their powerful swims. We also know that the salmon's muscles naturally break down during their journey releasing some of the Ax into their bloodstream acting like an antioxidant. This could also help boost their immune system, making them tougher against the bacteria and other parasites they encounter during their journey. To investigate this, we will raise two groups of Atlantic salmon with and without Ax in their diet, simulate the migration to see how the Ax levels affect their swimming stamina, oxidative stress and their immune response. This study will solve the mystery about Ax's precise role in these long-distance migrations. We could protect the salmon by protecting those little Ax-rich crustaceans they eat. It would also lead us to better ways of raising salmon in fish farms making them healthier, more resilient and resistant to infections.

SHORT DESCRIPTION OF THE RESEARCH PROJECT

1) Scientific goal of the project

Many members of the Salmonidae family are capable of traveling astounding distances (up to 3000 km) in order to reproduce in freshwater, a behaviour known as anadromy. This journey constitutes a major strain on their physiology, resulting in degradation of muscle and mobilization of lipids and proteins (Bowerman et al. 2017; Johnson et al., 1997). This may result in increased oxidative stress, and in turn increase susceptibility to pathogen infection by impairing the immune system (Wilson et al., 2014). Crucially to this work, muscle tissue is also the main storage of astaxanthin (Ax) (Rajasingh et al., 2007), a carotenoid attached to α-actinin in myofilament (Matthews et al., 2006), which gives salmon flesh its signature colouration.. Exercise-induced muscle degradation results in a release of Ax into the bloodstream. Ax possesses potent antioxidative properties, which may then be used to scavenge reactive oxygen species (ROS) released during the migration effort. While the role of carotenoids in the skin for mate choice is well-described (Lehnert et al., 2016), the biological role of Ax storage in muscle and its subsequent presence in blood remain poorly understood. Two questions crucial for filling this gap are (1) the effect of Ax on the muscle tissue, which we hypothesize to has a beneficial influence on endurance during migration by reducing exercise-induced oxidative stress (H1); (2) the utilization of Ax in the bloodstream - here we propose that Ax helps prevent oxidative stress by scavenging harmful ROS (H2a). Alternatively, Ax enhances the immune response of the fish, improving its resistance to pathogen infection (H2b). Given this, we predict that Ax-supplemented salmon, compared to Ax-deprived ones, will show higher swimming performance and lower oxidative stress during intense exercise (P1), as well as lower oxidative stress and higher immune activity and gene expression after prolonged exercise and fasting (P2a and P2b).

2) Significance of the project

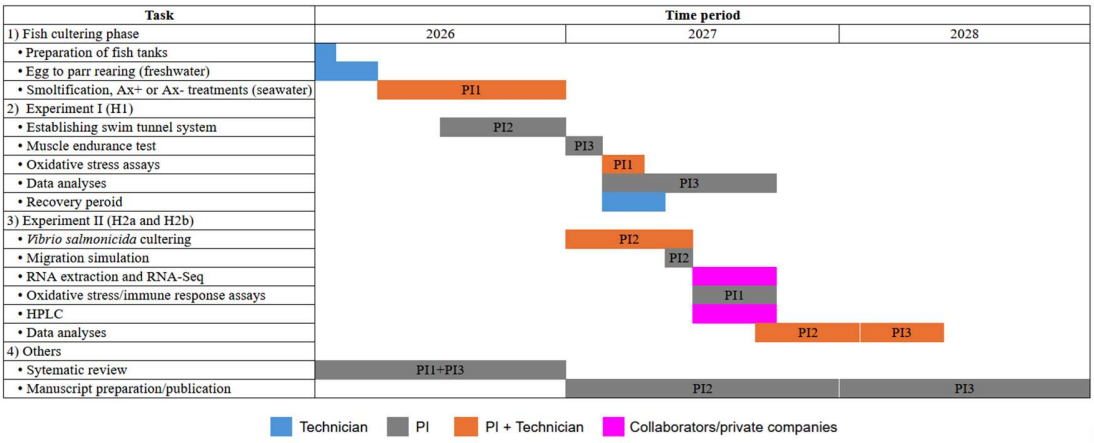
Salmonid fish hold a significant ecological and economic value. Their Ax-based vibrant pigmentation is an important trait whose evolutionary and physiological drivers remain sparsely understood. Studies in humans and rodents demonstrate the beneficial effects of Ax on muscle endurance (Polorov et al., 2014; Liu et al., 2018). These effects are attributed

to Ax's ability to prevent redox imbalance (Polorov et al., 2014), alongside its role in improving insulin sensitivity and glucose metabolism (Ishiki et al., 2013). However, the functional significance of the substantial Ax accumulation in the muscle tissue of salmonids remains poorly understood, especially in the context of their energetically demanding migratory behaviour, which leads to muscle degradation and a redistribution of energy reserves in the forms of lipid and protein (Jonsson et al., 1997; Bowerman et al., 2017). Only a few studies have addressed the potential association of Ax on salmonid muscle tissue, for example, by showing its role in upregulating the glycolytic gene *fbp1* (Schmeisser et al., 2021) and its binding capacity to α-actinin in myofilament (Matthews et al., 2006). Our project will advance this area of research using state-of-the-art swim tunnel experiments (Prescott et al., 2023; Remen et al., 2016) to test the effect of Ax on muscle endurance (H1).

Beyond its role in muscle physiology, Ax exhibits antioxidant effects in systemic circulation and may enhance immune responses (Park et al., 2010; Does et al., 2016). These properties not only contribute to disease prevention and delayed aging in humans (Ekpe et al., 2018), but also promote growth and health status in aquatic species (Lim et al., 2022). Evidence suggests that oxidative stress (Wilson et al., 2014) and pathogen burden (Dolan et al., 2016; Chapman et al., 2020) increase significantly during the spawning migration of salmonid fish. Our project will therefore explore Ax's protective role in reducing systemic oxidative stress (H2a) and enhancing immune function (H2b) under simulated migration conditions. This approach integrates various research fields, including behavioural evolution, life-history theory, physiological ecology, and immunology. This research may help in wild salmon conservation through the protection of Ax-rich crustacean populations, present at the well-mapped salmon feeding grounds. Additionally, our findings could have implications for aquaculture practices for reduction in antibiotic use through Ax supplementation.

3) Concept and work plan

General work plan: The project spans three years. The first phase will focus on culturing Atlantic salmon (*Salmo salar*) with or without astaxanthin (Ax) supplementation. After approximately one year of rearing, the hypothesis-testing phase will begin using salmon at the smolt stage, when they are physiologically prepared for migration. The same groups of fish will be used to test both H1 and H2a/H2b in chronological order. H1 aims to investigate the specific role of Ax within muscle tissue. Fish will be subjected to forced exercise in a swim tunnel to assess muscle endurance, and blood samples will be collected before and after the exercise to evaluate oxidative stress levels associated with short-term muscle activity. H2a and H2b will explore the systemic role of Ax outside the muscle. Migration will be simulated by generating a constant water flow in tanks over the course of one month, inducing muscle degradation and promoting the release of Ax into the bloodstream. Blood samples will then be analyzed to assess oxidative stress markers and immune responses. Tasks are allocated as shown in the Gantt chart.



Risk analysis: The list of possible risks and mitigation strategies is addressed in the below table.

Risk and description	Mitigation strategy
Biological variability Variation in individual fish response to dietary treatments and experimental conditions is a potential risk.	Mitigation strategies include using a robust statistical analysis.
Bacteria maintenance Maintaining a viable culture of *Vibrio salmonicida*	Establish a culturing protocol or plan for sourcing parasites from external laboratories
Technical issues Equipment malfunctions (e.g., swim tunnel, respirometer, ELISA reader) could delay experiments.	Regular maintenance and having backup equipment, great collaborators and access to alternative facilities will mitigate this risk.
Colony health issues Infections and other health concerns, machinery-related deaths or injuries to the animals	Comprehensive veterinary care for the fish (during and between experiments), all moving parts of the machinery will be sealed and protected from fish access
Procedure-related stress The experiments may cause stress or harm to the fish.	We will adhere to strict animal care protocols, including anesthesia and biopsy procedures, and monitor fish health closely. We will also optimize experimental conditions to minimize stress.
Dietary effects The experimental diets, while designed to isolate the effects of Ax, could have	We will carefully formulate and analyze the diets, and include control groups to account for any non-carotenoid effects.

unintended consequences on fish physiology.	
Sampling stress Repeated sampling (e.g., blood draws, muscle biopsies) could stress the fish and affect the results.	We will optimize sampling procedures to minimize invasiveness and frequency, and allow for adequate recovery periods.

Public engagement and collaboration: We intend to foster public engagement and knowledge sharing through collaborations with Polish and also Scandinavian universities (such as the Aquatic Food program at the Norwegian University of Science and Technology), offering students internships on the new techniques we will use in our projects. We would like to add science communication during Biologists' Night which happens every year in Poland. We also plan to share our results and write popular articles with robust social media engagement, through engaging lectures, interactive exhibits, and accessible online content.

4) Research methodology

Culturing: The study will be conducted on Atlantic salmon (*Salmo salar*). All animal procedures will be subject to ethical approval. The fish will be reared from the egg stage in four tanks (5 m^3) with: constant temperature (15 °C); filtered, recirculated water; 24:0 L:D photoperiod until smolt stage (1 year). To obtain 100 fish for the experiment, we will start with 5500 eggs, based on a 1.8% survival rate to the smolt stage in domesticated Atlantic salmon (Skaala et al., 2019). During the first 3 months, the fish will be fed with a standardised crushed cod pellet diet, twice per day, to satiation. After 3 months of growth, we will replace the water in the tank with seawater (35 ppt). Fish in two tanks will continue to be fed with a standardised diet (Ax- group), while the other two tanks will be supplemented with astaxanthin (Ax+ group, 100 mg/kg). After 1 year, we will tag the dorsal fin of each individual for identification and the fish will be ready for experimental procedures. We specifically chose non-lethal methods so **no fish will be euthanized** for this project. All assays will be done using commercially available kits. At the end of the project, the entire colony will continue being maintained.

Experiment I (H1): 50 Ax+ fish and 50 Ax- fish will be used in this experiment. 10 individuals per replicate, with 5 replicates for each group, will be randomly selected to assess muscle endurance, as swimming performance is appropriately measured in groups (Remen et al., 2016). Specifically, 10 individuals will be placed in a swim tunnel with internal diameter of 36 cm (Figure 1) left to acclimate overnight. Then, critical speed (*Ucrit*; Bret, 1964) and time-to-fatigue (Nikora et al., 2002) as well as maximum metabolic rate (MMR; Prescott et al., 2023) will be measured. Before and after exercise, blood samples will be taken (non-lethal sampling) for oxidative stress analysis: oxidative damage (dROMs), antioxidant capacity (OXY-adsorbent) and lipid peroxidation (TBARS). This procedure will be repeated until all 100 fish will be measured.

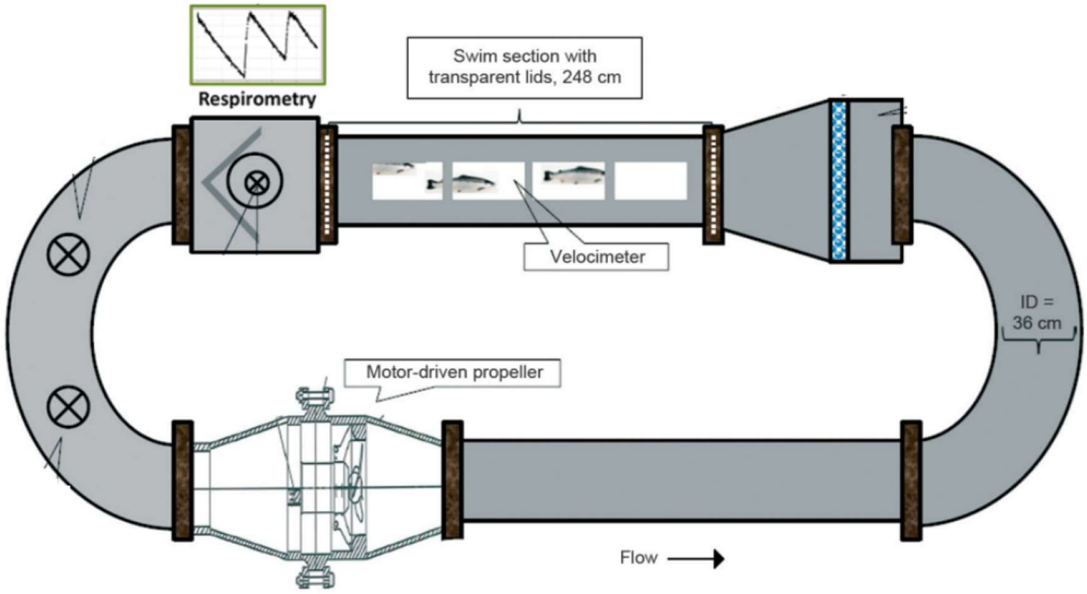


Fig. 1. Conceptual drawing of the large swim tunnel. ID: inner diameter

Experiment II-A (H2a): All fish used in experiment I will be anaesthetised and blood samples will be collected from the caudal vein. A muscle biopsy will be performed (dorsal muscle, non-lethal; Henderson et al., 2016) and the fish will be left to recover for 7 days. Then, all smolts will be placed into a common tank with a constant generated current (1.2 m/s) and fasted there for 2 weeks. These conditions mimic the effort required for long-period oceanic migration. After 2 weeks, blood and muscle sampling procedures will be repeated. Ax levels in muscle and blood will be assessed with the use of HPLC. This analysis will be outsourced to a commercial analytics lab, since equipment necessary for this analysis is prohibitively costly.

Oxidative stress will be assessed in the same way as in experiment I.

Experiment II-B (H2b): For this analysis, we will use blood obtained from experiment II-A (before and after experimental procedure). Activity of the immune system will be assessed for: (i) phagocyte peak respiratory burst using luminol-enhanced chemiluminescence; (ii) cytokine expression using ELISA; bacterial killing assay (BKA) using *Vibrio salmonicida*, a common salmon pathogen; (iii) transcriptomic changes of immune genes using RNA-seq. RNA-seq will be outsourced to a commercial analytics lab.

Statistical analyses:

Measured variables will be used as dependent variables in generalised linear mixed models (GLMM) with diet as a grouping factor (experiment I) or astaxanthin levels as a covariate (experiment II). Fish ID will be used as a random factor to account for repeated measurements. A multivariate analysis will also be performed to explore the relationships between our variables and identify patterns of interrelated variables, such as the oxidative stress affecting overall immune response. H1 (increased endurance) would be supported by a reduction in oxidative damage/the TBARS assay or an increase in antioxidant capacity. Increased critical swimming speed, time-to-fatigue and maximum metabolic rate would also support the first hypothesis. Ax as a bloodstream antioxidant (H2a) should result in

similar patterns in oxidative stress parameters as for H1 after exercise. Increased resistance to pathogens (H2b) would be supported by an increase in phagocyte respiratory burst, cytokine expression, decrease in bacteria survival rate (BKA) or increased immune system gene expression (RNAseq) after exercise. All these results should be correlated with an increased level of Ax in either muscle or blood samples.

5) Composition and qualifications of the research team,

PI1: Napat Emdee: 1st year PhD student with a background in stress responses in laboratory rats and tardigrades using RNA-seq

PI2: Rama Sarvani Krovi: 3rd year PhD student with experience in molecular techniques, lab management, data analysis and paper writing. Following is the publication list.
https://doi.org/10.1111/imb.12973
https://doi.org/10.1007/s10531-024-02940-8
https://doi.org/10.1038/s41598-018-20946-5

PI3: Jakub Płachta: 2nd year PhD student, experience in oxidative stress research in birds including oxidative stress assays and data analysis.

6) Project literature

Bowerman TE, Pinson-Dumm A, Peery CA & Caudill CC (2017) Reproductive energy expenditure and changes in body morphology for a population of Chinook salmon *Oncorhynchus tshawytscha* with a long distance migration. J*ournal of Fish Biology 90:* 1960–1979. https://doi.org/10.1111/jfb.13274

Brett JR (1964) The respiratory metabolism and swimming performance of young sockeye salmon. *Journal of the Fisheries Research Board of Canada 21*: 1183–1226. https://doi.org/10.1139/f64-103

Chapman JM, Teffer AK, Bass AL, Hinch SG, Patterson DA, Mikker Km & Cooke SJ (2020) Handling, infectious agents and physiological condition influence survival and post-release behaviour in migratory adult coho salmon after experimental displacement. *Conservation Physiology 8(1)*: coaa033. https://doi.org/10.1093/conphys/coaa033

Dolan BO, Fisher KM,Colvin ME (2016) Innate and adaptive immune responses in migrating spring-run adult chinook salmon, *Oncorhynchus tshawytscha*. *Fish & Shellfish Immunology 48*: 136–144. https://doi.org/10.1016/j.fsi.2015.11.015

Dose J, Matsugo S, Yokokawa H, Koshida Y, Okazaki S, Seidel Y, Eggersdorfer M, Rimbach G & Esatbeyoglu T (2016) Free radical scavenging and cellular antioxidant of astaxanthin. *International Journal of Molecular Sciences 17*: 103. https://doi.org/10.3390/ijms17010103

Epke L, Inaku K & Ekpe V (2018) Antioxidant effects of astaxanthin in various diseases—a review. *Journal of Molecular Pathophysiology 7(1)*: 1–6. https://doi.org/10.5455/jmp.20180627120817

Henderson CJ, Stevens TF & Lee SY (2016) Assessing the suitability of a non-lethal biopsy punch for sampling fish muscle tissue. *Fish Physiology and Biochemistry 42*: 1521–1526. https://doi.org/10.1007/s10695-016-0237-z

Ishiki M, Nishida Y, Ishibashi H, Wada T, Fujisaka S, Taikawa A, Urakaze M, Sasaoka T, Usui I & Tobe K. (2013) Impact of divergent effects of astaxanthin on insulin signaling in L6 cells. *Endocrinology 154*: 2600 –2612. https://doi.org/10.1210/en.2012-2198

Jonsson N, Jonsson B & Hansen LP (1997) Changes in proximate composition and estimates of energetic costs during upstream migration and spawning in Atlantic salmon *Salmo salar*. *Journal of Animal Ecology 66(3)*: 425–436. http://doi.org/10.2307/5987

Lehnert S, Pitcher TE, Devlin RH & Heath DD (2016) Red and white Chinook salmon: genetic divergence and mate choice. *Molecular Ecology 25*: 1259–1274. https://doi.org/10.1111/mec.13560

Lim KC, Yusoff FM, Karim M & Natrah FMI (2022) Carotenoids modulate stress tolerance and immune responses in aquatic animals. *Reviews in Aquaculture 15(2)*: 872–894. https://doi.org/10.1111/raq.12767

Liu SZ, Ali AS, Campbell MD, Kilroy K, Shankland EG, Roshanravan B, Marcinek DJ & Conley KE (2018) Building strength, endurance, and mobility using an astaxanthin formulation with functional training in elderly. *Journal of Cachexia, Sarcopenia and Muscle 9(5):* 826–833. https://doi.org/10.1002/jcsm.12318

Matthews SJ, Ross NW, Lall SP & Gill TA (2006) Astaxanthin binding protein in Atlantic salmon. *Comparative Biochemistry and Physiology, Part B 144*: 206–214. https://doi.org/10.1016/j.cbpb.2006.02.007

Nikora VI, Aberle J, Biggs BJF, Jowett IG & Sykes JRE (2003) Effects of fish size, time-to-fatigue and turbulence on swimming performance: A case study of *Galaxias maculatus*. *Journal of Fish Biology 63*: 1365–1382. https://doi.org/10.1111/j.1095-8649.2003.00241.x

Park JS, Chyun JH, Kim YK, Line LL & Chew BP (2010) Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans. *Nutrition & Metabolism 7:* 18*.* https://doi.org/10.1186/1743-7075-7-18

Polotov TG, Vardaris CV, Mihaliuc AR, Gonçalves MS, Pereira B, Ganini D & Barros MP (2014) Astaxanthin supplementation delays physical exhaustion and prevents redox imbalances in plasma and soleus muscles of Wistar rats. *Nutrients 6*: 5819–5838. https://doi.org/10.3390/nu6125819

Prescott LA, Symonds JE, Walker SP, Miller MR, Semmens JM & Carter CG (2023), Long-term sustained swimming improves swimming performance in Chinook salmon, *Oncorhynchus tshawytscha*, with and without spinal scoliosis. *Aquaculture 574*: 739629. https://doi.org/10.1016/j.aquaculture.2023.739629

Rajasingh H, Våge DI, Pavey SA & Omholt SW (2007) Why are salmonids pink?. *Canadian Journal of Fisheries and Aquatic Sciences 64(11)*:1614–1627. https://doi.org/10.1139/f07-119

Remen M, Solstorm F, Bui S, Klebert P, Vågseth T, Solstorm D, Hvas M & Oppedal F (2016) Critical swimming speed in groups of Atlantic salmon *Salmo salar*. *Aquaculture Environment Interactions 8*: 659 –664. https://doi.org/10.3354/aei00207

Schmeisser J, Verlhac-Trichet V, Madaro A, Lall SP, Torrissen O & Olsen RE (2021) Molecular mechanism involved in carotenoid metabolism in post-smolt Atlantic Salmon: Astaxanthin metabolism during flesh pigmentation and its antioxidant properties. *Marine Biotechnology 23*: 652–670. https://doi.org/10.1007/s10126-021-10055-2

Skaala Ø, Besnier F, Borgstrøm R, Barlaup BjørnTorgeir, Sørvik AG, Normann E, Østebø BI, Hansen MM & Glover KA (2019) An extensive common-garden study with domesticated and wild Atlantic salmon in the wild reveals impact on smolt production and shifts in fitness traits. *Evolutionary Applications 12*: 1001–1016. https://doi.org/10.1111/eva.12777

Van Den Thillart G, Van Ginneken V, Körner F, Heijmans R, Van Der Linden R & Gluvers A (2004) Endurance swimming of European eel. *Journal of Fish Biology 6 (2)*: 312–318 https://doi.org/10.1111/j.0022-1112.2004.00447.x

Wilson SM, Taylor JJ, Mackie TA, Patterson DA, Cooke SJ & Willmore WG (2014) Oxidative stress in Pacific salmon (*Oncorhynchus* spp.) during spawning migration. *Physiological and Biochemical Zoology 87(2)*: 346–352. https://doi.org/10.1086/674798

6) Table with budget of the project.

Item	**Funds for the each budget year (zł)**			
	2026	**2027**	**2028**	**Total**
1.Direct costs	369,177	187,588.50	187,588.50	744,354
1/Salaries and benefits	57,000	57,000	57,000	171,000
2/Equipment	123,000	0	0	123,000
3/Other direct costs	183,452	103,452	163,450	450,354
2.Indirect costs	72,634	36,318	36,318	145,270
Total costs (1+2)	441,811	223,906.50	283,906.50	949,624

7) Breakdown of project costs including justification and relevance for the tasks in the project

Salaries and benefits:

1. Salaries for three PIs: 36 months x 3 people x 1250 zł (Responsibilities: Constructing swim tunnel, performing experiments and biochemical assays, data analysis, manuscript writing, conference attendance)
   Total: **135 000 zł**

2. Technical assistant:

   Animal care: 36 months x 1000 zł (Responsibilities: maintaining fish colony, including experimental diet administration)
   Total: **36 000 zł**

Equipment:

1. Swim tunnel construction (respirometer, temperature, oxygen and flow rate control etc.):

a. Propeller = 20 000 zł

b. Respirometer = 5 000 zł

c. Camera = 3 000 zł

d. Flow meter, flow control, filter = 10 000 zł

e. Building materials and electronics = 5 000 zł

Total: = **43 000 zł**

Will allow for measuring endurance in salmon smout in Experiment I

2. Fish tanks (four grow tanks and one common tank with flow rate control): **80 000 zł**
   Necessary for housing the animals and performing exercise in Experiment II

Other Direct Costs:

1. Summary cost of oxidative stress analysis (dROMs, OXY-adsorbent, TBARS combined):
   a. Experiment I - 200 samples x 2 replications x 37 zł per sample = **14 800 zł**
   b. Experiment II - 200 samples x 2 replications x 37 zł per sample = **14 800 zł**
2. Analysis of astaxanthin concentration (HPLC, outsourced to a commercial analytics lab):
   a. Experiment I - 200 samples x 440 zł per sample = **88 000 zł**
   b. Experiment II - 200 samples x 440 zł per sample = **88 000 zł**
3. Summary cost of immunological assays (peak respiratory burst, cytokine expression, BKA):
   a. Experiment II - 100 samples x 2 replications x 120.5 zł per sample = **24 100 z**ł
4. Animal colony maintenance for 3 years (feed, electricity, repairs, cleaning materials, Ax supplement): **160 554 zł**
5. Qualified electronic signature: **610 zł**
6. PIT tags for fish identification: **1000 zł**
7. International conferences (2 conferences, attendance for all 3 PIs; travel, accommodation, conference fees): **60 000 zł**