Can a pet help to reduce allergy of its owner?

Do sponges have neurons?

Migratory and ontological consequences of artificial night lighting on Purple Martin

The mobilization of the glymphatic system in torpor and its association with the oxidative stress

Methodological Workshop in Evolutionary Biology for Ph.D. students - grant writing part

20-25 April 2023, Ochotnica Górna

Methodological Workshop in Evolutionary Biology- Grant Writing Part *is a course designed for doctoral students to develop the knowledge of the basic aspects of the preparation of a research project from the stage of hypothesis to writing applications. It will also bring awareness of how to assess research projects.*

In this course, the doctoral students will be able to formulate scientific hypotheses, develop a plan of a study and prepare its budget. They will learn skills to prepare a research project proposal to funding agencies, e.g. Preludium contest of the National Science Center. A student can write a review of the project.

In order to successfully complete this intensive course, the participants will be brought to Ochotnica Górna, where they will actively seek ways to solve scientific problems while working in interdisciplainary teams, valuing teamwork. Students will also learn to evaluate scientific projects of other participants in a constructive way and draws conclusions from the comments of others.

Cover prepared by Farzeen Saeed
Report prepared by Farzeen Saeed

TABLE OF CONTENTS

List of participants	01
Research topics proposed by participants	02
Can a pet help to reduce allergy of its owner?	05
Reviews	12
Final version	19
Do sponges have neurons? Insights into the Porifera 'neural' system through neuroimaging and genomics	27
Reviews	34
Final version	43
Migratory and ontological consequences of artificial night lighting on Purple Martin (*Progne subis*) - behavioral observations	51
Reviews	59
Final version	67
The mobilization of the glymphatic system in a state of torpor in an avian model and its association with the oxidative stress	76
Reviews	87
Final version	94

ORGANISER:

Prof. dr hab. Joanna Rutkowska	Institute of Environmental Sciences, JU

REVIEWERS:

Dr. Agata Plesnar-Bielak	Institute of Environmental Sciences JU
Prof. dr. hab. Pawel Koteja	Institute of Environmental Sciences JU
Dr. Edyta Sadowska	Institute of Environmental Sciences JU
Prof dr. hab. Mariusz Cichoń	Institute of Environmental Sciences, JU

PARTICIPANTS:

Mateusz Chechetkin	Institute of Environmental Sciences, JU
Martyna Gorkowska	Institute of Zoology and Biomedical Research, JU
Alaa Hseiky	Institute of Environmental Sciences, JU
Szymon Kantor	Institute of Zoology and Biomedical Research, JU
Martyna Marzec	Institute of Zoology and Biomedical Research, JU
Rebecca Miszczak	Institute of Botany, JU
Anastasiia Mykhailenko	Institute of Environmental Sciences, JU
Ekaterina Rostovskaya	Institute of Environmental Sciences, JU
Farzeen Saeed	Institute of Environmental Sciences, JU
Anbarieh Saadat	Institute of Zoology and Biomedical Research, JU
Patar Sinaga	Institute of Botany, JU
Michał Strączyński	Institute of Environmental Sciences, JU
Patrycja Dziurowicz	Institute of Botany, JU

RESEARCH TOPICS PROPOSED BY PARTICIPANTS

1. How to raise an alpha, neurobehavioral study on wolves (Anbarieh)
2. Comparison of pain pathways in Australian gympie-gympie stinging tree and New Zealand tree nettle toxins (Anbarieh)
3. What attracts Martins to our cars? (Michał)
4. Furred jaywalkers- assessing risk factors and levels for animals in heavily motorized urban area (Michał)
5. **The effect of light pollution on Great tits sleeping behavior during the breeding season (Martyna G)**
6. Long-term effects of repeated, but mild brain trauma – studies in an animal model (Martyna G)
7. Genotyping viruses in ticks (Martyna M)
8. Long-term effects of usage of hand sanitizers on the skin microbiome? (Martyna M)
9. What are you hiding? The secret life of (parasites in) large migrating carnivores (Ekaterina)
10. **Can a pet help to reduce allergy of its owner? (Ekaterina)**
11. **Do sponges have neurons? Insights into the Porifera 'neural' system through neuroimaging and genomics. (Anastasiia)**
12. How much biodiversity there should be in urban areas? (Anastasiia)
13. Effect of major histocompatibility complex genes on microbiome diversity – tradeoff or synergy? (Mateusz)
14. **Does stress promote hybridization? Effect of rapid environmental change on heterospecific mating preference (Mateusz)**
15. Characterizing the basis of transgressive segregation in *Sitpa* (Pattar)
16. Exploring the microbiome of rare *Mangifera* (mango) rhizosphere (Pattar)
17. Predatory behavior: a neurobiological study on artificially selected bank voles (Alaa)
18. Western diet and microbiome diversity. Obese versus non-obese prone animals (Alaa)
19. The low-gravity environment as a factor improving neuron regeneration process after traumatic spinal cord injury (Szymon)
20. Microplastic as a modern Trojan horse - a vehicle for more dangerous substances or a threat in itself? (Szymon)
21. The impact of microplastic and heavy metals on plant cell/tissues (Rebecca)
22. Influence of climate on cambium growth (Rebecca)
23. Conservation status, threats and perspectives of the Cladonia-Scots pine forest community in Poland (Patrycja)
24. Influence of environmental factors and seasonal changes of usnic acid in lichens Cl. mitis and Cl. uncialis occurring in forest habitats depending on high (Patrycja)
25. **The evolutionary consequences of artificial night lighting (Farzeen)**
26. **Knowledge gaps in understanding the mechanism of torpor (Farzeen)**

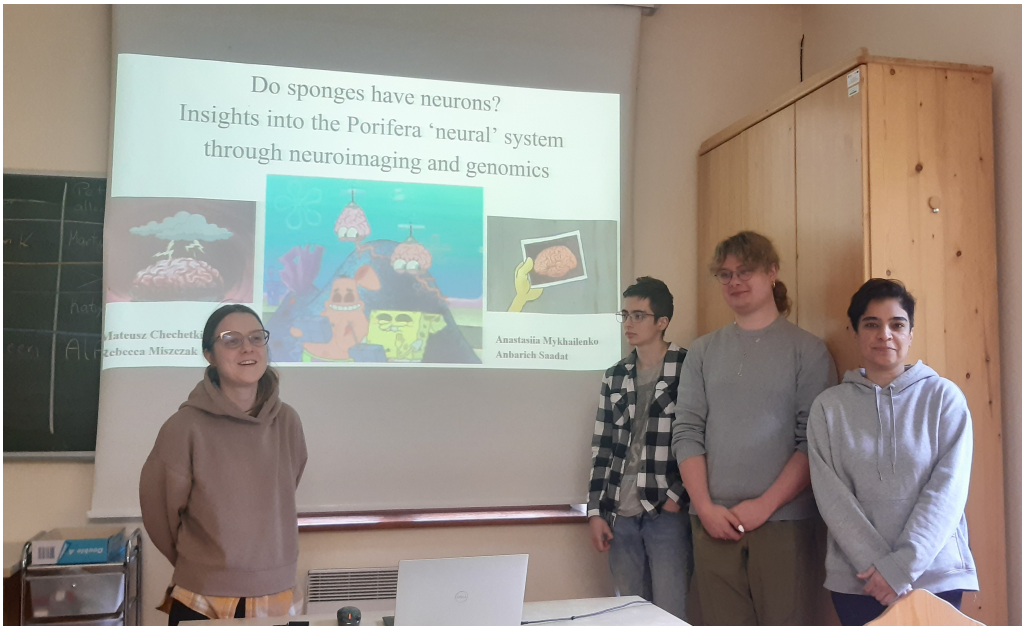
*Underlined titles were chosen by voting for further work during workshop

CAN A PET HELP TO REDUCE ALLERGY OF ITS OWNER?

Ekaterina Rostovskaya, Alaa Hseiky, Martyna Marzec

DO SPONGES HAVE NEURONS? INSIGHTS INTO THE PORIFERA 'NEURAL' SYSTEM THROUGH NEUROIMAGING AND GENOMICS

Anastasiia Mykhailenko, Mateusz Chechetkin, Rebecca Miszczak, Anbarieh Saadat



MIGRATORY AND EVOLUTIONARY CONSEQUENCES OF ARTIFICIAL NIGHT LIGHTING ON PURPLE MARTIN (*PROGNE SUBIS*)

Martyna Gorkowska, Patrycja Dziurowicz, Michał Strączyński

THE MOBILIZATION OF THE GLYMPHATIC SYSTEM IN A STATE OF TORPOR IN AN AVIAN MODEL AND ITS ASSOCIATION WITH THE OXIDATIVE STRESS

Szymon Kantor, Farzeen Saeed, Patar Sinaga

CAN A PET HELP TO REDUCE ALLERGY OF ITS OWNER?

Alaa Hseiky, Martyna Marzec, Ekaterina Rostovskaya

Abstract

Nowadays about 20-30% of population is allergic and this number is constantly increasing due to the climate change and air pollution. Allergic diseases decrease the life quality, may develop in negative symptoms, such as hay fever, conjunctivitis, asthma and anaphylaxis. Moreover, it increases chances of developing non-communicable diseases, such as cancer. Recent studies have shown positive impact of pet exposure on reducing food allergy of kids without genetic allergy background. Therefore, additional research on barely studied pollen allergies is required, in order to highlight the impact of pet presence on both children and adults. This study implements both sociological and biological approaches, with questionnaire highlighting the impact of various pet species and blood immunological test measuring its intensity. It can help to form public health policies and practices related to pet ownership and allergy prevention. The results can also provide guidance for families who are considering fostering pets from animal shelter.

SHORT DESCRIPTION OF THE RESEARCH PROJECT

1) Scientific goal of the project

Allergic reactions of a particular organism to typically harmless substances in the environment are caused by hypersensitivity of its immune system. It reacts to proteins of something common as it was a pathogen – by producing antibodies (Justiz Vaillant, Vashisht and Zito, 2023). Allergies, or allergic diseases, include rhinitis (hay fever); drug, food, and insect allergy; atopic dermatitis (eczema); allergic asthma and anaphylaxis (Pawankar *et al.*, 2013). The most common allergens include pollen and certain food products, such as egg, lactose or nuts (Okabe *et al.*, 2023).

Thus, we aimed to investigate the effect of pet presence onto human immune response on one of the least studied allergens – pollen. To meet this aim, we formulated the following hypotheses:

1. Pets reduce the incidence risk of pollen allergies.
2. Pet's effectiveness is dose-dependent, with negative correlation of pet number and/or weight with allergy risks.
3. Pet's effectiveness depends on the species, with cats and dogs reducing the incidence risk the most.
4. Pets decrease allergy probability in adulthood, but the effect is less than in infantile age.

2) Significance of the project

Nowadays, between 20-30% of the world's population suffers from some form of allergic disease, and their prevalence is rising dramatically worldwide. So, in Europe, one in four children is allergic (Pawankar *et al.*, 2013). Scientists are connecting this to increasing pollution of the environment and climate change (D'Amato and Cecchi, 2008; Lake *et al.*, 2017).

Climate change: Pollen allergies are highly seasonal, with most of the plant species releasing their pollen within the period of six month, from spring till autumn (*https://climate-adapt.eea.europa.eu/*, 2021). Aerobiological observations show that pollen map of Europe is changing due to human factor, such as introduction of new plant species and creation of green infrastructure, and climate change. It lead to the increase in the prevalence of pollen-induced allergies in Europe in the past decades (D'Amato *et al.*, 2007).

Climate change has been shown to influence pollen allergies in several ways. First of all, in response to global warming, plants shift their seasonal timing and release pollen earlier and for longer

pollination period. Moreover, in conditions of higher carbon dioxide concentration stimulating plant growth, plants may produce more pollen (D'Amato and Cecchi, 2008). Furthermore, climate change becomes the stimulus for plants' geographic shifts towards north, to the areas that were previously unaffected by allergens (D'Amato *et al.*, 2007). Additionally, climate change might affect atmospheric dispersion of pollen. Water droplets can capture pollen grains, causing them to fall to the ground reducing their concentration in the air. However, with more extreme weather and droughts resulting in decreased annual amount of rain (D'Amato and Cecchi, 2008; Lake *et al.*, 2017) more allergens stay in the air.

Molecular mechanisms of allergies: Allergic diseases are developing based on both genetic and epigenetic (environment-wide) mechanisms (Agache *et al.*, 2020). Ferreira *et al.* (2017) have shown genetic predisposition to the overreaction of the immune system (atopy). In response to allergen exposure, it secretes IgE antibodies (Pawankar *et al.*, 2013), which bind to specific receptors on mast cells and basophils, leading to the release of histamine and other inflammatory mediators. It causes various allergic symptoms such as
itching, swelling, and constriction of the bronchial tubes (Lambrecht and Hammad, 2015), for example, allergic rhinitis results from an IgE-mediated inflammation of the nasal mucosa (Pawankar *et al.*, 2013).

Type I immediate hypersensitivity reactions, mediated by IgE antibodies, are immune responses that are exaggerated or inappropriate against an antigen or allergen and occur within 24 hours. Their manifestations include systemic and local anaphylaxis such as hay fever, asthma, hives, food allergies, eczema (Justiz Vaillant, Vashisht and Zito, 2023). IgE antibodies are involved in the development of allergic sensitisation: repeated exposure to allergens leads to higher levels of IgE antibodies production and increase the risk of developing allergies. Therefore, elevated levels of allergen-specific IgE antibodies in blood serum can indicate sensitisation to specific allergens and are used to diagnose allergy diseases (Galli and Tsai, 2012).

IgA antibodies are involved in the regulation of the immune response to allergens. As component of the mucosal immune system, they play a vital role in protecting the body from pathogens and allergens. Studies have shown that allergen-specific IgA antibodies can decrease the risk of developing an allergy (Corthésy, 2013). By binding to antigens IgA antibodies prevent the attachment of allergens to mucosal surfaces and inhibit the activation of immune cells involved in allergic responses. If the production of IgA antibodies is dysregulated, the risk of allergy appear to increases (Schwitzguébel *et al.*, 2015), for example deficiencies in IgA production is associated with an elevated probability of developing food allergies.

Allergic diseases can be not only decreasing the quality of life, but also life-threatening, as in the case of asthma attack or anaphylactic shock. Asthma is a life-long chronic inflammatory disorder of the airways, which is still under-diagnosis and with inadequate treatment. Atopy is the strongest identifiable predisposing factor to the development of asthma (Pawankar *et al.*, 2013). When coming to anaphylaxis, it is a serious hypersensitivity reaction of both allergic and non-allergic etiologies. Systemic anaphylaxis is a massive allergic reaction (Justiz Vaillant, Vashisht and Zito, 2023), for which there is only emergency treatment by epinephrine (adrenaline) intramuscular injection (Pawankar *et al.*, 2013). In addition to symptoms, allergy causes long-term immune dysfunction, moreover, it has underlying inflammation which forms the factor for other non-communicable diseases (Pawankar *et al.*, 2013). As a result, people with allergies were shown to have an increased risk for some types of cancer (compared with the general population), such as lymphoma and myeloma (Merrill, Isakson and Beck, 2007; Cotterchio *et al.*, 2014). Nevertheless, for some types, including glioma and pancreatic cancer, risk rates decrease (Merrill, Isakson and Beck, 2007).

State of the art: To the best of our knowledge, many aspects of allergic diseases remain understudied (Pawankar *et al.*, 2013). For example, breastfeeding is known to play an important role in immune system of a baby, but still there is no strong evidence on its effectiveness in primary prevention of allergy (Mennini, Arasi and Fiocchi, 2021). So far, the main focus on allergy studies belonged to food allergies (Chen *et al.*, 2010) and asthma (Lodge *et al.*, 2011; Portnoy *et al.*, 2012),

which is especially surprising when counted that the incidence of food allergies in children is moreless 10% in developed countries (Okabe *et al.*, 2023) with pollen allergy symptoms registered in 40% of whole Europe population (Lake *et al.*, 2017). Food allergies and asthma are often studied in the context of cat and dog exposure (Ownby, Johnson and Peterson, 2002; Dharmage *et al.*, 2012). There are several reasons for this, and one of them is hygiene hypothesis (Okabe *et al.*, 2023), which sets a reverse link between presence of infections and immune disorders (Okada *et al.*, 2010). Longitudinal studies in urban populations suggest that perinatal pets exposure, especially dogs, may reduce the development of allergic disease in those without a family history of allergy (Lodge *et al.*, 2011). Nevertheless, in a complex of pet keeping and parental atopy to increase the risk of respiratory symptoms in children (Dong *et al.*, 2009), as a high-risk group to allergic diseases.

Despite sometimes people can be allergic to pet hair or saliva, in general having a home pet might decrease your immunological response to allergens. Pet-keeping reduces the risk of allergy in a dose-dependent fashion (Hesselmar *et al.*, 2018): allergic diseases were diagnosed in 32-49% kids of different age groups without pets exposure, and this percentage was decreasing with higher number of pets, up to zero allergic reactions in families with ≥5 pets.

There is little or no data about other than dog and cat pet species' influence on risk of allergy. However, such information can be crucial for better understanding factors and mechanism of therapeutic effect of pet exposure in developing allergies. The study performed in 2023 indicates that early life exposure to both cats and dogs reduce the risk of developing food allergies to specific products, for example egg or milk. In contrast such exposition to hamster significantly increased the incidence risk of nut allergy (Okabe *et al.*, 2023).

Pioneering nature of our research: 1) Pollen allergies remain majorly understudied, especially when taking into account the frequency of pollen allergies to food ones; 2) The majority of allergy studies are focused on kids, remaining unknown its development on adults and the factors affecting it; 3) There is a need for data on the influence of multiple pet species exposure, as so far there is data mostly for dogs, and cats.

This research seeks to study the effect of pets on reduction of immunological response to allergens, such as pollen, on both children and adults. It will prove the possibility of pet treatment for allergic diseases in adulthood and determine the most effective pet species for this.

3) Concept and work plan

Our previously defined hypotheses (1-4) will be tested in the context of 3 specific research objectives 1-3: (i) to identify the effect of various species exposure in reducing the incidence of pollen allergy, (ii) to determine the effectiveness of pet exposure in increasing IgA secretion in adults and kids, and (iii) to access the impact of pet exposure in IgE concentration in children and adults. To accomplish objective 1, there will be a questionnaire conducted, and followed by experiment with performed blood tests to meet objectives 2 and 3 (Fig.1). The experiment will last one pollination season (approximately from Mach till August).

To begin with, we develop a comprehensive questionnaire to gather data on various aspects related to pets, and children and adult allergies. The questionnaire will be designed based on existing templates utilized in similar studies. However, we will improve the questionnaire to address aspects previously overlooked, including a broader range of pets and both young and adult participants. Once the questionnaire is finalized, we will create a poster that displays the QR code to access the survey.

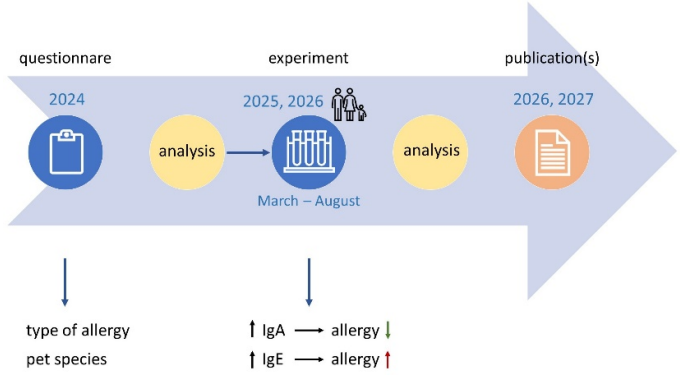


Fig. 1: The timeline of our project studying the protective effects of different species on the allergy of both adults and children

A survey allows us to collect a large amount of data, but it also has limitations in terms of control. Therefore, we plan to conduct a more controlled experiment (Fig. 2).

To recruit participants, we will add a question to our questionnaire asking families who have never fostered a pet and are either planning to foster a cat or a dog from an animal shelter or not planning to own a pet to contact us if they would like to participate. To encourage participation, we will register participants for a lottery and award a monetary prize to 10%
of the winners. The experiment will compare the protective effects of cats and dogs to pet absence on pollen allergy in both children and adults. This approach was planned because
dogs and cats are the most common pets, and studies on pollen allergies, particularly in adults, are not widespread.
Fig. 2: The experimental groups of our controlled experiment.
The expect that pets will decrease allergies, and this will be assessed by the increase in the concentration of immunoglobulin A while the decrease in the concentration of immunoglobulin E. This effect could be more detectable in the young compared to adults, and in females compared to males.
Preliminary research: The authors have prior experience conducting various types of questionnaires, although they have not previously worked specifically in the field of pets and allergies. In addition, they have extensive experience designing experiments and conducting complex statistical analyses, which will enable them to effectively analyze the data collected in this study. Furthermore, the authors have a diverse background that includes positions requiring strong communication, persuasion, and negotiation skills, which will be important in recruiting and engaging participants in the study. In addition, the authors have an extensive network of contacts and collaborators who they can consult for additional support and expertise if needed.
Risk assessment: To meet legal and ethical requirements, the study needs to be approved by the Human Research Ethics Committee and the permission to process human data by RODO. Also, written informed consent from all parents needs to be obtained. Nevertheless, there is no need in permits and licenses obtained from Ethics Committee, as no study on animals is directly conducted. Our study involves a potential risk of obtaining smaller number of blood samples in children, which may pose challenges in obtaining accurate data. Additionally, due to the nature of our registration process, we might not be able to find enough participants to achieve adequate statistical power for our analysis, which could limit the validity of our results. Another risk is that we may not achieve equal representation of all groups, which could impact the generalizability of our findings. Furthermore, there could be a possibility that families fostering the pet be sensitized to pet allergens. Questionnaires are based on the participants' responses.
We will carefully consider these risks and take necessary steps to minimize them, including ensuring proper consent procedures, monitoring enrolment efforts, and following all legal and ethical guidelines.

4) Research methodology

A questionnaire: Hypotheses 1-3 will be tested by using questionnaire on family allergy and their exposition to pets, which will be prepared based on knowledge of experimenters and similar questionnaires used in research papers referring topic of allergies. The questionnaire will be addressed to parents of toddlers and will be distributed to kindergartens localized in highly urbanized areas: Warsaw, Cracow, Katowice, Gdansk, Wroclaw, and non-urbanized areas: villages evenly distributed around Poland. Total number of 100 kindergartens will be involved in the study: 50 in urbanized areas and 50 in non-urbanized areas, 10 in each city/village respectively. The questionnaire will refer to 1) the first 3 years of the child life; 2) the parent's childhood and current status. It will include questions concerning: **i) allergy**: whether allergic or not, if allergic, what is the type (food/pollen), symptoms and severity of the allergy, and at what age it started manifesting; **ii) prenatal/postnatal conditions**: time of breastfeeding, maternal smoking; **iii) pet**: how many pets (0-4, or more), pet species, age of kid when exposed to the pet (natal/prenatal or if postnatal specify); **iv) sociological part**: place of residence (city/village), presence of siblings, family medical history of allergies (atopy).

In our questionnaire we enrich the number of pet species from several taxonomic classes. Respondents will choose from mammals, birds, reptiles and amphibians (with example illustrations) and later specify the species. The completed questionnaires will be collected when at least 1500 answers are noted or after a maximum of 6 months period after distribution. The data cleaning will be performed in appropriate program and statistical analysis will be performed in SAS software.

Experiment on plasma concentration of immunoglobulins IgE and IgA: This method will be used to test hypothesis 1 and 4 by assessing the effect of having a pet on the immunological response of pollen allergic individuals. Three timepoints will be checked to assess pet exposure time related effects. Our experiment will include 90 families (mother, father and 1-3 children) hiving indicated symptoms of pollen allergies but have never hosted an animal pet before. The families will be chosen in a way that 30 families are planning to foster a cat, 30 families are planning to foster a dog, while 30 animals are not planning to foster any pet and will be considered our control group. The pets to be fostered should come from animal shelters.

Three blood samples will be collected per individual by Venepuncture. This will be performed by a specialist private company with which we are going to collaborate with. The samples will be collected before fostering the pet (t=0 minutes), 1 week, and 1 month after fostering the pet. For the control group, t = 0 minutes will be determined so that the time of collecting the blood is consistent among the three groups. The blood will be stored in dry ice, transferred immediately to our collaborator's lab, and analysed to measure the concentration of immunoglobulins IgE and IgA. The protective effects of the pets would be assessed by the increase in the concentration of IgA, and the decrease in the concentration of IgE. To encourage people to participate in our experiment, and support their willingness to foster animals from shelter, we will make a lottery so that around 10 % of the families will get a monetary award in the form of a pre-paid credit card. Furthermore, to analyse the concentration of the immunoglobulins, linear mixed model will be used with the concentration as the dependent variable, pet fostering (cat, dog, none) and sex as the independent factors age and time of blood collection as covariate, and the family number as the random factor.

In the follow-up project, we are planning to repeat similar experiment but using the most effective animal species (not cats and dogs) on reducing allergy. This species will be determined using the results of our questionnaire. This will allow us not only to compare different mammalian species, but also to compare mammal class to other classes as Aves (birds) which have feathers and not fur. Furthermore, we are planning to check whether the protective effect of the pet exposure would be consistent among the years, and whether its mechanism would be used as allergy treatment.

5) Project literature

Agache, I. *et al*. (2020) 'Genetics and epigenetics of allergy', *Current Opinion in Allergy and Clinical Immunology*, 20(3), pp. 223–232.

Chen, C.-M. *et al*. (2010) 'The role of cats and dogs in asthma and allergy – a systematic review', *International Journal of Hygiene and Environmental Health*, 213(1), pp. 1–31.

Corthésy, B. (2013) 'Role of secretory IgA in infection and maintenance of homeostasis', *Autoimmunity Reviews*, 12(6), pp. 661–665.

Cotterchio, M. *et al*. (2014) 'Association between Allergies and Risk of Pancreatic Cancer', *Cancer Epidemiology, Biomarkers & Prevention*, 23(3), pp. 469–480.

D'Amato, G. *et al*. (2007) 'Allergenic pollen and pollen allergy in Europe', *Allergy*, 62(9), pp. 976–990.

D'Amato, G. and Cecchi, L. (2008) 'Effects of climate change on environmental factors in respiratory allergic diseases', *Clinical & Experimental Allergy*, 38(8), pp. 1264–1274.

Dharmage, S.C. *et al*. (2012) 'Exposure to Cats: Update on Risks for Sensitization and Allergic Diseases', *Current Allergy and Asthma Reports*, 12(5), pp. 413–423.

Dong, G.-H. *et al*. (2009) 'Pets keeping in home, parental atopy, asthma, and asthma-related symptoms in 12,910 elementary school children from northeast China', *Indoor Air*, 19(2), pp. 166–173.

Ferreira, M.A. *et al*. (2017) 'Shared genetic origin of asthma, hay fever and eczema elucidates allergic disease biology', *Nature Genetics*, 49(12), pp. 1752–1757.

Galli, S.J. and Tsai, M. (2012) 'IgE and mast cells in allergic disease', *Nature Medicine*, 18(5), pp. 693–704.

Hesselmar, B. *et al*. (2018) 'Pet-keeping in early life reduces the risk of allergy in a dose-dependent fashion', *PLOS ONE*, 13(12), pp. 1-13.

https://climate-adapt.eea.europa.eu/ (2021). European Climate and Health Observatory: European Environment Agency. Available at: https://climate-adapt.eea.europa.eu/en/observatory/evidence/health-effects/aeroallergens/.

Justiz Vaillant, A.A., Vashisht, R. and Zito, P.M. (2023) 'Immediate Hypersensitivity Reactions', in *StatPearls*. Treasure Island (FL): StatPearls Publishing.

Lake, I.R. *et al*. (2017) 'Climate Change and Future Pollen Allergy in Europe', *Environmental Health Perspectives*, 125(3), pp. 385–391.

Lambrecht, B.N. and Hammad, H. (2015) 'The immunology of asthma', *Nature Immunology*, 16(1), pp. 45–56.

Lodge, C.J. *et al*. (2011) 'Perinatal Cat and Dog Exposure and the Risk of Asthma and Allergy in the Urban Environment: A Systematic Review of Longitudinal Studies', *Journal of Immunology Research*, 2012, pp. 1-11.

Mennini, M., Arasi, S. and Fiocchi, A.G. (2021) 'Allergy prevention through breastfeeding', *Current Opinion in Allergy and Clinical Immunology*, 21(2), p. 216.

Merrill, R.M., Isakson, R.T. and Beck, R.E. (2007) 'The association between allergies and cancer: what is currently known?', *Annals of Allergy, Asthma & Immunology: Official Publication of the American College of Allergy, Asthma, & Immunology*, 99(2), pp. 102–116; quiz 117–119, 150.

Okabe, H. *et al*. (2023) 'Associations between fetal or infancy pet exposure and food allergies: The Japan Environment and Children's Study', *PLOS ONE*, 18(3), pp. 1-14.

Okada, H. *et al*. (2010) 'The "hygiene hypothesis" for autoimmune and allergic diseases: an update', *Clinical and Experimental Immunology*, 160(1), pp. 1–9.

Ownby, D.R., Johnson, C.C. and Peterson, E.L. (2002) 'Exposure to Dogs and Cats in the First Year of Life and Risk of Allergic Sensitization at 6 to 7 Years of Age', *JAMA*, 288(8), pp. 963-971.

Pawankar, R. *et al*. (eds) (2013) *White Book on Allergy: Update 2013*. World Allergy Organization.

Portnoy, J.M. *et al*. (2012) 'Environmental assessment and exposure control: a practice parameter—furry animals', *Annals of Allergy, Asthma & Immunology*, 108(4), pp. 1-27.

Schwitzguébel, A.J.-P. *et al*. (2015) 'Immunoglobulin deficiency in patients with chronic rhinosinusitis: Systematic review of the literature and meta-analysis', *The Journal of Allergy and Clinical Immunology*, 136(6), pp. 1523–15.

6)Table with budget of the project.

	Amount in PLN
Direct costs, including	346 979
- personnel costs and scholarships	244 500
- research equipment/device/software cost	13 979
- other direct costs	88 500
Indirect costs, including:	76 335
- indirect costs of OA	6 940
- other indirect costs	69 396
Total costs	**423 315**

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

Equipment

Laptop 4000 PLN

Biorender registration 99$/month= 99 x 4,20 PLN x 24 months= 9979 PLN

Consumables

Printing 12 PLN/poster x 100 kindergarten = 1200 PLN
Lottery prize 100 PLN x 9 prizes = 900 PLN
Blood analysis = IgA-40 PLN + IgE-30 PLN = 70 PLN/person x 4 family members x 90 families x 3 tests/person = 75600 PLN

Personal and Field Travel/Accommodation/Subsistence

Medical personal (blood samples collection) = 2500 PLN x 3 months = 7500 PLN
Travel costs for samples collection = 3300 PLN

Employment

Salaries for PI = 2000 PLN/month x 36 months x 3 PI = 216000 PLN
Publication 3,000 PLN
Conference = 8500 PLN per PI, each person presents at different conferences; total of 25500 PLN.
We have explored the possibility of performing the immunoglobulin tests in-house but found it to be more expensive. For instance, an Elisa kit required to measure the concentration of IgE for only 96 tests costs around $700 (3360 PLN), which translates to a cost of 35 PLN per test, exclusive of equipment, electricity, or labor expenses. Instead of this, we sign a contract with a company, and pay for the blood collection and analysis.

REVIEWS

Dr. Agata Plesnar Bielak

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 project explores an important topic of allergies. The authors intend to investigate how fostering a pet influences severity of pollen allergies. The project extends previous research on that topic done in the context of food allergies, but does not go beyond correlative description of the problem. The authors do not discuss any hypotheses about the mechanisms behind protective effect of pets on allergies (except from hygiene hypotheses, which is also only mentioned and no causal link is provided). No potential mechanisms are going to be tested. In consequence, the project will improve our understanding of the investigated phenomenon to a limited extend. On the other hand, the results project could deliver data that may serve to formulate such hypotheses, as it will allow for the comparison of patterns observed for two different types of allergies (inhalant vs. food). It would be good to know what are the predictions here: are the effects on of pets on pollen allergies expected to be higher or lower compared to food allergies? Given the fact that pollen and animal fur are both inhalants, do you expect that the protective mechanisms of owning a pet would be different than in case of food allergies? Shall we expect people with pollen allergies to develop fur allergies more often?

Comment about a structure of the proposal: Description of the field is quite extensive, but a little bit outside the context of the proposed research (for example the part about the climate change). The aims of the study are clear, although ho they will be obtained is not known from the first parts of the proposal.

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 project has some innovative elements, including investigation of age impact on the association between keeping a pet and suffering from allergies. The results can potentially be published in a recognizable, middle-impact journal. The research on pollen allergies (rather than food allergy) is an important aspect of the proposal.
Also, the project does not test for casual links and molecular mechanisms (see above), except from measuring IgA and IgE levels.

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 and the planned tasks can be realized on time. I am not sure if one season (and three blood samples within only a month) is enough for the project to fully

fulfil its aims. A longer time framework would certainly be helpful in seeing time patterns of the studied relationships.
Project team is well prepared for conducting the project and they have necessary skills to analyze data and interpret the results.
The proposal lacks a risk management strategy. Although potential risks are enumerated, but the authors do not write how they will be addressed. Preliminary research is also not described. Instead, research experience of the PIs is discussed at this point.

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

Yes.
However, conference costs are not employment. It is also not clear what you mean by "Publication" cost.

5. **Strengths of the proposal**
   1. Addressing pollen allergy – one of the most common types of allergies
   2. Incorporating adults into the investigation

6. **Weaknesses of the proposal**
   1. Short timeframe of the experiment (also the authors do not describe how the time of the experiment will correspond the pollen season)
   2. Not addressing casual links and mechanisms behind the observed patterns
   3. Lack of risk management plan

Rebecca Miszczak

The general aim of the project is to investigate the effect of the presence of pets on the human immune response to pollen. The study will use a combination of sociological and biological approaches, including questionnaires and blood immunological tests, to determine the effect of various species of pets and their intensity on the human immune response to pollen. The authors raise the following questions: Do the presence of pets reduce the incidence risk of pollen allergies? Does pet effectiveness depend on dose? Do the effectiveness of the pet depends on the species? Does age matter in reducing the likelihood of having allergies when you own a pet? These questions are well defined and supported by the relevant literature. The project appears to have a strong scientific foundation. The authors provided a moderately comprehensive review (see Section 3) of the current state of knowledge on allergic diseases and their molecular mechanisms, as well as the role of climate change, demonstrating a good understanding of the background information necessary for the project.
The results of the project may potentially have an international impact on the scientific community and health care. The knowledge in this field is very scarce, as (the authors stated) main focus on allergy studies is concentrated around food allergies and asthma. However, in situation, where impact of pet/s occurs to be non-significant, results will not be able to be published in high-impact journals. Moreover, as is planned now, not all aims may be possible to achieve (see next section).

The project is very ambitious and requires very careful planning and extensive logistics. In my opinion, there are several issues that have to be tackled:
- The information provided in the questionnaire is too general. The choice of using a questionnaire is good as it will provide valuable information and this is a common method to use in this type of research. However, authors must take into account that

questionnaires can provide incorrect feedback (due to: people lying, questions can be interpreted differently, survey fatigue). The use of complicated language can also decrease the chances of acquiring quality data. It would be a good idea to add a part of the questionnaire with sample questions. However, due to the limited writing time of the grant, the choice made by the authors is acceptable.
- Part of the study has already been done. Chen et al. (2008; doi: 10.1183/09031936.00092807) investigated the effect of dog ownership during childhood on the development of allergy in the first 6 years of living. A sensitisation screening test was used to detect the presence of specific IgE antibodies against inhaled allergens. Having a dog during early childhood was associated with a lower occurrence of combined pollen and inhalant sensitisation. However, no correlation was found between dog ownership and dog sensitisation or allergy-related symptoms and illnesses up to the age of 6. Contact with dogs on a regular basis during childhood, without actually owning one, was not found to have any impact on these health outcomes. Additionally, no connections were discovered between exposure to endotoxins from house dust during childhood and sensitisation results.
- The sample size of the experiment on plasma concentration of immunoglobulins IgE and IgA (30 families per pet) is very small. Especially the age of the children may have a great effect on the response variable. As I am not an expert, I compare it to the sample size presented by Chen et al. (2008). The group cooperated with the study on the Human Immune System and the Development of Allergies in Childhood (LISA) and the German Infant Nutritional Intervention Programme (GINI). In total, there were 2252 babies with parents of siblings having allergy history. My suggestions would be to collaborate with a private company (it is already done in the project, but only by taking samples) to acquire more data. Private companies such as DIAGNOSTYKA have cooperation with many medical places where specialists take blood samples. Giving very small questionnaires such as 3-4 questions would not be a chore to a patient but is a possibility to increase sample size (Authors would have the possibility to contact such patients).
- In my opinion, using lottery as an incentive is not ethical - especially since the project has unlimited budget. In sociological science, it is standard to pay around 50 PLN per participant test. I would change this accordingly – all participants should get paid.
- There is also a possibility of collaboration with animal shelters and/or animal aid foundations to increase the number of sample size (especially for the IgE experiment, but also for the questionnaire). Before adoption, people can get a small questionnaire with invitation to participate in the project.
In conclusion, authors need to expand sample size using different methods, need to adjust ethical consideration, and make sure to produce simple, easy-to-understand questionnaire.
The cost of project are mostly justified. The choice of hiring an external company to take blood samples is very well designed. There are few things that authors should change in their budget. First of all, using Biorender for the whole project time is not reasonable, as it is only needed to produce high quality schemes/graphs. I would reduce to 3-4 months. As mentioned above, lottery is, at least in my opinion, not ethical and cost should be changed accordingly. Authors want a salary of 2000 PLN per month per PI. This sums up to 216000 PLN, which is about 50% of the total project costs. Considering that authors will only: make a questionnaire and distribute it online, hire a company to take blood tests, and data analysis – this, in my opinion, is too much. I would change it to 1000 PLN per month per PI.

The project tackles important questions and has the potential to produce high-impact results. It is rather well written, especially given a very short time period and carrier stage of authors and PIs not working in this field of science previously.

As weaknesses of the proposal, I recognise the small sample size (IgE experiment), moderate risk of not obtaining high quality data out of the questionnaire. The logistics of the methodology need to be improved.

Michał Strączyński

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

The proposal describes how changes in climate and lifestyle increases the risk of developing allergies and backs it up with sufficient sources in lines 31-48. The proposal explains the scientific importance of studying allergies in general as well as the potential biological impact for human health well in my opinion.
As much as a basic description of allergy types is useful for giving context, I do not believe going such detail of the molecular mechanisms of allergies as presented in lines 49-69 was necessary as upon reading the rest of the proposal I fail to see the connection between the proposed research and the details of allergic diseases.
The first mentions of species names appear in line 28 and lack the appropriate scientific names to go with them .The idea of testing the allergy-preventing nature of exposure to pet is not novel, however the proposal to expand the scope of animals tested beyond cats and dogs does sound like a reasonable progression from existing knowledge. Including the possibility of pet exposure reducing allergic reactions is a far reaching and risky but makes for an interesting possibility. Combining social and hard science approaches through the use of questionnaire and laboratory analysis of collected blood samples should allow for the desired comprehensive study of multiple groups, allergic and protective factors as well as possible symptoms, even if not amazingly original.
The planned use of a lottery (line 136) in order to entice participation seems problematic on legal and ethical grounds to me. Firstly, I have never seen lottery being used in this manner so I'm unsure if it factually increases engagement in a meaningful way and the project doesn't offer any evidence of this. Secondly, the use of a random lottery with no clearly communicated procedures raises questions of legitimacy and transparency of the rewarding process. Did the researchers consider the legal framework of organizing such lottery? Thirdly, I believe that in order for the participants to be treated fairly and to avoid potential legal risks, either all of the families should get something, or none should get anything. Is all this necessary? Authors thoughtfully describe most other potential risks and problems I could think of.

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)

Because most previous studies regarding the influence of pets was done with food allergies in mind, the focus this project brings to previously understudied pollen allergies increase its relevance and potential for future work upon its results. As shown in the references, the problem is widely known yet not very well studied. While not being particularly grand in its implications and expected results, the project is sufficient for producing a paper in a reputable journal. The experiments are at risk of not really proving anything, depending on the outcome but even in such case, the resulting paper might focus on exploring the use of combined sociological and biological methodologies in exploring important daily problems.

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, because of the proposed method of acquiring data through questionnaire and the authors expectation to cooperate with a private company (line 191) who will handle the blood collection for experimental part. This should allow them to accomplish the tasks in time. The general plan of the questionnaire is well structured, and the steps planned for laboratory analysis are achievable and explained well enough.
While the project provides a rough timeline (Fig 1), no precise timing or framework was given. We do not know how each step is supposed to take and when it is supposed to take place.
There is no literature provided for the sample sizes and I'm not sure if the ones proposed will be sufficient given the number of locations tested (Only 1500 questionnaires and 90 families spread over more than 5 highly populated areas) (Line 158).
I am unsure whether only collecting blood samples from what looks to be a group biased towards cat and dog owners will work well when confronted with the questionnaire answers which do not have such a limitation. Furthermore, the methodology does not mention or describe specific tools and reagents which can be used in the laboratory work part of the project. Lastly, there are no statistical models proposed, but there is mention of cleaning the data acquired, which shows some consideration of statistics was done.

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

The project predicts to enlist help from a private company which will reduce the workload placed on the research team.
The cost breakdown is in line with the steps described. Assigning most of the budget to salaries is also undesirable and the authors also want to make use of the biorender website for two full years which seems as an excessive duration.

5. Strengths of the proposal

Using and interesting methodology combining different approaches, the project touches on an increasingly vital subject and may produce results useful in future research on therapeutic methods for allergic people.

6. Weaknesses of the proposal

The scientific background, while sufficiently detailed could be made briefer, the sample sizes seem too small and the methodology in general is described in not enough detail.
The proposed cost structure raises some concerns and could be refined.

Patar Sinaga

This project aims to find out whether pets have the possibility of a reduced immune response to pollen as an allergen in human. In my opinion, this is a promising first step considering that this topic is quite popular, growing and complex in society. The authors describes the situation clearly how research on pollen as an allergen is still in small quantities and its relation to climate change is enough to show that this allergen is a crucial concern. What I understand about the methodology is that the authors want to

see the differences in allergen reduction in adults and children, but it is better to mention the range or age profile of the two groups from the start so as to provide a comprehensive picture for readers, especially non-specialists that could probably have no idea about the possible differences. The combination of having sociological and biological is a new approach and could give a broader perspective about immunological response, while being able to compare it within population profile. It seems that this research will receive considerable attention from the multinational community both for those who are classified as vulnerable to pollen as an allergen and is quite promising as an alternative treatment especially if the results obtained support the hypothesis offered by the authors.
The selection of this topic will provide fairly comprehensive data, in addition to sociological profiles, testing with the aim of validating whether antibody data supports that certain pet species can help reduce the immunological response to pollen allergies. Knowledge gaps and hypotheses are well delivered and can have a significant impact on allergy therapy.
There are several notes that I do not understand about the methodology that the authors will use. In lines 115-117, "to determine the effectiveness of pet exposure in increasing IgA secretion in adults and kids" and "to access the impact of pet exposure in IgE concentration in children and adults" are there differences in the age profile of kids and children here? or not. A more detailed description of the age profile should be put in this section. And if the authors can predict the increase in IgA, then justification of the expectation (as written in the description of Figure 2): decreasing in IgE, not stated in the first paragraph of concept and workplan. The choice of research locations in highly-urbanized areas and non-urbanized areas for me is not clear, whether this is related to the statistical distribution of people who have certain pets or whether this is related to the distribution of people with types of allergies. There are some typos, such as in lines 187 (hiving) and 189 (animals). It may also be necessary to explain the importance of collaboration with third parties for IgA and IgE analysis in methodology if the matter related to expenses. In my mind, When there are funds that can be allocated to do this analysis in-house, there are a lot more possibilities for further research that can be done compared to considering spending on a single project. In general, the overall work plan and methodology can be well understood, especially for those who are not familiar with this topic, but there are several decisions taken by researchers that are not well justified so that the information delivered can be confusing.
I suggest displaying a breakdown of project costs in table form so that it is more uniform with the table budget and easier to follow. I didn't find any justification for the allocation of funds for buying a laptop, maybe you can explain more about the urgency. It would be better if there was an explanation of the possible types or names of journals and conferences to be attended, whether interdisciplinary or not, bearing in mind the authors have different research backgrounds. Despite my confusion regarding collaboration with third parties for IgA and IgE analysis (see above), the additional explanation at the end of the proposal regarding the justification of price comparisons for in-house and collaborators is very detailed and quite convincing.
I am quite enthusiastic to see the authors raise a topic that is very relevant to many people, related to pollen allergies which are still unknown. The topic is very interesting and hopefully will attract the attention of many people, considering that this condition is close to people's lives. Proposals are written in a straightforward, systematic manner and use a diction approach that is easy for non-specialists to understand. Text formatting in the proposal that divides each point also looks straightforward and provides directions for moving from one topic of discussion to another. The formulation of hypotheses and some expectations is well done and can be tested. If this research gives significant results in

reducing the response to pollen allergies, of course this will be used as a reference that can be applied to its function.
There are several explanations that should be explained, especially in the methodology section so as not to cause bias and misinterpretation. Writing can still be improved, especially the choice of words and writing errors. An explanation of the details of the work carried out during the 3 years of research may be described in the proposal.

FINAL VERSION

Can A Pet Help To Reduce Allergy Of Its Owner?

Alaa Hseiky, Martyna Marzec, Ekaterina Rostovskaya

Summary:

Nowadays about 20-30% of population suffers from allergies and this number is constantly increasing due to the climate change and air pollution. Allergic diseases reduce the quality of life and can result in negative symptoms, such as hay fever, conjunctivitis, asthma and anaphylaxis. Moreover, it increases the risk of developing non-communicable diseases, such as cancer. Recent studies have shown a positive effect of pet exposure on reducing food allergy in children without genetic allergy background. Therefore, additional research on barely studied pollen allergies is needed to highlight the impact of pet presence on both children and adults. This study implements both sociological and biological approaches, with a questionnaire highlighting the impact of various pet species and a blood immunological test measuring its intensity. It can help to form public health policies and practices related to pet ownership and allergy prevention. The results can also provide guidance to families who are considering adopting pets from animal shelters.

SHORT DESCRIPTION OF THE RESEARCH PROJECT

1) Scientific goal of the project

Allergic reactions of a particular organism to typically harmless substances in the environment are caused by hypersensitivity of its immune system. It reacts to proteins of something common as it was a pathogen – by producing antibodies (Justiz Vaillant, Vashisht and Zito, 2023). Allergies, or allergic diseases, include rhinitis (hay fever); drug, food, and insect allergy; atopic dermatitis (eczema); allergic asthma and anaphylaxis (Pawankar *et al.*, 2013). The most common allergens include pollen and certain food products, such as egg, lactose or nuts (Okabe *et al.*, 2023).

Thus, we aimed to investigate the effect of pet presence on human immune response to one of the least studied allergens – pollen. To achieve this goal, we formulated the following hypotheses:

a). Pets reduce the incidence risk of pollen allergies.

b). The effect of pets is dose-dependent, with a negative correlation between the number and/or weight of pets and allergy risks.

c). Pet's effectiveness depends on the species, with cats (*Felis catus*) and dogs (*Canis lupus familiaris*) reducing the incidence risk the most.

d). Pets decrease allergy probability in adulthood, but the effect is less than in childhood.

2) Significance of the project

Nowadays, between 20-30% of the world's population suffers from some form of allergic disease, and their prevalence is increasing dramatically worldwide. In Europe, one in four children is allergic (Pawankar *et al.*, 2013). Scientists link this to increasing environmental pollution and climate change (D'Amato and Cecchi, 2008; Lake *et al.*, 2017).

Climate change: Pollen allergies are highly seasonal, with most of the plant species releasing their pollen within the period of six month, from spring to autumn (*https://climate-adapt.eea.europa.eu/*, 2021). Climate change has been shown to influence pollen allergies in several ways. First of all, in response to global warming, plants shift their seasonal timing, releasing pollen earlier and for a longer pollination period (D'Amato and Cecchi, 2008). Additionally, climate change may affect the geographical distribution and atmospheric dispersion of pollen. Water droplets can trap pollen grains, causing them to fall to the ground reducing their concentration in the air. However, with more extreme weather and droughts resulting in decreased annual rainfall (D'Amato and Cecchi, 2008; Lake *et al.*, 2017) more allergens remain in the air.

Molecular mechanisms of allergies: Allergic diseases are developing on the bases of both genetic and epigenetic (environmental) mechanisms (Agache *et al.*, 2020). Ferreira *et al.* (2017) have shown genetic predisposition to immune system overreaction (atopy). In response to allergen exposure, it secretes IgE antibodies (Pawankar *et al.*, 2013), which bind to specific receptors on mast cells and basophils, leading to the release of histamine and other inflammatory mediators. This leads to a variety of allergic symptoms such as itching, swelling, and bronchoconstriction (Lambrecht and Hammad, 2015). For example, allergic rhinitis results from an IgE-mediated inflammation of the nasal mucosa (Pawankar *et al.*, 2013).

Type I immediate hypersensitivity reactions, mediated by IgE antibodies, are immune responses that are exaggerated or inappropriate against an antigen or allergen and occur within 24 hours. Their manifestations include systemic and local anaphylaxis such as hay fever, asthma, hives, food allergies, eczema (Justiz Vaillant, Vashisht and Zito, 2023). IgE antibodies are involved in the development of allergic sensitisation: repeated exposure to allergens leads to higher levels of IgE antibodies production and increase the risk of developing allergies. Therefore, elevated levels of allergen-specific IgE antibodies in blood serum can indicate sensitisation to specific allergens and are used to diagnose allergic diseases (Galli and Tsai, 2012).
IgA antibodies are involved in regulating the immune response to allergens. As component of the mucosal immune system, they play a vital role in protecting the body from pathogens and allergens. Studies have shown that allergen-specific IgA antibodies can reduce the risk of developing an allergy (Corthésy, 2013). By binding to antigens, IgA antibodies prevent allergens from adhering to mucosal surfaces and inhibit the activation of immune cells involved in allergic responses. If the production of IgA antibodies is dysregulated, the risk of allergy appears to increase (Schwitzguébel *et al.*, 2015), for example, deficiencies in IgA production are associated with an elevated probability of developing food allergies.

Allergic diseases can not only reduce quality of life, but can also be life-threatening, as in the case of an asthma attack or anaphylactic shock. Asthma is a lifelong chronic inflammatory disorder of the airways, that remains underdiagnosed and undertreated. Atopy is the strongest identifiable predisposing factor for the development of asthma (Pawankar *et al.*, 2013). Anaphylaxis is a severe hypersensitivity reaction of both allergic and non-allergic etiology.

Systemic anaphylaxis is a massive allergic reaction (Justiz Vaillant, Vashisht and Zito, 2023), for which there is the only emergency treatment is intramuscular injection of epinephrine (adrenaline)(Pawankar *et al.*, 2013). In addition to symptoms, allergy causes long-term

immune dysfunction, moreover, it has underlying inflammation which forms the factor for other non-communicable diseases (Pawankar *et al.*, 2013). As a result, people with allergies have been shown to have an increased risk of some cancers (compared to the general population), such as lymphoma and myeloma (Merrill, Isakson and Beck, 2007; Cotterchio *et al.*, 2014). Nevertheless, for some cancers, including glioma and pancreatic cancer, risk rates decrease (Merrill, Isakson and Beck, 2007).

State of the art: To the best of our knowledge, many aspects of allergic diseases remain understudied (Pawankar *et al.*, 2013). For example, breastfeeding is known to play an important role in immune system of a baby, but there is still no strong evidence of its effectiveness in primary prevention of allergy (Mennini, Arasi and Fiocchi, 2021). So far, the main focus of allergy studies has been on food allergies (Chen *et al.*, 2010) and asthma (Lodge *et al.*, 2011; Portnoy *et al.*, 2012), which is particularly surprising when one considers that the incidence of food allergies in children is less than 10% in developed countries (Okabe *et al.*, 2023), while pollen allergy symptoms are registered in 40% of the total European population (Lake *et al.*, 2017).

Food allergies and asthma are often studied in the context of exposure to cats and dogs (Ownby, Johnson and Peterson, 2002; Dharmage *et al.*, 2012). There are several reasons for this, one of which is *hygiene hypothesis* (Okabe *et al.*, 2023), which proposes an inverse relationship between the presence of infections and immune dysfunction (Okada *et al.*, 2010).

Longitudinal studies in urban populations suggest that perinatal exposure to pets, especially dogs, may reduce the development of allergic disease in those without a family history of allergy (Lodge *et al.*, 2011). Another explanation for the effect of pets is related to the *molecular mechanisms* of allergy. Pet exposure causes the secretion of IgA, which prevents allergens from binding to nasal and oral mucosal surfaces and the secretion of IgE (Schwitzguébel *et al.*, 2015), i.e. it is effective in case of both pollen and food allergies. Nevertheless, the combination of pet keeping and parental atopy to increases the risk of respiratory symptoms in children (Dong *et al.*, 2009), making them a high-risk group to allergic diseases.

Although people can sometimes be allergic to pet hair or saliva, in general, having a pet at home can reduce the immunological response to allergens. Pet-keeping reduces the risk of allergy in a dose-dependent manner (Hesselmar *et al.*, 2018): allergic diseases were diagnosed in 32-49% of children of different age groups without pet exposure, and this percentage decreased with a higher number of pets, up to zero allergic reactions in families with ≥5 pets.

There is little or no data on the influence of pet species other than dogs and cats on allergy risk. However, such information may be crucial to better understand the factors and mechanisms of therapeutic effect of pet exposure in the development of allergies. The 2023 study suggests that early life exposure to both cats and dogs reduces the risk of developing food allergies to certain products, such as egg or milk. In contrast, such exposure to hamsters significantly increased the risk of developing nut allergy (Okabe *et al.*, 2023). Moreover, it was shown that children exposed to dogs had less symptoms of pollen allergy (Chen *et al.*, 2008).
The pioneering nature of our research: 1) Pollen allergies remain majorly understudied, especially when taking into account the frequency of pollen allergies to food ones; 2) The

majority of allergy studies are focused on children, remaining unknown its development on adults and the factors affecting it; 3) There is a need for data on the influence of multiple pet species exposure, as so far there is data mostly for dogs, and cats. This research seeks to study the effect of pets on reducing the immunological response to allergens, such as pollen, in both children and adults. It will prove the possibility of pet treatment for allergic diseases in adulthood and identify the most effective pet species for this.

3) Concept and work plan

Our previously defined hypotheses (1-4) will be tested in the context of 3 specific research objectives 1-3: (i) to determine the effect of exposure to various species in reducing the incidence of pollen allergy, (ii) to determine the effectiveness of pet exposure in increasing IgA secretion in adults and children, and (iii) to access the impact of pet exposure on IgE concentration in children and adults. To accomplish objective 1, a questionnaire will be conducted in 2024, followed by an experiment with blood tests to meet objectives 2 and 3 (Fig. 1). The experiment will last for two pollination seasons (approximately from Mach to August in the years 2025 and 2026).

To begin with, we will develop a comprehensive questionnaire to gather data on various aspects related to pets and allergies in children and adults. The questionnaire will be designed based on existing templates used in similar studies. However, we will improve the questionnaire to cover aspects that have been previously overlooked, including a broader range of pets and both young and adult participants. Once the questionnaire is finalised, we will create a poster with the QR code to access the survey.

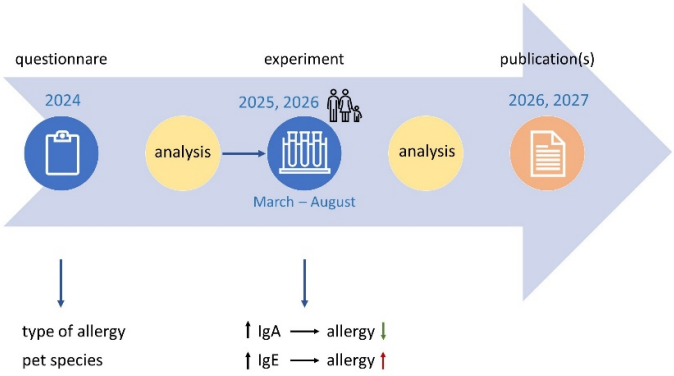


Fig. 1: The timeline of our project studying the protective effects of different species on the allergy of both adults and children

A survey allows us to collect a large amount of data, but it also has limitations in terms of control. Therefore, we plan to conduct a more controlled experiment. To recruit participants, we will add a question to our questionnaire asking families who have never fostered a pet and who either plan to adopt a cat or a dog from an animal shelter or do not plan to get a pet to contact us if they would like to participate. Furthermore, we will collaborate with animal shelters to ask people who are planning to foster a pet to take part in our experiment. The experiment will compare the protective effects of cats and dogs versus the absence of pets on pollen allergy in both children and adults. This approach has been planned because dogs and cats are the most common pets, and studies on pollen allergies, particularly in adults, are not widespread. The results of the questionnaire and the immunological experiment will lead to at least 2 publications in a high or high-medium impact factor journal such as the "Journal of Allergy and Clinical Immunology".

Authors' experience: The authors have prior experience in conducting various types of questionnaires, although they have not previously worked specifically in the field of pets and allergies. In addition, they have extensive experience in experimental design and complex statistical analyses, which will enable them to effectively analyse the data collected in this

study. Furthermore, the authors have diverse backgrounds, including positions requiring strong communication, persuasion, and negotiation skills, which will be important in recruiting and involving participants in the study. In addition, the authors have an extensive network of contacts and collaborators who can be called upon for additional support and expertise as required.

Risk assessment: In order to meet legal and ethical requirements, the study must be approved by the Human Research Ethics Committee and the permission to process human data by RODO. Also, written informed consent from all parents needs to be obtained. However, there is no need for permits and licenses obtained from Ethics Committee, as no study on animals is directly conducted. Even if the parents consent and agree to participate, we could expect that some of the answers will be biased by the individuals where in some cases we might obtain wrong information from them.

There is a potential risk that our study will have a lower number of blood samples from children, which may pose challenges in obtaining accurate data. Additionally, due to the nature of our registration process, we may not be able to find enough participants to achieve adequate statistical power for our analysis, which may limit the validity of our results. Another risk is that we may not achieve equal representation of all groups, which could affect the generalisability of our findings. Furthermore, there is the possibility that families fostering the pet get sensitised to pet allergens. We will carefully consider these risks and take the necessary steps to minimise them, including ensuring proper consent procedures, monitoring enrolment efforts, and following all legal and ethical guidelines. To adress all of the above risks, we have increased our sample size which will reduce any bias.

4) Research methodology

A questionnaire: Hypotheses 1-3 will be tested by using a questionnaire on family allergies and their exposure to pets, designed on the basis of the experimenters' knowledge and similar questionnaires used in research papers related to allergies. The questionnaire will be addressed to parents of children and will be distributed to kindergartens, animal shelters and clinics equally distributed either in highly urbanised areas or in non-urbanised areas: villages. The two areas were selected to take into account the amount of pollen in the air, with the expectation that the amount of pollen in the urbanised areas would be lower than in the non-urbanised villages, leading to the difference in allergy prevalence between the areas. The questionnaire will be detailed and easy to understand but will generally cover: 1) the first 3 years of the child's life; 2) the parent's childhood and current status. It will include questions concerning: **i) allergy**: whether allergic or not, if allergic, what is the type (food/pollen), symptoms and severity of the allergy, and at what age it started manifesting; **ii) prenatal/postnatal conditions**: duration of breastfeeding, maternal smoking; **iii) pet**: number of pets (0-4, or more), pet species, age of child when exposed to the pet (natal/prenatal or if postnatal specify); **iv) sociological part**: place of residence (city/village), presence of siblings, family medical history of allergies (atopy).

In our questionnaire we enrich the number of pet species from several taxonomic classes. Respondents will choose from mammals, birds, reptiles and amphibians (with illustrative samples) and specify the species later. The questionnaires will be collected for 6 months. Data cleaning will be performed in a suitable program and statistical analysis will be performed in R, SAS software, etc. Several models including adjusted logistic regression models etc. will be used to describe the associations between pet ownership and the parameters collected. In the statistical models, parental history of allergic diseases, prenatal condition and sex will be

adjusted. *Experiment on the plasma concentration of immunoglobulins IgE and IgA:* This method will be used to test hypotheses 1 and 4 by assessing the effect of having a pet on the immunological response of pollen allergic individuals. Our experiment will include several families (about 250 with a mother, father and 1-3 children) who have shown symptoms of pollen allergies but have never had an animal pet before. The families will be selected so that 33% of the families plan to foster a cat, 33% families plan to foster a dog, while 33% of the families do not plan to foster any pet and will be our control group. The pets to be fostered should come from animal shelters.

Three blood samples will be collected per person in the first year (2025) by Venepuncture. This will be performed by a specialist private company with which we are going to collaborate with. The samples will be taken before the animal is adopted (day 0), 1 week, and 1 month after adoption. For the control group, day 0 will be determined so that the time of blood collection is consistent among the three groups. The blood will be stored on dry ice and immediately transferred to our collaborator's laboratory for the concentration of immunoglobulins IgE and IgA to be analysed using Human IgE and IgA Elisa Kits, respectively. The same procedures will be repeated on the same individuals but one year later (2026). The protective effect of the pets would be assessed by the increase in the concentration of IgA, and the decrease in the concentration of IgE. In order to encourage people to participate in our experiment and to support their willingness to adopt animals from shelters, we will hold a lottery so that around 10 % of the families will receive a financial award in the form of a pre-paid credit card. Furthermore, to statistically analyse the concentration of the immunoglobulins, linear mixed model will be used with the concentration as the dependent variable, pet fostering (cat, dog, none) and sex as the independent factors, age and time of blood collection as covariates, and the family number as a random factor.

In the follow-up project, we plan to repeat a similar experiment, but using the most effective animal species (not cats and dogs) for reducing allergy. This species will be determined by the results of our questionnaire. This will allow us not only to compare different mammalian species, but also to compare the mammal class with other classes such as Aves (birds), which have feathers and not fur. We also plan to test whether the protective effect of the pet exposure would be consistent across the years, and whether its mechanism could be used as an allergy treatment.

5) Project literature

Agache, I. *et al.* (2020) 'Genetics and epigenetics of allergy', *Current Opinion in Allergy and Clinical Immunology*, 20(3), pp. 223–232.

Chen, C.-M. *et al.* (2010) 'The role of cats and dogs in asthma and allergy – a systematic review', *International Journal of Hygiene and Environmental Health*, 213(1), pp. 1–31.

Corthésy, B. (2013) 'Role of secretory IgA in infection and maintenance of homeostasis', *Autoimmunity Reviews*, 12(6), pp. 661–665.

Cotterchio, M. *et al.* (2014) 'Association between Allergies and Risk of Pancreatic Cancer', *Cancer Epidemiology, Biomarkers & Prevention*, 23(3), pp. 469–480.

D'Amato, G. and Cecchi, L. (2008) 'Effects of climate change on environmental factors in respiratory allergic diseases', *Clinical & Experimental Allergy*, 38(8), pp. 1264–1274.

Dharmage, S.C. *et al.* (2012) 'Exposure to Cats: Update on Risks for Sensitization and Allergic Diseases', *Current Allergy and Asthma Reports*, 12(5), pp. 413–423.

Dong, G.-H. *et al.* (2009) 'Pets keeping in home, parental atopy, asthma, and asthma-related symptoms in 12,910 elementary school children from northeast China', *Indoor Air*, 19(2), pp. 166–173.

Ferreira, M.A. *et al.* (2017) 'Shared genetic origin of asthma, hay fever and eczema elucidates allergic disease biology', *Nature Genetics*, 49(12), pp. 1752–1757.

Galli, S.J. and Tsai, M. (2012) 'IgE and mast cells in allergic disease', *Nature Medicine*, 18(5), pp. 693–704.

Hesselmar, B. *et al.* (2018) 'Pet-keeping in early life reduces the risk of allergy in a dose-dependent fashion', *PLOS ONE*, 13(12), pp. 1-13.

https://climate-adapt.eea.europa.eu/ (2021). European Climate and Health Observatory: European Environment Agency. Available at: https://climate-adapt.eea.europa.eu/en/observatory/evidence/health-effects/aeroallergens/.

Justiz Vaillant, A.A., Vashisht, R. and Zito, P.M. (2023) 'Immediate Hypersensitivity Reactions', in *StatPearls*. Treasure Island (FL): StatPearls Publishing.

Lake, I.R. *et al.* (2017) 'Climate Change and Future Pollen Allergy in Europe', *Environmental Health Perspectives*, 125(3), pp. 385–391.

Lambrecht, B.N. and Hammad, H. (2015) 'The immunology of asthma', *Nature Immunology*, 16(1), pp. 45–56.

Lodge, C.J. *et al.* (2011) 'Perinatal Cat and Dog Exposure and the Risk of Asthma and Allergy in the Urban Environment: A Systematic Review of Longitudinal Studies', *Journal of Immunology Research*, 2012, pp. 1-11.

Mennini, M., Arasi, S. and Fiocchi, A.G. (2021) 'Allergy prevention through breastfeeding', *Current Opinion in Allergy and Clinical Immunology*, 21(2), p. 216.

Merrill, R.M., Isakson, R.T. and Beck, R.E. (2007) 'The association between allergies and cancer: what is currently known?', *Annals of Allergy, Asthma & Immunology: Official Publication of the American College of Allergy, Asthma, & Immunology*, 99(2), pp. 102–116; quiz 117–119, 150.

Okabe, H. *et al.* (2023) 'Associations between fetal or infancy pet exposure and food allergies: The Japan Environment and Children's Study', *PLOS ONE*, 18(3), pp. 1-14.

Okada, H. *et al.* (2010) 'The "hygiene hypothesis" for autoimmune and allergic diseases: an update', *Clinical and Experimental Immunology*, 160(1), pp. 1–9.

Ownby, D.R., Johnson, C.C. and Peterson, E.L. (2002) 'Exposure to Dogs and Cats in the First Year of Life and Risk of Allergic Sensitization at 6 to 7 Years of Age', *JAMA*, 288(8), pp. 963-971.

Pawankar, R. *et al.* (eds) (2013) *White Book on Allergy: Update 2013*. World Allergy Organization.

Portnoy, J.M. *et al.* (2012) 'Environmental assessment and exposure control: a practice parameter—furry animals', *Annals of Allergy, Asthma & Immunology*, 108(4), pp. 1-27.

Schwitzguébel, A.J.-P. *et al.* (2015) 'Immunoglobulin deficiency in patients with chronic rhinosinusitis: Systematic review of the literature and meta-analysis', *The Journal of Allergy and Clinical Immunology*, 136(6), pp. 1523–153.

6. **Table with budget of the project**.

	Amount in PLN
Direct costs, including	1 027 583
- personnel costs and scholarships	252 000
- research equipment/device/software cost	8 983
- other direct costs	766 600
Indirect costs, including:	226 068
- indirect costs of OA	20 552
- other indirect costs	205 517
Total costs	1 253 651

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

Direct costs:

Employment

Salaries for PI = 2000 PLN/month x 36 months x 3 PI = 216 000 PLN
Congress of the European Academy of Allergy and Clinical Immunology = 12 000 PLN per PI, each person presents at different conferences = 36 000 PLN

Personnel and Field Travel/Accommodation/Subsistence

Medical personnel (blood samples collection) = 2500 PLN x 6 months x 2 seasons = 30 000 PLN
Travel costs for samples collection = 6600 PLN

Equipment

High-performance laptop 8000 PLN
BioRender registration 39$/month= 39 x 4,20 PLN x 6 months= 983 PLN

Consumables

Printing 12 PLN/poster x 5 000 kindergartens and shelters = 60 000 PLN
Lottery prize 10000 PLN x 25 prizes = 250 000 PLN
Blood analysis = IgA-40 PLN + IgE-30 PLN = 70 PLN/person x 4 family members x 250 families x 3 tests/person x 2 years = 420 000 PLN

We have explored the possibility of performing the immunoglobulin tests in-house but found it to be more expensive. For instance, an Elisa kit required to measure the concentration of IgE for only 96 tests costs around $700 (3360 PLN), which translates to a cost of 35 PLN per test, exclusive of equipment, electricity, or labour expenses. Instead of this, we sign a contract with a company, and pay for the blood collection and analysis.

Do Sponges Have Neurons? Insights Into The Porifera 'Neural' System Through Neuroimaging And Genomics.

Mateusz Chechetkin, Rebecca Miszczak, Anastasiia Mykhailenko, Anbarieh Saadat

Summary

Sponges are the oldest living animals and a sister group to all modern eumetazoans. They do not possess fully differentiated tissues or a developed nervous system. So how do they respond to mechanical stimuli and neurotransmitters? And why do they have genes responsible for parts of neuronal cells and the differentiation of the nervous system?

Despite recent progress, such as identification of a potential candidate for a neuronal-like cell in sponges, little is known about the ways in which they are able to generate responses to environmental and chemical stimuli. Furthermore, there are two competing explanations for the apparent mismatch between sponges anatomy, physiology, and genetics. One possibility is that sponge nerve-like cells represent the simplest possible structure that did not develop further. The other potential explanation is that the common ancestor of sponges and eumetazoa possessed a more complex nervous system, and some functions were lost overtime.

This project aims to unravel the mechanisms by which sponges are able to act as if they have neurons and elucidate their evolutionary origins. We will use neuroimaging techniques to visualize the reaction of sponge cells to selected neurotransmitters and identify the receptors or receptor-like structures that bind them. We will also use comparative genomics to search for pseudogenes - sequences similar to those of more complex animals that were damaged by mutation. Transcriptome analysis and methylation analysis will allow us to trace the developmental path of neuron-related genes and detect silenced sequences. Presence of pseudogenes and/or genes that are deactivated in adult animals would support the loss of function hypothesis, while their absence would support the simplest form hypothesis.

This research has the potential to discover ways of neuronal-like communication, function and neurotransmitter response not yet known to science. It could also settle a decades-long debate on the evolutionary origin of sponges and their position in the metazoan family tree. The results of the research will constitute a substantial contribution to evolutionary biology, developmental biology, and neuroscience.

1) Scientific goal of the project

The project is directed to study **one of the most archaic neural systems** in the animal kingdom, detected in Sponges (Porifera).

Sponges are the 'elder sister' of the whole Metazoan group and the most ancient animal forms on the planet (oldest fossils are dated to 890-Million yo) (Turner et al. 2021). Sponges are considered to be the oldest living Metazoans, possessing the simplest morphology known among multicellular animals (Hooper et al. 2021). They have only two germ layers and do not have fully differentiated tissue systems, including the nervous system (Musser et al. 2021). However, coordinated movements (locomotion) was detected

multiple times in sponges with a siliceous skeleton (Demospongia) (Bond and Harris 1988, Nickel et al. 2004, Nickel et al. 2010). Moreover, there are signs that some sponge cells resemble the electrochemically dominated integration (nervous) system: on the level of tissue organization, behavior, and genes (Nickel et al. 2010).

We know that sponges have structures that are most likely involved in the neural-like responses. We also know that sponges have genes homologous to gene groups involved in the differentiation and functioning of nervous cells. Nevertheless, the picture remains incomplete. The microstructural analysis reveals convincing candidate cells to play a role in neural-like coordination of movements, but there is no description of how these cells react to the neuromediator induction. Also, it is still unclear whether identified genes and transcription factors represent simplified remnants of a more complex nervous system or an early precursor that did not develop further in sponges. **In this study we are aiming to identify the selected neurotransmitter receptors that initiate the responses to stimuli. We will also determine whether the sponge's basic nerve-like response is the result of losing function in their evolutionary past.**

This project has potential to reveal the mechanisms by which sponges produce a nervous cell-like response without nervous tissues and to clarify what evolutionary process underlies the formation of neural-like system in sponges.

2) Significance of the project

Justification:

The evolution of the central nervous systems of the Bilateria is generally better understood than the evolutionary origin of the nervous system. However, in order to understand the origin of nervous systems in the widest context, pre-nervous systems should also be investigated. Morphological, physiological, and genomic evidence (Bond and Harris 1988, Nickel et al. 2004, Nickel et al. 2010, Ellwanger et al. 2006, Francis et al. 2017, Musser et al. 2021) suggests that a poriferan nervous system comprises modules that might be homologous to modules in nervous systems of other animal groups.

For instance, there are studies that suggest slow signaling pathways that include small molecule transmitters or neuropeptides could be a path of communication between sponge tissues. Some of these molecules have been shown to mediate the contraction behavior (Leys 2015). Neurotransmitters like glutamate and GABA are shown to both trigger and inhibit contraction in sponges. Interestingly, GABA induces contraction in freshwater sponges and inhibits contraction in saltwater sponge *Tethya wilhelma* (Ellwanger et al. 2006), and the mechanism that underlies this difference is unclear. The presence of receptors for metabotropic glutamate and GABA and their physiological response has been studied, but to our knowledge, there has been no study that tried to visualize and pinpoint the described receptors.

The evolutionary position of sponges remains uncertain. What we know of sponge anatomy and physiology suggests that they split from the common ancestor of all animals before the development of any neuronal cells or a centralized nervous system. However, it has been demonstrated (Wong et al. 2019) that sponges possess genes responsible for the development and functioning of nerve cells. Genes associated with synapse cell structures, receptors of neurotransmitters (e.g. GABA and glutamate) as well as neurotransmitter synthesis have been found in sponge genomes (Leys 2015, Perovic et al. 1999). Additionally, sponges appear to have developmental transcription factors associated with nervous system function, such as Pax and Sox (Fortunato et al. 2012, Rivera et al. 2013). It is still unclear whether those genes represent simplified remnants of a more complex

nervous system or an early precursor that did not develop further in sponges. Detection of so-called 'pseudogenes', or genes that used to be functional in the more complex ancestor and have degraded, could provide evidence for the secondary loss of neural structures in sponges.
Furthermore, to this day, there is no conclusive research tracing the developmental trajectory of genes in sponges. It is unclear whether genes that have been identified in whole genome sequencing are expressed in larval stages, and how many of them are inactive in adult individuals. If it is discovered that some genes are silenced during the development of the sponge, this would support the hypothesis of evolutionary loss of function. In contrast, the absence of pseudogenes or silenced genes would support the hypothesis of simplest possible structure.
State of art:
There are existing well-developed methods of imaging internal structure and activity of tissues including transmission electron microscopy and immunofluorescence techniques (Winey et al. 2014, Burkhardt et al. 2017, Musser et al. 2021). For *Spongilla lacustris*, there are specifically identified markers for imaging all known types of tissues and cells (Musser et al. 2021) and the imaging via transmission electron microscopy has been already used to investigate sponge tissues before and has been described by Burkhardt et al., 2017.

Currently, the two biggest achievements in addressing the evolution of the neural system in sponges are: whole genome sequencing of sponges which identified genes potentially involved in nervous system functioning (Srivastava et al. 2010); and RNA sequencing of adult tissues to identify cell types and gene expression profiles (Musser et al. 2021). As of 2023, there have not been studies on nervous function-related pseudogenes or developmental changes regarding gene expression in sponges. Nevertheless, methodology for the detecting pseudogenes is extensively developed (Abrahamsson et al. 2022) and RNA sequencing techniques were developed specifically for the species of our interest (Musser et al. 2021).
Impact:
Study of the nervous system in such a primitive organism that has an efficient yet simple organisation would be beneficial to understand the evolution of the nervous system as we see it today. Proving the existence of receptors or receptor-like structures to glutamate and GABA will constitute evidence for the presence of protosynaptic structure. By understanding what mechanisms sponges use to coordinate movements, we gain a unique animal model to acquire insights into the neurosystem disorders.
This research would also greatly contribute to a fundamental question in evolutionary biology and could potentially settle a decades-long debate on the evolutionary origin of sponges. Apart from the significance for fundamental research, it could improve the methodology of pseudogene identification and detection of gene methylation during

development, which has implications for research of human disease, such as developmental disorders and cancers.
In conclusion, we expect to publish the results of the research in one of the highly-ranked interdisciplinary journals and we project great visibility of the research among several diverse fields of biology, including but not limited to neurobiology, evolution, and developmental biology.
3) Concept and work plan
In this study we address the protoneural system in sponges on three fundamental levels (Suppl. 1: https://docs.google.com/document/d/1G4WJ2cO5utlfVfJaXWkbkMX9c-uH4ur2H_tjsN1UGoQ/edit):

- Investigating the presence of neurotransmitter receptors and communication pathways of neuron-like cells in two species of sponges: *S. lacustris* and *T. wilhelma*. To be able to image larvae of *S. lacustris*, blastomers that develop into flagellated cells will be collected. We are going to focus on imaging overall microstructure and label receptors or receptor-like structures to which glutamate and GABA bind. (PI responsible: Anbarieh Saadat, Rebecca Miszczak)
- Comparative genomics, functional genomics and epigenetics. Identifying pseudogenes in *S. lacustris* and *T. wilhelma*. Acquired mRNA data and data from NCBI will be used to find broken genes or methylated (silenced) genes (PI responsible: Mateusz Chechetkin, Anastasiia Mykhailenko)
- Developmental biology. Studying larval and adult stage of *S. lacustris* in order to distinguish patterns of gene expression related to individual development (PI responsible: Mateusz Chechetkin, Anastasiia Mykhailenko, Rebecca Miszczak)

Risk analysis:
There are several potential risks that we are going to address. First of all, to increase the chances of acquiring larva from *S. lacustris* specimens, we are going to sample for two years, first year from the natural habitat and second years from the artificial pond. There is also a risk that manufactured antibodies for immunogold labeling will not work and will require time for optimizing antibodies or reordering. To avoid losing time we will make this step a priority at the beginning of the project and will be able to correct the process in time. Additionally, due to the sponges being a very diverse and isolated animal group, many homologous genes that are known in other animal groups could be extremely divergent. Being aware of this issue, we will do an extensive literature search in order to find methods that help us detect gene sequences that only partially overlap.

4) Research methodology
Sampling and cultivation
The adult individuals of *T. wilhelma* will be ordered from the aquarium of the botanical zoological garden Wilhelma Stuttgart (Germany). Individuals (adults and larvae) of *S. lacustris* will be collected from the site in an artificial pond in Poleski National Park (Poland). Within the first summer (June-July) of the experiment around 100 samples (parts of sponges) will be collected from the river. Half of the samples will be planted in a pond. The rest will be used in the following analysis. In next seasons sponges cultivated in ponds will be used for the analysis.

Imaging
First, we will use transmission electron microscopy to image the interior of the cells using ultrathin sections. The samples collected from both larva and adult sponges will be contrasted and analyzed using transmission electron microscopy to localize internal structures. Post-embedding immunogold labelling is used to localize the receptors using primary antibodies and secondary antibodies which are coupled with gold particles. Number of receptors for neurotransmitters glutamate and GABA will be investigated. Second, we will use immunofluorescence that allows visualization of the distribution of the target molecule through fluorescent dyes with a fluorescence microscope. Amoeboid-neuroid markers to be used are Peroxidase A (choano-neuroid), c85989_g1 (Apopylar cells), c103466_g1 (Amoeboid cells), Acp5 (pinaco-neuroid). Membrane stains are CellBrite Fix and Fm143-Fx; cyan – nuclei (DAPI).

Identification of pseudogenes and silenced genes

To identify potential pseudogenes, we will take advantage of existing genetic databases and whole assembled genome sequences of sponges (Srivastava et al. 2010, Francis et al. 2017, Kenny et al. 2020). We will then identify putative pseudogenes by comparing to genomes of more complex animals (Harrison et al. 2003; Zhang et al. 2006). These tasks are performed entirely using bioinformatics techniques. To trace the developmental history of the target genes, transcriptome sequencing is performed at both larval and adult stage using RNAseq (Hrdlickova et al. 2017). Additionally, silenced genes are identified with methyl CpG binding domain analysis on the Illumina NGS platform (Yong et al. 2016) and further processed with bioinformatics methods (Cedoz et al 2018).

Statistical analysis
All statistical analysis will be performed in the R statistical computing environment (R Core Team, 2021). The package 'patternize' (Van Belleghem et al. 2017) will be used to quantify the variation of the color pattern in fluorescence imaging. Next, the Kolmogorov-Smirnov test will be used to compare the distributions of fluorescent dye between images. Principal Component Analysis (PCA) will be calculated to compare sets of data. Transcriptome data will be analyzed using differential expression analysis with the "DESeq2" package (Love et al. 2014).

5) Project literature

Abrahamsson, S., Eiengård, F., Rohlin, A., Dávila López, M. (2022). PΨFinder: a practical tool for the identification and visualization of novel pseudogenes in DNA sequencing data. *BMC bioinformatics*, ***23*(1)**, 1-11.
Bond, C., Harris, A. K. (1988). Locomotion of sponges and its physical mechanism. *Journal of Experimental Zoology*, **246(3)**, 271–284.
Burkhardt, P., Sprecher, S.G. (2017) Evolutionary origin of synapses and neurons - bridging the gap, *BioEssays*, **39(10)**, 1700024.
Cedoz, P. L., Prunello, M., Brennan, K., Gevaert, O. (2018). MethylMix 2.0: an R package for identifying DNA methylation genes. *Bioinformatics*, **34(17)**, 3044-3046.
Ellwanger, K. Eich, A., Nickel, M. (2006) GABA and glutamate specifically induce contractions in the sponge *Tethya Wilhelma*, *Journal of Comparative Physiology A*, **193(1)**, 1–11.
Francis, W. R., Eitel, M., Vargas, S., Adamski, M., Haddock, S. H., Krebs, S., ... & Wörheide, G. (2017). The genome of the contractile demosponge *Tethya wilhelma* and the evolution of metazoan neural signalling pathways. *BioRxiv*, 120998.
Kenny, N. J., Francis, W. R., Rivera-Vicéns, R. E., Juravel, K., de Mendoza, A., Díez-Vives, C., ... Leys, S. P. (2020). Tracing animal genomic evolution with the chromosomal-level assembly of the freshwater sponge *Ephydatia muelleri*. *Nature communications,* **11(1)**, 3676.
Harrison, P. M., Milburn, D., Zhang, Z., Bertone, P., Gerstein, M. (2003). Identification of pseudogenes in the *Drosophila melanogaster* genome. *Nucleic acids research*, **31(3)**, 1033-1037.
Hoper, J. N., Wörheide, G., Hajdu, E., Erpenbeck, D., De Voogd, N. J., Klautau, M. (2021). Zootaxa 20 years: Phylum Porifera. *Zootaxa*, **4979(1)**, 38-56.
Leys, S.P. (2015) Elements of a 'nervous system' in sponges, *Journal of Experimental Biology*, **218(4)**, 581–591.
Love MI, Huber W, Anders S (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, **15**, 550.
Musser, J. M., Schippers, K. J., Nickel, M., Mizzon, G., Kohn, A. B., Pape, C., ... Arendt, D. (2021). Profiling cellular diversity in sponges informs animal cell type and nervous system evolution. *Science*, **374(6568)**, 717-723.

Nickel, M. (2004). Kinetics and rhythm of body contractions in the sponge *Tethya wilhelma* (Porifera: Demospongiae). *Journal of Experimental Biology*, **207(26)**, 4515–4524.
Nickel, M. (2010). Evolutionary emergence of synaptic nervous systems: what can we learn from the non-synaptic, nerveless Porifera? *Invertebrate Biology*, **129(1)**, 1–16.
Perovic, S., Krasko, A., Prokic, I., Müller, I. M., & Müller, W. E. (1999). Origin of neuronal-like receptors in Metazoa: cloning of a metabotropic glutamate/GABA-like receptor from the marine sponge *Geodia cydonium*. *Cell and tissue research*, **296**, 395-404.
R Core Team. *R: A Language and Environment for Statistical Computing*; R foundation for statistical computing: Vienna, Austria, 2021
Rivera, A., Winters, I., Rued, A., Ding, S., Posfai, D., Cieniewicz, B., … Hill, A. (2013). The evolution and function of the Pax/S ix regulatory network in sponges. *Evolution & Development*, **15(3)**, 186-196.
Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M. E., Mitros, T., … & Rokhsar, D. S. (2010). The Amphimedon queenslandica genome and the evolution of animal complexity. *Nature*, **466(7307)**, 720-726.
Turner, E.C. (2021.) Possible poriferan body fossils in early Neoproterozoic microbial reefs. *Nature*, **596**, 87–91 .
Van Belleghem, S. M., Papa, R., Ortiz-Zuazaga, H., Hendrickx, F., Jiggins, C. D., Owen McMillan, W., Counterman, B. A. (2018). patternize: An R package for quantifying colour pattern variation. *Methods in Ecology and Evolution*, **9(2)**, 390-398.
Winey, M., Meehl, J. B., O'Toole, E. T., Giddings Jr, T. H. (2014) Conventional transmission electron microscopy, *Molecular Biology of the Cell*, **25(3)**, 319–323
Wong, E., Mölter, J., Anggono, V., Degnan, S. M., Degnan, B. M. (2019). Co-expression of synaptic genes in the sponge *Amphimedon queenslandica* uncovers ancient neural submodules. *Scientific Reports*, **9(1)**, 1-15.
Yong, W. S., Hsu, F. M., Chen, P. Y. (2016). Profiling genome-wide DNA methylation. *Epigenetics & chromatin*, **9(1)**, 1-16.
Zhang, Z., Carriero, N., Zheng, D., Karro, J., Harrison, P. M., Gerstein, M. (2006). PseudoPipe: an automated pseudogene identification pipeline. *Bioinformatics*, **22(12)**, 1437-1439.

6. Table with budget of the project.

	Amount in PLN
Direct costs, including	918150
- personnel costs and scholarships	725250
- research equipment/device/software cost	15000
- other direct costs	192900
Indirect costs, including:	201993
- indirect costs of OA	18363
- other indirect costs	183630
Total costs	1120143

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

Direct costs include:

1. Remuneration for the research team: Principal investigator salary, 4 people for 36 months, 5000 thousand per month = 720000 PLN
2. Renumeration for the satellite personnel: marine biologist/hydrobiologist/zoologist for the two field seasons = 3000 PLN (1000 per month, 1.5 month per season). Master students stipend: first season, two students = 1500 PLN (500 per month, 1.5 month per season); second season, one master student = 750 PLN.
3. Purchase or research equipment: light microscope = 15000 PLN
4. Neuroimaging: test tubes, plastic and glass containers, glass slides, lab knives, Poly/bed 812 embedding kit and TEM grids = 5000 PLN. Two antibody kits, price per kit 2000+3000 = 5000 PLN. Immunofluorescent markers: 15000 PLN
5. Genetic analysis costs: RNA extraction kit = 3000 PLN.
6. Living costs for the field research: First season: 1.5 month, 300 PLN per person daily, 3 people =41850 PLN; second season: 1,5 month, 2 people = 27900
7. Cultivation of *Spongilla lacustris* cost: rent of pond for 1 year = 8000 PLN
8. Ordering samples of *Tethya wilhelma* cost: 3 packages cost (150 PLN per 1), eppendorfs (200 PLN), glutaraldehyde (250 PLN) and formaldehyde (250 PLN) = 1150 PLN
9. Outsourced services; RNAseq = 15000 PLN, methylation analysis = 16000 PLN
10. National and international conferences: Abstract submission, flight, accommodation one person =10000 PLN, total = 40000 PLN

REVIEWS

Prof dr. hab. Pawel Koteja

General comments:
1) I have been warned to "bear in mind, that the authors had 48 hours to write it. Also, they are usually not experts in the field on which they worked." On my side I ask to consider that I am not in this area either, and that (because of other duties on Monday) I had practically only an hour or so to prepare the review. Thus, I could not really check the merit correctness of most of the information provided in the work; specifically, I could not check whether you correctly represent information in the literature cited, and even to carefully check whether you have not skipped some important publications – which in real live would certainly undermine credibility of the proposal. Therefore, in this review I rather judge the internal consistency of the reasoning; in other words I judge how I would judge the project assuming that al the base information provided is true.
2) Joasia instructed us to name the file with review as "*title-revXY, where "title" is the project id and "XY" are reviewers' initials*. The project does not have any explicit ID, and I can only assume that the project file name – "Sponges" – works as its it. Let me make the comment that this is not a very good choice for a file name, especially if it should serve as project ID. The fie name does not indicate that it contains a research project (not to say that it has been prepared for a specific course), and the single word does not really indicates the real objective of the work. Note also, that the file content does not inform that the text is a research proposal, either, or that it is a text prepared for a specific course (so, the is no way to learn from the file content that it is an exercise/bogus project, and that the content of the file should be not treated as a source of information for usage outside of the course. It has also no time stamp (and we know well that the "time" information attached to the file by operating system is for several reasons not reliable and may by highly misleading). I make the comment because I see a general problem with the students' (and not only students) approach to preparing documents – and the course such as this one can be also a platform to learn how to do this correctly.
Paweł

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)

Positive:
The project undertakes ambitious goals and seems to provide convincing information on this importance.

Critical remarks:
- The main part of the project title, "Do sponges have neurons," does not match with the background and objectives presented in paragraph 2 and 3 (lines 13-27) of Summary and further in point 2.
- The declaration in Summary (lines 30-31) of the plausible contribution of the project to "settle a decades-long debate on the evolutionary origin of sponges and their position in the metazoan family tree", and the declaration in point 2 that "the evolutionary position of sponges remains uncertain" (line 96) is not quite compatible with the declarations concerning the focal objective, which appears to be built on an assumption that the phylogeny is known. If this is not known or doubted, the doubt must be resolved before you attempt to resolve the more focal issue – whether neuronal system the lineage of sponges has never (yet) developed or got lost. You would be on a methodologically

slippery ground if you used the same information to resolve the two questions simultaneously.
- Line 107 and further – including analysis of "developmental trajectory…" – this makes the project definitely too ambitions.
- Generally, the hierarchical structure of the objectives (which is not clearly recognized!) means that some of the questions may become irrelevant in the light of data concerning other aspects. If, hypothetically, analysis of phylogeny would show that sponges evolved as "simplification" of some more complex organisms, which already possessed fully developed nervous system, it would not make the main question not relevant – and you would know it without attempts to fine genetic remnants of a more developed nervous system. Also, if the results would show that sponges do not have "neurons", it would not make sense to plan a research aimed at an attempt to discover their developmental trajectory".

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)

In NCN system this aspect has been typically evaluated as answering the question where/how the outcome could be published. From this perspective it seems to have a very high potential, and – in the case of successful realization and especially getting nontrivial results, the outcome could be published in the very top level journals. However, this opinion is subject to the reservation I made in the general comments and criticism in point 1.

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 part 3 – "Concept and work plan" is not very specific. For example, nothing in the text indicates an indicated scale of the work. Thus, it is difficult to assess whether the prospective results may be strong enough to provide reliable answers. Most of the main part of the text (lines 144-156) is just naming/listing the research technologies that would be applied (so the text duplicates partly the contents of point 4 – Research methodology), rather than present the actual research plan. Again – the hierarchical nature of the questions is not well reflected in the plan. The relatively large section on the Risk analysis (8 out of 20 or so lines of point 3) mentions only technical issues, but not possible problems that may arise when basic assumptions are not met. There is no information about time schedule of the project – or even how long it would take.

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

I cannot judge this aspect because the scale of the project is not described even approximately. There is no information on the intended number of observations, and not even a declaration of the total duration. Thus, I do not even know whether the 725k for stipend is justified. However, my impression is that the cost of such a project is GROSSLY underestimated.

5. **Strengths of the proposal**
The project is very ambitious

6. **Weaknesses of the proposal**
The project is too ambitious

Minor comments, concerning technical editing, language etc.:
It is a really bad habit to force moving the text to a next page by filling the page with empty lines. We teach students on the 1st year BSc level not to do so.
The Summary is written in a style that something between the "Summary for general audience" (popular level) and the scientific summary. For the latter it is not specific enough: it does not state what specific results will be consider as supporting/rejecting the working hypothesis.
Line 20: "are able to act as if they **have**…" - it should be "as if they had".
Line 70: "In this study we **are aiming**…": it should be "we aim".

Martyna Gorkowska

The project rises an important issue of studying sponges' mechanisms of neuronal-like communication, as well as the evolutionary origins of the nervous-like structures in sponges.
The presented state of the art indicates the gap in the knowledge that such a project can potentially fill. It can stand as a starting point for future studies which the authors of the project mention in the supplement. However, for me, the project itself lacks at least a short description of potential future studies; the short statement in the supplement is too short.
The research planned in the project follows the logical path and the chosen methodology is well-planned to answer the raised questions. The "Scientific goal of the project" part is clearly explaining the goals of the studies, however, the first two sentences (lines 55-57) are twisted.
I suggest the correction of this part, as the information about whether sponges are the 'elder sister' of the whole Metazoan group or 'the oldest living Metazoan' presented in it is inconsistent. Moreover, in the same part of the proposal, in lines 64-68, there are several facts presented, but there is a lack of sources for that information.
The scientific goal and the questions that the study is going to answer are clear and well-underlined. Additionally, in lines 109-112, the authors are signalizing how the possible outcome of experiments will support one of the proposed hypotheses.
The authors extensively describe and justify the reason to conduct the planned research through
a detailed presentation of the available information on the subject. The literature chosen to base on is international, multidisciplinary, and recent. However, the sentence starting in line 92 and ending in 94 states: 'receptors for metabotropic glutamate and GABA' which seems like a mental shortcut. Glutamate and GABA are neurotransmitters while receptors are the ones that can be metabotropic. GABA and glutamate act through metabotropic receptors.
Moreover, I would suggest at least a short explanation of the chosen sponges species (*Tethya wilhelma* and *Spongilla lacustris*) as the reason is possible to figure out, but not clear enough to be sure. Moreover, in terms of the species planned to be used, in the research methodology, the authors point that around 100 samples of *S.lacustris* is going to

be collected and further processed, but they do not present any information about the number of species of *T.wilhelma* to be used.

The project undoubtedly has a huge potential impact for identifying neuronal-like communication, as well as the evolutionary origins of the nervous-like structures in sponges. The authors underline the pioneering character of their experiments by presenting a variety of previous studies in the fields of concern. The character of possible results might fill the gap in very basic biology but possibly in the future may impact broader issues concerning the evolutionary origins of the nervous system or neuronal-like structures and the neurotransmitters' influence on it. The fact, the authors plan to conduct experiment on two sponges species is an advantage, however further verification of the results and comparison between species would be needed to complete the whole issue.

The experiments planned to conduct in the course of the project are logically planned. Also, the analytical part of the task is extensively described and it is evident that the authors are aware of the steps that they are planning to do. The risk assessment part of the proposal is very logical and the addressed issues are reasonable. The ways planned to prevent potentially problematic aspects of the experiments are well-suited. The fact that the authors planned the collaboration with marine biologist/hydrobiologist/zoologist provides control over different stages of sample collection which reduces the risk of potential problems in this part of the experiment.

The costs to be incurred are mainly well justified, however, in the budget breakdown there is the cost of the light microscope, glutaraldehyde, and formaldehyde which are not mentioned even once in the whole project. These positions of the budget should be better explained in the methodological part of the proposal.
The form of the breakdown of the cost is inconsistent as in some points (e.g. 8) the authors presented the total amount of costs whereas in others (e.g. 4) the costs are only presented individually for each item/reagent. The form of this direct costs breakdown would be more understandable if the total amounts will be presented for each of the points.
Additionally, there is some mistake in counting direct costs (in the table). The amount of PLN in 'other direct costs' is 192900, whereas when I counted (multiple times) the other direct costs based on the breakdown I got the 177900 as a result. The difference is 15 000, which I assume may be a result of accidentally adding the cost of the light microscope. The total amount of direct costs in the table is inconsistent with the rest of the table.

The authors clearly present the questions to be answered thanks to the planned experiments. The methodological approaches are logical and well-planned. Justification and state-of-art parts are very impressive as it consists of plenty of data based on multidisciplinary and recent publications. Principal investigators are aware of possible problems during the project and reasonably plan risk-avoiding activities. The figure presented as a supplement is well prepared and it makes it easier to understand the planned experiments.
The issue raised in that project is indeed interesting and worth studying as it can fill the gap in the knowledge concerning sponge's nervous-like communication, structures, and its origin.

In my opinion, even though the expenses planned to be incurred, the biggest weakness of the project is the budget in terms of technical issues described in point 4 of the review.

Moreover, there are a few aspects mentioned in the course of the review that should be clarified better or more consistently.

Ekaterina Rostovskaya

The project focuses on neural-like responses of sponges (Porifera), what are the neurotransmitter receptors and how they have been evolutionally formed. Hypotheses are not formulated, but the rows 74-76 inform us of determining the mechanisms of nervous cell-like response and evolutionary process of neural-like system's formation in sponges.
Neurotransmitters mediating the contraction behaviour (triggering, inhibition) have already been shown, but the mechanism of their work and reasons why neurotransmitters might have different effect on freshwater and saltwater sponges remains unclear. Though the presence of receptors for these neurotransmitters and their physiological response have been studied, no visualisation and pinpoint of receptors were done.
Authors claim evolutionary position of sponges to remain uncertain (raw 96), but it is not confirmed by any references. In sponges' genome were shown the genes responsible for development and functioning of nerve cells and nervous system function, but it has not been proven if these genes remained undeveloped or were secondarily simplified parts of a more complex nervous system. Moreover, it is understudied which of these genes are being expressed and if they are active in both larval and adult stages.

This study will enrich the knowledge on evolution of nervous system and its possible origins, such as protosynaptic structures. In addition to this, the project is defined as revealing the evolutionary origin of sponges and improving the methodology of identification of pseudogenes and genes silenced during development. Authors claim the study of movement coordination's mechanisms to be applicable in the context of neurosystem disorders, and presudogenes and silenced genes – in research of developmental disorders and cancers. In my opinion as of a non-specialist, for such predictions authors should provide more explanations and/or citations.
This study has a potential impact in the field of basic science, both in the structure and evolutional development of nervous system in the context of sponges and other taxa. The information on sponge's ecology and distribution would have been helpful in understanding the international context, but I still consider this research important in the field of invertebrates.
Despite diverse rankings of references (with IF from 0 for *Journal of Comparative Physiology* and *Zootaxa*, IF 0.9 for *Invertebrate Biology* – and up to 32.2 for *Science* and 47.5 for *Nature*), the average of 10.73 and median of 4.4 shows that this project has a perspective of publishing from good to high quality journals. The research field of invertebrate biology itself is quite specific, but implementation of the research outcomes in the field of evolutionary genomics increases the importance.

Concept and work plan are supplemented with a figure of research design. The project focuses on two sponge species, *Tethya Wilhelma* and *Spongilla lacustris*, one of which will be ordered from zoological botanical garden and the other will be collected from natural habitat and artificial pond. The project includes two experimental branches – search for the receptors and communication pathways (using transmission electron microscopy and immunofluorescent imaging) and genetic analysis of gene expression.
Authors divide responsibilities in the project concept, which provides us with information of their input into the study and decreases potential risks of work disorganisation. Risk analysis is provided, but in my opinion, it is lacking the estimations for the evolutionary

component of the study. It is also lacking information on risks of sample transportation from Germany.
The research methodology is detailed, up to amoeboid-neuroid markers provided (rows 185-187), but without a citation. In general, the project seems to be planned realistically, with procedures and equipment named. The source of the equipment in some cases is not given, for example, availability of the Illumina NGS platform.
Noteworthy, it is not provided how the authors will estimate the evolutionary origins of sponges' neural-like transmitters, which is stated in the aims.
It is not given any information on international cooperation, though satellite personnel (marine biologist, hydrobiologist, zoologist) might be there. Suggestion for a future project is provided.

The total project costs are 1 120 143 PLN, 64% of which go for the principal investigators' salary (5000 PLN / month). When comes to satellite personnel salary, the salary of 1000 PLN per month is suggested for marine biologist / hydrobiologist / zoologist. To the best of my knowledge, it is a very low salary for such specialisations, so either the research methodology and budget require explanation of the working tasks, or the salary is unjustifiably low.
The travelling costs till the place of field research are missing, as well as the delivery costs for samples ordered. If not given other information, renting costs of using the Illumina NGS platform are not provided.
The total sum of direct costs provided (918 150 PLN) does not match to the sum of personnel costs, research equipment and other direct costs (933 150 PLN in total). Noteworthy, the cost of open access publication (indirect cost-1) will not cover potential publication in Nature (5000$).

The project is well structured and has a large potential. After some minor corrections are provided, it has chances of making findings worth presenting on international conferences and publishing in high-impact journals.

In my opinion, this project lacks more structured background for non-specialistic audience. For example, in the rows 136-137 it is possible to improve the accessibility of the project by explaining: gene methylation during development takes part in gene silencing (though it is mentioned in the row 153). Terminology is not consistent: in the rows 52-53 authors aim to "study one of the most archaic neural systems", but in the raw 64 they name sponges responses "neural-like". The definitions "nervous cell-like" and "neural-like" are being used as interchangeable, but no comment on this is provided. It would be helpful to have at least a bit of information on sponges' ecology, ecosystem impact and geographical distribution.
In my opinion, for logical flow "State of the art" (raw 113) should go before "Justification" (raw 79).
Hypotheses are not formulated, but I do consider they can be provided. In absence of clear statements on hypotheses, the phrase "species of our interest" (row 126) is confusing and it takes time to come back looking for the same citation as cited – and later in the supplementary figure get known there are two species.
Minor comments to the editing, such as left and right margins of 2,01 cm, additional spacing, shortage "890-Million yo" (row 56). Preprint citation needs to be corrected (rows 222-224). The supplementary figure requires some clarification, spelling corrections, and also its quality is decreased.

Szymon Kantor

Neuroscientists and evolutionary biologists do discuss and debate quite a controversial topic of the evolution and development of the nervous system in Porifera, and there is ongoing research and exploration in this area, which the authors of the project pay attention to.
One area of debate centers on the nature of the neural-like structures in sponges and whether they represent a true nervous system. While sponges do possess specialized cells that can detect and respond to stimuli, the extent to which these cells function in an integrated and coordinated manner to generate complex behaviors is still a matter of investigation and debate.
Another area of debate concerns the evolutionary origins of the nervous system and whether it evolved independently in different animal lineages or whether it has a common origin that can be traced back to a single ancestral group of organisms. Understanding the evolutionary history of the nervous system is important for unraveling the mechanisms of neural development and the emergence of complex behaviors and cognitive abilities in all groups of animals.
However, the topic has not yet been thoroughly explored, which makes it an incredibly interesting area of research at the intersection of neurobiological, physiological, and evolutionary sciences. The proposed scope of the topic indeed allows for the expectation of highly valuable information to be obtained for many fields of contemporary science.

As noted by the authors of the project, the issue of the evolution and functioning of the nervous system in Porifera representatives is a highly discussed issue. The current achievements of research, trying to find answers in the above matter, are published in reputable peer-reviewed journals and scientific periodicals with a worldwide reach (e.g., Nature, Science, Journal of Experimental Biology; examples from the literature provided by the authors of the application). Taking into account the relevance of the subject, it gives hope that the results of this project will also have a chance to be published in reputable and well-known journals.
However, the importance of the research, which is also proposed in this project, generates great interest from numerous scientific groups. This makes me remain cautious as to the extent of the possible successes of the authors of this project. The authors do not envisage establishing cooperation with foreign research groups or research centers, which would allow me to look more optimistically at the issue of, for example, the priority of publication of truly groundbreaking results, which the authors seem to not necessarily have fully justified certainty about. The feasibility assessment of the project is high. The presented diagram of the sequence of experimental procedures (Suppl. 1) is clear and allows you to find yourself in the planned research. The research techniques proposed by the authors (e.g. transmission electron microscopy, immunofluorescent staining of biological material, transcriptome sequencing) are well described, documented and constantly developed, which minimizes the risk associated with their implementation. The choice of animal model for the experiments was well justified; they seem to be in fact the best possible option in the proposed studies. The animals used in the research will be partly purchased from a reputable breeder [botanical zoological garden Wilhelma Stuttgart (Germany)]. Genomic research will be conducted based on open access databases and published information on sequenced genomes.
From the reservations, I will only point out that, in the case of providing markers for different types of sponge cells, it would be advisable to provide examples of producers of primary antibodies and product identification numbers. Ready, prefabricated, and standardized antibodies give hope for trouble-free implementation of immunohistochemical tests. However, the need to create them yourself or order them

from manufacturers may result in a significant delay in starting the work. In the extreme case of not being able to create them, this part of the project may be unfeasible.
What deserves special mention is that the authors made a convincing risk analysis and proposed possible ways to solve possible problems that they are aware of.

The proposed cost estimate covers the needs of all proposed research tasks. Unfortunately, it is very poorly presented in itself. First of all, there is no justification for the direct costs incurred (neither verbally in the cost estimate nor in the form of references to the planned research plan). There is no suggestion of specific manufacturers and identification numbers of individual materials and reagents (in the case of disposable materials, such as laboratory plastic or microscope slides, it is unnecessary, but in the case of highly specialized materials, such as kits for RNA extraction or immunohistochemical tests, it seems to me to make the cost estimate more credible and entire project). The estimated costs of some items (such as glutaraldehyde or paraformaldehyde) appear to be underestimated and may not be enough to purchase them over the course of the entire project. The authors offered PLN 40,000 for participation in conferences, without specifying which ones they plan to attend or which members of the research team will be representatives of the group. The authors also did not foresee expenses for such trivial but still critically necessary items for the implementation of the project, such as the purchase of an electronic certified signature (necessary to keep product documentation), personal protective equipment (necessary to maintain occupational health and safety in the biological laboratory), spare parts and operational equipment for research and scientific equipment, postal, courier, and transport services. Intriguingly, in the breakdown of direct costs, the greater part is taken up by salaries (principally PI's salaries) and travel to conferences. As a result, research funding is highly limited. Errors are also at the level of basic mathematics (the requested sum for direct costs is different than the sum obtained after the reviewer has independently summarized the components of direct costs).
To sum up, I consider the project's budget as the weakest part of the entire application, clearly standing out in its entirety and completely unsatisfactory.
Certainly, the authors have managed to demonstrate that the research topic undertaken is interesting, interdisciplinary and important. They presented a convincing risk analysis, proposed an adequate methodology and planned research techniques that could be implemented, which proves their high competence to implement the entire project. The authors of the project did not make clear hypotheses or predictions regarding the subject matter. These can be deduced by carefully reading the application, however, this should not be the task of the reviewer of the application.

Line 65:
We know that sponges have structures that are most likely involved in the neural-like responses.
This sentence is insufficient because it lacks specificity and detail. While it is reliable that sponges have structures that are involved in neural-like responses, simply acknowledging this fact does not provide a detailed understanding of the mechanisms involved.

Lines 65, 66, 67:
We also know that sponges have genes homologous to gene groups involved in the differentiation and functioning of nervous cells.
Again, lack of specificity and detail. When providing this type of information, simply posting it seems to me insufficient, because the lack of providing a credible source for this information raises my concerns about its accuracy.

Line 67:
The microstructural analysis reveals convincing candidate cells to play a role in neural-like coordination of movements…
No analysis source. The authors do not mention whether it was their research or a third party.

Line 93:
The presence of receptors for metabotropic glutamate…
There is no such thing as "metabotropic glutamate". However, I believe this is most likely an unintentional error in word order.

Line 93, 94:
The presence of receptors for metabotropic glutamate and GABA and their physiological response has been studied…
The source of the information is not given.

Line 186, 187, 188:
Amoeboid-neuroid markers to be used are Peroxidase A (choano-neuroid), c85989_g1 (Apopylar cells), c103466_g1 (Amoeboid cells), Acp5 (pinaco-neuroid). Membrane stains are CellBrite Fix and Fm143-Fx; cyan – nuclei (DAPI).
Presumption (maybe unjustified?) of plagiarism:
https://www.biorxiv.org/content/10.1101/758276v1.full (Figure 3 description)

FINAL VERSION

Do sponges have neurons? Insights into the Porifera 'neural' system through neuroimaging and genomics.

Mateusz Chechetkin, Rebecca Miszczak, Anastasiia Mykhailenko, Anbarieh Saadat

Summary

Sponges are the oldest living animals and a sister group to all modern eumetazoans. They do not possess fully differentiated tissues or a developed nervous system. So how do they respond to mechanical stimuli and neurotransmitters? And why do they have genes responsible for parts of neuronal cells and the differentiation of the nervous system?

Despite recent progress, such as identification of a potential candidate for a neuronal-like cell in sponges, little is known about the ways in which they are able to generate responses to environmental and chemical stimuli. Furthermore, there are two competing explanations for the apparent mismatch between sponges' anatomy, physiology, and genetics. One possibility is that sponge nerve-like cells represent the simplest possible structure that did not develop further. The other potential explanation is that the common ancestor of sponges and eumetazoa possessed a more complex nervous system, and some functions were lost overtime.

This project aims to unravel the mechanisms by which sponges are able to act as if they have neurons and elucidate their evolutionary origins. We will use neuroimaging techniques to visualize the reaction of sponge cells to selected neurotransmitters and identify the receptors or receptor-like structures that bind them. We will also use comparative genomics to search for pseudogenes - sequences similar to those of more complex animals that were damaged by mutation. Transcriptome analysis and methylation analysis will allow us to trace the developmental path of neuron-related genes and detect silenced sequences. Presence of pseudogenes and/or genes that are deactivated in adult animals would support the loss of function hypothesis, while their absence would support the simplest form hypothesis.

This research has the potential to discover ways of neuronal-like communication, function and neurotransmitter response not yet known to science. It could also settle a decades-long debate on the evolutionary origin of sponges and their position in the metazoan family tree. The results of the research will constitute a substantial contribution to evolutionary biology, developmental biology, and neuroscience.

1) Scientific goal of the project

The project is directed to study **one of the most archaic neural systems** in the animal kingdom, detected in Sponges (Porifera).

Sponges are the 'elder sister' of the whole eumetazoan group and the most ancient animal forms on the planet (oldest fossils are dated to 890-Million years ago) (Turner et al. 2021). Sponges are considered to be the oldest living Metazoans, possessing the simplest

morphology known among multicellular animals (Hooper et al. 2021). They have only two germ layers and do not have fully differentiated tissue systems, including the nervous system (Musser et al. 2021). However, coordinated movements (locomotion) was detected multiple times in sponges with a siliceous skeleton (Demospongia) (Bond and Harris 1988, Nickel et al. 2004, Nickel et al. 2010). Moreover, there are signs that some sponge cells resemble the electrochemically dominated integration (nervous) system: on the level of tissue organization, behavior, and genes (Nickel et al. 2010).

We know that sponges have structures that are most likely involved in the neural-like responses (Simpson 2012, Ryen et. al 2015, Ellwanger et al. 2006a/2006b). We also know that sponges have genes homologous to gene groups involved in the differentiation and functioning of nervous cells (Ryan et al. 2015, Francis et al. 2017). Nevertheless, the picture remains incomplete. The microstructural analysis reveals convincing candidate cells to play a role in neural-like coordination of movements (Leys 2015), but there is no description of how these cells react to the neuromediator induction. Also, it is still unclear whether identified genes and transcription factors represent simplified remnants of a more complex nervous system or an early precursor that did not develop further in sponges. **In this study we are aiming to identify the selected neurotransmitter receptors that initiate the responses to stimuli. We will also determine whether the sponge's basic nerve-like response is the result of losing function in their evolutionary past.**

There are **three main hypothesis** we are going to address:

a). H1 Sponges possess receptors or receptor-like structures that are responsible for neuromediator induced contractions.

b). H2 Sponges possess pseudogenes - genes similar to those of more complex animals but broken by mutation - that used to be involved in the development and functioning of the nervous systems.

c). H3 Sponges have genes responsible for the functioning and development of the nervous system that are methylated (silenced, not expressed) in adult animals.

For each hypothesis, confirming it would support secondary loss of function explanation, while falsifying it would support the simplest possible system explanation.

This project has potential to reveal the mechanisms by which sponges produce a nervous cell-like response without nervous tissues and to clarify what evolutionary process underlies the formation of neural-like system in sponges.

2) Significance of the project

Justification:

The evolution of the central nervous systems of the Bilateria is generally better understood than the evolutionary origin of the nervous system. However, in order to understand the origin of nervous systems in the widest context, pre-nervous systems should also be investigated. Morphological, physiological, and genomic evidence (Bond and Harris 1988, Nickel et al. 2004, Nickel et al. 2010, Ellwanger et al. 2006a/2006b, Francis et al. 2017, Musser et al. 2021) suggests that a poriferan nervous system comprises

modules that might be homologous to modules in nervous systems of other animal groups.

For instance, there are studies that suggest slow signaling pathways that include small molecule transmitters or neuropeptides could be a path of communication between sponge tissues. Some of these molecules have been shown to mediate the contraction behavior (Leys 2015). Neurotransmitters like glutamate and GABA are shown to both trigger and inhibit contraction in sponges. Interestingly, GABA induces contraction in freshwater sponges and inhibits contraction in saltwater sponge *Tethya wilhelma* (Ellwanger et al. 2006a), and the mechanism that underlies this difference is unclear. The presence of metabotropic glutamate and GABA receptors and their physiological response has been studied (Leys 2015), but to our knowledge, there has been no study that tried to visualize and pinpoint the described receptors.

The evolutionary path of sponges' neuronal response remains uncertain. What we know of sponge anatomy and physiology suggests that they split from the common ancestor of all animals before the development of any neuronal cells or a centralized nervous system (Moroz, L. L., & Romanova, D., 2022). However, it has been demonstrated that sponges possess genes responsible for the development and functioning of nerve cells (Wong et al. 2019). Genes associated with synapse cell structures, receptors of neurotransmitters (e.g. GABA and glutamate) as well as neurotransmitter synthesis have been found in sponge genomes (Leys 2015, Perovic et al. 1999). Additionally, sponges appear to have developmental transcription factors associated with nervous system function, such as Pax and Sox (Fortunato et al. 2012, Rivera et al. 2013). It is still unclear whether those genes represent simplified remnants of a more complex nervous system or an early precursor that did not develop further in sponges. Detection of so-called 'pseudogenes', or genes that used to be functional in the more complex ancestor and have degraded, could provide evidence for the secondary loss of neural structures in sponges.
Furthermore, to this day, there is no conclusive research tracing the developmental trajectory of genes in sponges. It is unclear whether genes that have been identified in whole genome sequencing are expressed in larval stages, and how many of them are inactive in adult individuals. If it is discovered that some genes are silenced during the development of the sponge, this would support the hypothesis of evolutionary loss of function. In contrast, the absence of pseudogenes or silenced genes would support the hypothesis of simplest possible structure
As of today, there are two species that are relatively well described in literature: fresh water sponge *Spongilla lacustris* and sea sponge *Tethya wilhelma. T. wilhelma* is known to respond to induction with neurotransmitters and its contractive abilities and tissue organisation are relatively well described (Ellwanger et al. 2006a/2006b,). Moreover, there is a whole genome of *T. wilhelma* that was recently published with the annotation of functioning genes (Francis et al. 2017). The tissue structure and types of cells of *S. lacustris* was also been recently published along with its transcriptome (Musser et al. 2021). Both of them are good candidates to further the existing research.

State of art:
There are existing well-developed methods of imaging internal structure and activity of tissues including transmission electron microscopy and immunofluorescence techniques (Winey et al. 2014, Burkhardt et al. 2017, Musser et al. 2021). For *Spongilla lacustris*, there are specifically identified markers for imaging all known types of tissues and cells (Musser et al. 2021) and the imaging via transmission electron microscopy has been already used to investigate sponge tissues before and has been described by Burkhardt et al., 2017.

Currently, the two biggest achievements in addressing the evolution of the neural system in sponges are: whole genome sequencing of sponges which identified genes potentially involved in nervous system functioning (Srivastava et al. 2010); and RNA sequencing of adult tissues to identify cell types and gene expression profiles (Musser et al. 2021). As of 2023, there have not been studies on nervous function-related pseudogenes or developmental changes regarding gene expression in sponges. Nevertheless, methodology for the detecting pseudogenes is extensively developed (Abrahamsson et al. 2022) and RNA sequencing techniques were developed specifically for the species of our interest (Musser et al. 2021).

Impact:

Study of the nervous system in such a primitive organism that has an efficient yet simple organisation would be beneficial to understand the evolution of the nervous system as we see it today. Proving the existence of receptors or receptor-like structures to glutamate and GABA will constitute evidence for the presence of protosynaptic structure. By understanding what mechanisms sponges use to coordinate movements, we gain a unique animal model to acquire insights into the neurosystem disorders.

This research would also greatly contribute to a fundamental question in evolutionary biology and could potentially settle a decades-long debate on the evolutionary origin of sponge neuronal response. Apart from the significance for fundamental research, it could improve the methodology of pseudogene identification and detection of gene methylation during development. Better understanding of sponge transcription factors and gene silencing has implications for human disease research, as these mechanisms are deeply conserved and are involved in some developmental disorders and cancers (Lee, T. I., & Young, R. A., 2013).

In conclusion, we expect to publish the results of the research in one of the highly-ranked interdisciplinary journals and we project great visibility of the research among several diverse fields of biology, including but not limited to neurobiology, evolution, and developmental biology. Furthermore, if we would identify silenced genes in sponges, future experiments could be done to induce artificial differentiation of their cells into proto-neurons and better understand their evolutionary origin.

3) Concept and work plan

In this study we address the protoneural system in sponges on three fundamental levels (Suppl. 1: https://docs.google.com/document/d/1G4WJ2cO5utlfVfJaXWkbkMX9c-uH4ur2H_tjsN1UGoQ/edit):

1. Investigating the presence of neurotransmitter receptors and communication pathways of neuron-like cells in two species of sponges: *S. lacustris* and *T. wilhelma*. We are going to focus on imaging overall microstructure with transmission electronic microscopy, label receptors or receptor-like structures to which glutamate and GABA bind, and visualise tissues with immunofluorescent imaging. This stage will be implicated during the last two years (PI responsible: Anbarieh Saadat, Rebecca Miszczak)

2. Comparative genomics. Identifying pseudogenes in sponges using fully assembled genomes. Data acquired from NCBI will be used to find broken genes. This part will be processed during all three years in parallel to the rest of the study (PI responsible: Mateusz Chechetkin, Anastasiia Mykhailenko)

3. Developmental biology and epigenetics. Studying larval and adult stage of *S. lacustris* in order to distinguish patterns of gene expression related to individual development. RNAseq and methylation analysis will be used to identify patterns of gene expression and methylation (silencing) in sponges. This part will be initiated after the first season of

sampling or the second, depending on the sampling success (PI responsible: Mateusz Chechetkin, Anastasiia Mykhailenko, Rebecca Miszczak)

Risk analysis:
There are several potential risks that we are going to address. First of all, to increase the chances of acquiring larva from *S. lacustris* specimens, we are going to sample for two years, first year from the natural habitat and second years from the artificial pond. There is also a risk that manufactured antibodies for immunogold labeling will not work and will require time for optimizing antibodies or reordering. To avoid losing time we will make this step a priority at the beginning of the project and will be able to correct the process in time. Additionally, due to the sponges being a very diverse and isolated animal group, many homologous genes that are known in other animal groups could be extremely divergent. Being aware of this issue, we will do an extensive literature search in order to find methods that help us detect gene sequences that only partially overlap.

4) Research methodology
Sampling and cultivation
Fifty adult individuals of *T. wilhelma* will be ordered from the aquarium of the botanical zoological garden Wilhelma Stuttgart (Germany). For the firther analysis they will be preserved in the formaldehyde-glutaraldehyde mix. Individuals (adults and larvae) of *S. lacustris* will be collected from the site in an artificial pond in Poleski National Park (Poland). Within the first summer (June-July) of the experiment around 100 samples (parts of sponges) will be collected from the river. Half of the samples will be planted in a pond. The rest will be used in the following analysis. In order to identify samples that contain embryos light microscopy will be used. In the next season sponges cultivated in pond will be used for the analysis. Samples of *S. lacustris* will also be preserved in formaldehyde-glutaraldehyde mix for the neuro imaging and in stored in liquid nitrogen.

Imaging
First, we will use transmission electron microscopy to image the interior of the cells using ultrathin sections. The samples collected from both larva and adult sponges will be contrasted and analyzed using transmission electron microscopy to localize internal structures. Post-embedding immunogold labelling is used to localize the receptors using primary antibodies and secondary antibodies which are coupled with gold particles. Number of receptors for neurotransmitters glutamate and GABA will be investigated.
Second, we will use immunofluorescence that allows visualization of the distribution of the target molecule through fluorescent dyes with a fluorescence microscope. Amoeboid-neuroid markers to be used are Peroxidase A (choano-neuroid), c85989_g1 (Apopylar cells), c103466_g1 (Amoeboid cells), Acp5 (pinaco-neuroid). Membrane stains are CellBrite Fix and Fm143-Fx; cyan – nuclei (DAPI) (Musser et al. 2021). Around 50 individuals from each species will be taken for the neuroimaging.

Identification of pseudogenes and silenced genes
To identify potential pseudogenes, we will take advantage of existing genetic databases and thre existing whole assembled genome sequences of sponges (Srivastava et al. 2010, Francis et al. 2017, Kenny et al. 2020). We will then identify putative pseudogenes by comparing to genomes of more complex animals (Harrison et al. 2003; Zhang et al. 2006). These tasks are performed entirely using bioinformatics techniques. To trace the developmental history of the target genes, transcriptome sequencing is performed at both larval and adult stage using RNAseq (three larval and threee adult individuals of *S. lacustris*) (Hrdlickova et al. 2017). Additionally, silenced genes are identified with methyl

CpG binding domain analysis on the Illumina NGS platform (Yong et al. 2016) and further processed with bioinformatics methods (Cedoz et al 2018).

Statistical analysis
All statistical analysis will be performed in the R statistical computing environment (R Core Team, 2021). The package 'patternize' (Van Belleghem et al. 2017) will be used to quantify the variation of the color pattern in fluorescence imaging. Next, the Kolmogorov-Smirnov test will be used to compare the distributions of fluorescent dye between images. Principal Component Analysis (PCA) will be calculated to compare sets of data. Transcriptome data will be analyzed using differential expression analysis with the "DESeq2" package (Love et al. 2014).

5) Project literature

Abrahamsson, S., Eiengård, F., Rohlin, A., Dávila López, M. (2022). PΨFinder: a practical tool for the identification and visualization of novel pseudogenes in DNA sequencing data. *BMC bioinformatics*, ***23*****(1)**, 1-11.
Bond, C., Harris, A. K. (1988). Locomotion of sponges and its physical mechanism. *Journal of Experimental Zoology*, **246(3)**, 271–284.
Burkhardt, P., Sprecher, S.G. (2017) Evolutionary origin of synapses and neurons - bridging the gap, *BioEssays*, **39(10)**, 1700024.
Cedoz, P. L., Prunello, M., Brennan, K., Gevaert, O. (2018). MethylMix 2.0: an R package for identifying DNA methylation genes. *Bioinformatics*, **34(17)**, 3044-3046.
Ellwanger, K. Eich, A., Nickel, M. (2006) GABA and glutamate specifically induce contractions in the sponge *Tethya Wilhelma, Journal of Comparative Physiology A*, **193(1)**, 1–11.
Ellwanger, K. Nickel, M. (2006) Neuroactive substances specifically modulate rhythmic body contractions in the nerveless metazoon Tethya wilhelma (Demospongiae, Porifera), *Frontiers of Zoology,* **3(7)**
Francis, W. R., Eitel, M., Vargas, S., Adamski, M., Haddock, S. H., Krebs, S., … & Wörheide, G. (2017). The genome of the contractile demosponge *Tethya wilhelma* and the evolution of metazoan neural signalling pathways. *BioRxiv*, 120998.
Kenny, N. J., Francis, W. R., Rivera-Vicéns, R. E., Juravel, K., de Mendoza, A., Díez-Vives, C., … Leys, S. P. (2020). Tracing animal genomic evolution with the chromosomal-level assembly of the freshwater sponge *Ephydatia muelleri*. *Nature communications,* **11(1)**, 3676.
Harrison, P. M., Milburn, D., Zhang, Z., Bertone, P., Gerstein, M. (2003). Identification of pseudogenes in the *Drosophila melanogaster* genome. *Nucleic acids research*, **31(3)**, 1033-1037.
Hoper, J. N., Wörheide, G., Hajdu, E., Erpenbeck, D., De Voogd, N. J., Klautau, M. (2021). Zootaxa 20 years: Phylum Porifera. *Zootaxa*, **4979(1)**, 38-56.
Lee, T. I., & Young, R. A. (2013). Transcriptional regulation and its misregulation in disease. Cell, 152(6), 1237-1251.
Leys, S.P. (2015) Elements of a 'nervous system' in sponges, *Journal of Experimental Biology*, **218(4)**, 581–591.
Love MI, Huber W, Anders S (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, **15**, 550.
Moroz, L. L., & Romanova, D. (2022). Alternative Neural Systems: What is a neuron?(Ctenophores, Sponges, and Placozoans). *Frontiers in Cell and Developmental Biology*, *10*, 2446.

Musser, J. M., Schippers, K. J., Nickel, M., Mizzon, G., Kohn, A. B., Pape, C., … Arendt, D. (2021). Profiling cellular diversity in sponges informs animal cell type and nervous system evolution. *Science*, **374(6568)**, 717-723.
Nickel, M. (2004). Kinetics and rhythm of body contractions in the sponge *Tethya wilhelma* (Porifera: Demospongiae). *Journal of Experimental Biology*, **207(26)**, 4515–4524.
Nickel, M. (2010). Evolutionary emergence of synaptic nervous systems: what can we learn from the non-synaptic, nerveless Porifera? *Invertebrate Biology*, **129(1)**, 1–16.
Perovic, S., Krasko, A., Prokic, I., Müller, I. M., & Müller, W. E. (1999). Origin of neuronal-like receptors in Metazoa: cloning of a metabotropic glutamate/GABA-like receptor from the marine sponge *Geodia cydonium*. *Cell and tissue research*, **296**, 395-404.
R Core Team. *R: A Language and Environment for Statistical Computing*; R foundation for statistical computing: Vienna, Austria, 2021
Rivera, A., Winters, I., Rued, A., Ding, S., Posfai, D., Cieniewicz, B., … Hill, A. (2013). The evolution and function of the Pax/S ix regulatory network in sponges. *Evolution & Development*, **15(3)**, 186-196.
Ryan, J.F., Chiodin, M. (2015) Where is my mind? how sponges and placozoans may have lost neural cell types, *Philosophical Transactions of the Royal Society B: Biological Sciences*, **370(1684)**, 20150059.
Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M. E., Mitros, T., … & Rokhsar, D. S. (2010).
The Amphimedon queenslandica genome and the evolution of animal complexity. *Nature*, **466(7307)**, 720-726.
Simpson, T.L., 2012. *The cell biology of sponges*. Springer Science & Business Media.
Turner, E.C. (2021.) Possible poriferan body fossils in early Neoproterozoic microbial reefs. *Nature*, **596**, 87–91 .
Van Belleghem, S. M., Papa, R., Ortiz-Zuazaga, H., Hendrickx, F., Jiggins, C. D., Owen McMillan, W., Counterman, B. A. (2018). patternize: An R package for quantifying colour pattern variation. *Methods in Ecology and Evolution*, **9(2)**, 390-398.
Winey, M., Meehl, J. B., O'Toole, E. T., Giddings Jr, T. H. (2014) Conventional transmission electron microscopy, *Molecular Biology of the Cell*, **25(3)**, 319–323
Wong, E., Mölter, J., Anggono, V., Degnan, S. M., Degnan, B. M. (2019). Co-expression of synaptic genes in the sponge *Amphimedon queenslandica* uncovers ancient neural submodules. *Scientific Reports*, **9(1)**, 1-15.
Yong, W. S., Hsu, F. M., Chen, P. Y. (2016). Profiling genome-wide DNA methylation. *Epigenetics & chromatin*, **9(1)**, 1-16.
Zhang, Z., Carriero, N., Zheng, D., Karro, J., Harrison, P. M., Gerstein, M. (2006). PseudoPipe: an automated pseudogene identification pipeline. *Bioinformatics*, **22(12)**, 1437-1439.

6. Table with budget of the project.

	Amount in PLN
Direct costs, including	955995
- personnel costs and scholarships	736500
- research equipment/device/software cost	15000
- other direct costs	204495
Indirect costs, including:	210319
- indirect costs of OA	19112

- other indirect costs	191199
Total costs	1166314

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

Direct costs include:

1. Remuneration for the research team: Principal investigator salary, 4 people for 36 months, 5000 thousand per month = 720000 PLN
2. Renumeration for the satellite personnel: marine biologist/hydrobiologist/zoologist for the two field seasons = 12000 PLN (4000 per month, 1.5 month per season). Students stipend: first season, two students = 3000 PLN (1000 per month, 1.5 month per season); second season, one master student = 1500 PLN.
3. Purchase or research equipment: light microscope = 15000 PLN
4. Neuroimaging: test tubes, plastic and glass containers, glass slides, lab knives, Poly/bed 812 embedding kit and TEM grids = 15000 PLN. Two antibody kits, price per kit 2000+3000 = 5000 PLN. Immunofluorescent markers: 15000 PLN
5. RNA sequencing costs: RNA extraction kit = 3000 PLN, consumables (different tubes = 100 PLN pipette tips = 450 PLN, gloves = 100 PLN, liquid nitrogen=500, dry ice = 100 PLN) = 1250.
6. Living costs for the field research: First season: 1.5 month, 300 PLN per person daily, diet = 50 PLN, 3 people =47250 PLN; second season: 1,5 month, 2 people = 31500
7. Cultivation and sampling of *Spongilla lacustris* cost: rent of pond for 1 year = 8000 PLN, fishing nets = 500 PLN, diving equipment = 4000
8. Ordering samples of *Tethya wilhelma* cost: 3 packages cost (150 PLN per 1), eppendorfs (200 PLN), glutaraldehyde (250 PLN) and formaldehyde (250 PLN) = 1150 PLN
9. Outsourced services; RNAseq = 15000 PLN, methylation analysis = 16000 PLN
10. National and international conferences: Abstract submission, flight, accommodation for one person =10000 PLN, total = 40000 PLN (calculated for four people). Potential conferences: World sponge conference, Symposium for Invertebrate neuroscience, EuroEvoDevo
11. Electronic signature needed to sign documents. Electronic signature is valid for 2 years and costs approximately 205 PLN. Estimated cost for 3 years per person = 3 x 205 PLN, for four people = 1845.

MIGRATORY AND EVOLUTIONARY CONSEQUENCES OF ARTIFICIAL NIGHT LIGHTING ON PURPLE MARTIN (*PROGNE SUBIS*)

Martyna Gorkowska, Patrycja Dziurowicz, Michał Strączyński

Summary:

Artificial lightning at night (ALAN) is an alarming and growing problem for wildlife including birds. It negatively influences their biological rhythms, reproduction, and migration. Even though there are many studies concerning ALAN impact on birds there are still many gaps in the knowledge in this field. Our research project aims to answer several questions concerning the subject. What is the influence of ALAN on the reproductive success of Purple Martins and the offspring development? How strongly ALAN disturbs the photoperiod and changes the timing and pattern of bird migration? Are individuals exposed to more ALAN more stressed in comparison to the ones living in the less light polluted places? Does the level of stress hormones correlate with the survival rate of Purple Martin population? Does living in more ALAN and an elevated level of stress potentially lead to the weakness of birds and shortening of migration distance? To answer these questions, we will conduct several field experiments on Purple Martins populations in 15 research areas with differing intensity of ALAN. Thanks to the ringing of big groups of birds and GPS tracking, we will observe their migration patterns and survival rates. Observations of brood size and nestling development will help answer the question about reproduction success. Furthermore, measurements of corticosterone levels in both offspring and adults will enable the corelation between stress levels induced by ALAN and survival as well as migration patterns.
We believe that the extensive analysis of the obtained results will allow researchers to fill the gap in the current understanding of evolutionary consequences of ALAN's negative influence on birds' migration patterns and development. Additionally, because of the large spread of light pollution worldwide and its known effects on birds, it is crucial to focus on this subject to determine the threat to biodiversity posed by light pollution.

1) Scientific goal of the project

Artificial lighting at night (ALAN) is an alarming and growing problem for animal lives, as it can negatively alter individual behaviour, biological rhythms, and reproduction (Da Silva et al., 2015; Dominoni, 2015; Grunst et al., 2020). There is also some evidence that it may negatively influence bird migration patterns. Nestlings exposed to artificial light at night were shown to grow slower which delay colony departure (Smith et al. 2021). It is not known whether such disturbed development of nestlings may influence their migration patterns and what the evolutionary consequences of artificial night lightning. The gap in the knowledge raises several questions concerning these issues. What is the influence of ALAN on the reproductive success of Purple Martins and the offspring development? How strongly ALAN disturbs photoperiod and changes the timing and pattern of their migration? Are individuals exposed to more ALAN more stressed in comparison to the ones living in the less light polluted places? Does living in more ALAN and elevated level of stress potentially lead to the weakness of birds and shortening of migration distance? In this research project, we plan to answer those questions and based on the current knowledge and observations we state the following hypotheses:

a). The nestlings exposed to (more) artificial light at night, are smaller as their ontological development becomes altered.

b). More artificial light at night causes migratory pattern shifted in time and migration distance is shortened for adults and nestlings.
c). The exposure to artificial lighting at night creates long term migration shifts that persist from generation to generation.
d). Exposure to artificial lighting at night increases the level of stress hormone in offspring and adults.

To test these hypotheses, we will study Purple Martin communities in 15 research areas with differing levels of light pollution, understood as the intensity of artificial light expressed in Luxes (lx). We will introduce different observations and measurements of eggs amount, nestlings' development, birds' behaviour and survival rate. Moreover, thanks to birds tracking system we will be able to observe the migration pattern and route of individuals. We believe that the extensive analysis of the obtained results will allow to fill the gap in the current understanding of evolutionary consequences of the ALAN negative influence on birds' development and migration patterns.

2) Significance of the project

Light pollution caused by artificial light at night is rising worldwide and is increasingly seen as a problem for wildlife (Da Silva et al., 2015; Grunst et al., 2020). Artificial light at night (ALAN) is one of the anthropogenic disruptors of natural environmental conditions and, therefore, can disrupt biological systems and cause physiological stress (Grunst et al., 2020). In birds, in addition to collisions with artificially lit structures, one of the most commonly reported effects of light pollution is a change in temporal behaviour (Dominoni, 2015). As shown in previous studies, free ranging birds can be negatively influenced by light pollution regardless of sex, with 1.6 Luxes (lx) being enough to postpone the time the birds fall asleep. Higher intensities may also possibly worsen the effects, although there is not enough evidence to show this conclusively (Raap et al., 2017). Such disturbed sleeping may lead to chronic consequences for the birds (Johnson et al., 2022). The problem becomes worse during the nesting period, when the mother bird and her young are especially vulnerable to light pollution, causing them to lack night-time sleep and increased begging behavior in the offspring. The additional energy costs of this begging behavior, combined with a lack of sleep may lead to reduced fitness of the offspring and the parent (Raap et al., 2016). Migration timing can be crucial for migratory birds - if arrival and departure times are mismatched with optimal breeding conditions, this can result in reduced fitness and ultimately population decline (Both et al., 2006; Neufeld et al., 2021). According to Smith et al. (2021) adult Purple Martins (*Progne subis*) who experienced ALAN for more than 10 nights, initiated spring migration 8 days earlier than others who experienced natural darkness. This advance in timing was not compensated for during migration and birds experiencing ALAN that had left earlier also arrived at their breeding grounds eight days earlier, suggesting the potential for mismatch between bird timing and the availability of resources in early spring. Purple Martin is one of the largest migratory birds belonging to the family Hirundinidae, measuring 19-20 cm and weight 45-60 g. This species is found in North America during the breeding period from January to August, while during the non-breeding period it is found in South America, mainly in Brazil (Brown, 1997; Santos et al., 2021). The pre-migratory period is characterized by the formation of flocks numbering thousands of individuals that congregate at specific sites used as roosts. Importantly as long as conditions are suitable, Purple Martins will return to the exact same breeding site each year (Bridge et al.,2016). According to information from the Connecticut state government, this species is characterized by sexual dimorphism and a monogamous lifestyle. The female lays an average of four to six eggs for 15 to 18 days. After hatching, the young remain in the nest

for 24 to 28 days and are fed insects by both adults (portal.ct.gov., 2016). Two years after hatching, they reach sexual maturity (Stutchbury, 1991).

Another aspect is that exposure to ALAN can disrupt hormone production, activity, sleep, and recovery cycles and the health of wildlife (Grunts et al., 2020; Romero, 2004). One of the hormones that birds produce is corticosterone (CORT), which is responsible for adaptations to stressful situations (Blas, 2015). Elevated CORT concentrations have complex and adaptive functions in glucose metabolism and energy balance, flight responses, and the immune system. In turn, long-term elevation of CORT can result in chronic stress, impaired health, and decreased performance (Grunts et al., 2020; Romero, 2004; Sapolsky et al., 2000). There is a critical level of knowledge about whether exposure to ALAN results in elevated CORT concentrations in developing chicks and adults. Based on the fact that stress lowers the whole organism's fitness, we posit that this diminished fitness might lead to shortened distance of migration in both the parents and the offspring.

Although there are various publications concerning the influence of ALAN on birds functioning in different aspects, our research project is the first that will study the effect on migration survival rate, distance and timing. The study is pioneering, because it will be done in the field while previous studies in this issue were performed in the laboratory. Moreover, we plan to study the evolutionary consequences of ALAN on the population through reproduction and migration success, survival rates, and offspring development. Through the study of CORT levels in the birds, this will also be the first study to outline the effects of ALAN on the long-lasting health of free ranging migratory birds. We believe that such a study is worth conducting over a longer period of time in order to more accurately determine the effects of artificial light intensity at night on the migration and evolution of the species studied. This, in turn, will make it possible to determine the threat to biodiversity posed by light pollution.

3) Concept and work plan

General work plan

The experiment will be conducted for 36 months (3 breeding seasons), starting in the spring, after the return of Purple Martins to the breeding area. The chosen areas will differ in the intensity of the light. Because 1.6 Luxes can be enough to cause sleep interference in nesting females and behavioural changes of their nestlings (Raap et al., 2016), areas with 1.6 Luxes and less will be considered as the least light polluted. The gradient of intensity experienced in the research areas will be in range from 1.6 Luxes to 10, as it is a common intensity experienced in cities (Wigan Council FAQ). First, in the 15 chosen areas, we will prepare the experimental setup which will include installation of temperature loggers in 40 nests (for each experimental area) inhabited by Purple Martins pairs. The change in nest temperature will identify when the first offspring departed from the nest and the exact date when the birds departed on migration. In each of 15 areas, we will distinguish three experimental groups. All individuals studied will be ringed. The first group will consist of 200 Purple Martins that will provide information about migratory success. The second group will consist of 40 pairs (generation I) living in the prepared nests and their reproductive success and nestling (offspring I) development will be observed. The breeding season of birds will be observed to precisely determine the number of eggs laid in each nest. After hatching of the eggs, the nestlings will be counted, observed and weighted every week. The time of their nest leaving will be noted. The last group will consist of 20 individuals (per each experimental area) that emerged from pairs from the second group. The ones will be chosen based on their fitness and will be

equipped with GPS trackers, which will allow following of their migration distance and route. During the whole experiment the survival rate of the population will be monitored. The whole experiment will be repeated for the three following breeding seasons (Fig. 1).

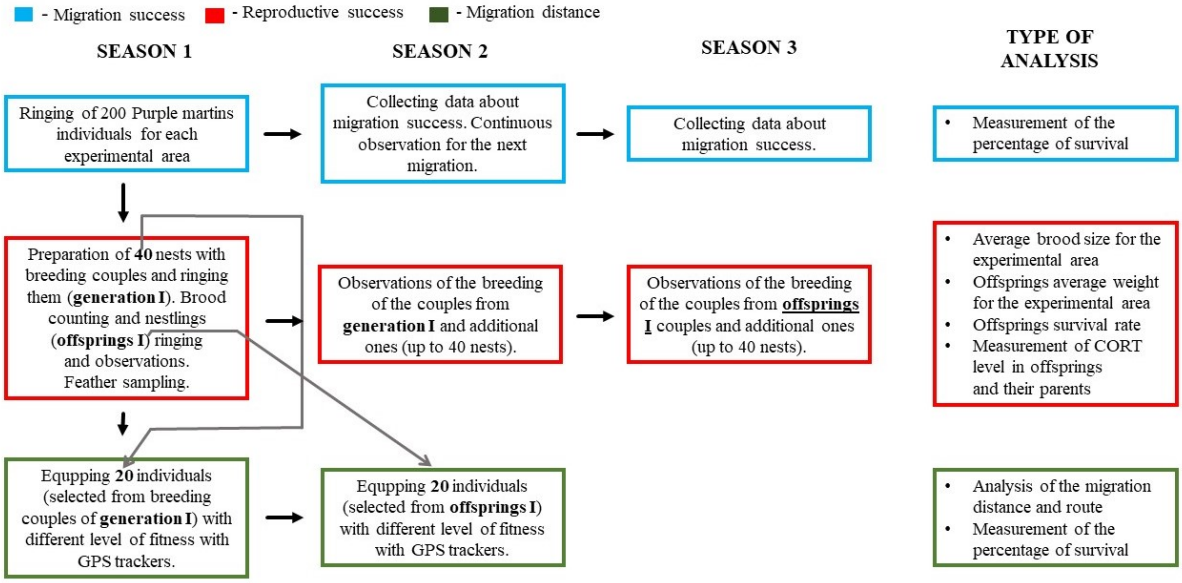


Figure 1: Diagram of the general work plan.

Specific research goals

Our research project aims to examine the following specific goals:

1. Monitoring of the reproduction success of different populations of Purple Martins in the chosen areas characterized with different intensity of ALAN.
2. Determination of the survival rate of nestlings and adult individuals during migration.
3. Observation and precise tracking of Purple Martin migration time, pattern, and route.
4. Monitoring of cortisol level in the studied individuals.

Due to the fact that experiments will be carried out in multiple areas that vary in intensity of ALAN, the results will allow us to compare the groups with each other and to correlate the influence of ALAN on the studied aspects of Purple Martin's life.

Risk analysis

Due to the fact that in the course of experiment we are going to install temperature logs in the nests and a group of birds will be equipped with GPS trackers, there is a risk of the equipment damage. To avoid this, and the loss of data, we will purchase devices of good quality and set in on animals in a very precise way. As all principal investigators do not experienced in bird studies, collaboration is planned with experts in this research. Moreover, laboratory tests of CORT levels will be performed by a qualified laboratory technician. The people who will take care of birds handling and ringing of birds will be trained and experienced in it and possess the necessary licences. We will acquire the ethical permissions needed for the installation of GPS tracking devices and feather collection. To minimise project risk, all stages will be tested in a smaller sample size.

4) Research methodology

Study area We will study Purple Martin (*Progne subis*) communities in 15 research areas. The locations will be designated from existing bird box colonies used for breeding and

grouped according to the intensity of light pollution. Pollution will be measured in Luxes (lx), using Benetech GM1020 before each breeding season. Each location will have a total of 20 birds with GPS trackers installed on them. The bird boxes inhabited by the chosen 40 pairs will have temperature and moisture loggers (HOBO Temperature/RH data logger MX20301A) installed.

Offspring development
The offspring and their parents inhabiting the 40 designated boxes will be counted and weighed using electronic scales for birds. Every three days, the boxes will be manually controlled by specialized ornithologists.

Migration
We will use a mini-GPS tracker "PinPoint 10", manufactured by the company Lotek Wireless which weighs 1.1 g. The trackers will be strapped to the bird using leg-loop made of Teflon ribbon (Thaxter et al. 2014). The birds used for GPS tracking will be taken from the 40 breeding pairs and separated into four categories (cat 4 to 1) with five birds in each, based on their physical condition measured by the amount of fat built around the chest muscles responsible for flight (the more fat, the higher the category). From each research area 200 birds will be randomly selected for ringing. The birds will be caught using an ornithological net.

CORT analysis
Feather collection for corticosterone analysis will take place after two weeks of chick life, then after a month and every month thereafter until the birds migrate. The young will have five contour feathers (breast) removed, and one wing feather will be extracted from the adults. At three weeks of age, the young birds will also be ringed. CORT will be extracted from feathers using methanol, with samples placed in a sonicating water bath at room temperature for 30 min and then incubated overnight in a shaking 50°C water bath. After methanol from feather using vacuum filtration, the methanol extracts will be dried under nitrogen gas and reconstituted in a saline buffer.

Survival rates
Each season when the birds return, they will be caught using an ornithological net from 5 am in the morning. The number of birds returning with rings will be counted and compared to the initial 200 to calculate their death rates when migrating.

Repetition
The migration survival rates, weighing of birds and CORD analysis steps will be repeated for each of three seasons, while GPS marking will be carried out for two years during the project. Schematical diagram presenting experiments to carry out during the project is presented in Figure 1.

Data analysis
The collected data will be statistically analyzed (for example redundancy analysis (RDA), Generalized linear model (GLM), General Unified Threshold for Survival (GUTS Framework)). The numbers of eggs, and the nestlings as well as weight of the birds and corticosterone levels from nest boxes will be used to calculate mean values for each box.

Migration survival rates for each study site will be noted in the following season. We will compare these results between the study sites to determine the influence of ALAN exposure on the measured aspects.

5) Literature

Blas, J. (2015). Stress in birds. In Sturkie's avian physiology (pp. 769-810). *Academic Press*.
Both, C., Bouwhuis, S., Lessells, C. M., and Visser, M. E. (2006). Climate change and population declines in a long-distance migratory bird. *Nature,* 441(7089), 81-83. https://doi.org/10.1038/nature04539
Bridge, E.S., Pletschet, S.M., Fagin, T., Chilson, P.B., Horton, K.G., Broadfoot K.R. and Kelly J.K. (2016). Persistence and habitat associations of Purple Martin roosts quantified via weather surveillance radar. *Landscape Ecol* 31, 43–53. https://doi.org/10.1007/s10980-015-0279-0
Brown, C R. (1997). Purple Martin (Progne subis), The Birds of North America Online (A. Poole, Ed.). Ithaca: *Cornell Lab of Ornithology*; Retrieved from the Birds of North America Online: http://bna.birds.cornell.edu/bna/species/2
Da Silva, A., Valcu, M., and Kempenaers, B. (2015). Light pollution alters the phenology of dawn and dusk singing in common European songbirds. Philosophical Transactions of the Royal Society B: *Biological Sciences*, 370(1667), 20140126. https://doi.org/10.1098/rstb.2014.0126
Dominoni, D.M. (2015). The effects of light pollution on biological rhythms of birds: an integrated, mechanistic perspective. *J Ornithol* 156 (Suppl 1), 409–418. https://doi.org/10.1007/s10336-015-1196-3
Grunst, M. L., Raap, T., Grunst, A. S., Pinxten, R., Parenteau, C., Angelier, F., and Eens, M. (2020). Early-life exposure to artificial light at night elevates physiological stress in free-living songbirds. *Environmental Pollution*, 259. https://doi.org/10.1016/j.envpol.2019.113895
Johnsson, R.D., Connelly, F., Gaviraghi Mussoi, J., Vyssotski, A., Cain, E., Roth II, T. and Lesku, J. (2022). Sleep loss impairs cognitive performance and alters song output in Australian magpies. *Sci Rep* 12, 6645. https://doi.org/10.1038/s41598-022-10162-7
LED Street Lights Frequently Asked Questions Wigan Council https://www.wigan.gov.uk/Docs/PDF/Resident/Parking-Roads-Travel/LED-street-lights-FAQ.pdf (accessed on 22.04.2023)
Neufeld, L.R., Muthukumarana, S., Fischer, J.D., Ray, J.D., Siegrist, J. and Fraser, K.C. (2021). Breeding latitude is associated with the timing of nesting and migration around the annual calendar among Purple Martin (Progne subis) populations. *J Ornithol* 162, 1009–1024 https://doi.org/10.1007/s10336-021-01894-w
Purple Martin, Department of Energy and Environmental Protection https://portal.ct.gov/DEEP/Wildlife/Fact-Sheets/Purple-Martin (accessed on 22.04.2023)
Raap, T., Pinxten, R., and Eens, M. (2016). Artificial light at night disrupts sleep in female great tits (Parus major) during the nestling period and is followed by a sleep rebound. *Environmental Pollution*, 215, 125-134. https://doi.org/10.1016/j.envpol.2016.04.100
Raap, T., Sun, J., Pinxten, R., and Eens, M. (2017). Disruptive effects of light pollution on sleep in free-living birds: Season and/or light intensity-dependent?. *Behavioural processes*, 144, 13-19. https://doi.org/10.1016/j.beproc.2017.08.011
Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in ecology and evolution*, 19(5), 249-255. https://doi.org/10.1016/j.tree.2004.03.008
Santos, C. O., Branco, J. M., Belotti, M. C., Abilleira, P., Siegrist, J., Fischer, J., Lima, L.M., Cohn-Haft, M. and Hingst-Zaher, E. (2021). Distribution and migration phenology of Purple Martins (Progne subis) in Brazil. *Ornithology Research*, 29(4), 213-222. https://doi.org/10.1007/s43388-021-00071-0
Sapolsky, R. M., Romero, L. M., and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine reviews*, 21(1), 55-89. https://doi.org/10.1210/edrv.21.1.0389

Smith, R. A., Gagné, M., and Fraser, K. C. (2021). Pre-migration artificial light at night advances the spring migration timing of a trans-hemispheric migratory songbird. *Environ. Pollut*. 269:116136. doi: 10.1016/j.envpol.2020.116136

Stutchbury, B. J. (1991). Floater behaviour and territory acquisition in male purple martins. *Animal Behaviour*, 42(3), 435-443.

Thaxter, C. B., Ross-Smith, V. H., Clark, J. A., Clark, N. A., Conway, G. J., Marsh, M., Leat, E. and Burton, N. H. (2014). A trial of three harness attachment methods and their suitability for long-term use on Lesser Black-backed Gulls and Great Skuas. *Ringing & Migration*, 29(2), 65-76. https://doi.org/10.1080/03078698.2014.995546

6) Table with budget of the project

	Amount in PLN
Direct costs, including	**2 574 446,00**
- personnel costs and scholarships	684 000,00
- research equipment/device/software cost	977 200,00
- other direct costs	913 246,00
Indirect costs, including:	**566 378,00**
- indirect costs of OA	51 489,00
- other indirect costs	514 889,00
Total costs	**3 140 824,00**

7) Breakdown of project costs and their relevance in the project

Category	**Description**	**Amount in PLN**	
Salaries and benefits	Scholarships	3 persons*2500 PLN*36 months	270 000
	Ornitologists	3 persons*3500 PLN*36 months	378 000
	Laboratory technician	1 person*12000 PLN*3 season	36 000
Equipment	GPS- PinPoint 10	20 individuals*15 research stands*2 season*630 PLN	378 000
	Loggers HOBO MX20301A	40 nests*15 research stands*950 PLN	570 000
	Leg-lopp harness	20 individuals*15 research stands*2 season*8 PLN	4 800
	Ornithological net	2 nets*15 research stans*400 PLN	12 000
	Luxometr Benetech GM1020	2*150 PLN	300
	Avian scales	15 research stands*450 PLN	6 700
	Rings for adults	200 birds*15 research stans*3 seasons* 0,3 PLN	2 700
	Rings for offspring	40 pair*5 eggs*15 research stands*3 seasons*0,3 PLN	2 700
Chemical reagents	Methanol	60 l per 3 seasons	1 800
	Nitrogen gas	60 l per 3 seasons	3 700

	Saline buffer	30 l*530 PLN	15 900
Trip	Plane tickets	3 persons*8000 PLN (two ways)*3 seasons	72 000
	Accomodations	6535 PLN*8 months*3 seasons for 3 persons	157 860
	Diet	4160 PLN*8 months*3 seasons for 3 people	99 840
	Car rental	1344 PLN*8 months*3 seasons	32 256
	Petrol	40 l*5,76 PLN*8 months*4 weeks*3 seasons	21 749,76
	Insurence	3 persons*1500 PLN for 4 months*2*3 seasons	27 000
	Visa	3 persons*380 PLN	1 140
	Conferences	2*3 persons*5000 PLN	30 000
	Maintenance and rental	15 research stands*10000 PLN*3 seasons	450000
		Total	**2 574 446**

REVIEWS

Dr. Edyta Sadowska

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 is large-scale comprehensive project that aims to test whether artificial lighting at night (ALAN) can affect bird populations; migration, reproduction and survival of parents and their offspring reared under different levels of light pollution. The authors also want to test whether stress hormones could be the mechanism behind the changes. The specific research questions to be asked are interesting and clearly outlined. These questions are important because urbanisation is an ongoing process that causes loss of biodiversity, and there is still a lively debate about the main causes.
However, there are several issues that affect its overall quality.
Firstly, although the overall aim is explicitly stated, it is not entirely clear to me how the study will answer the specific questions. The first two hypotheses are written in a correlative way, while the last two hypotheses are more in the way of experimental way (exposed to light vs not exposed). From the methods part, it seems to be whole correlative study. Moreover, the statistical part does not provide a clue as to how the goes will be achieved as it is stated "We will compare these results between the study sites to determine the influence of ALAN exposure on the measured aspects". This means that the 15 study sites will be compared to each other, but it is not known how ALAN exposure will be taken into account.
Secondly, at the very beginning (lines 35-36), the proposed question is very specifically related to Purple Martins. This gives the impression of a case study that is not designed to answer a more general question. Unfortunately, the part about Purple Martins in "Significance of the project" did not convince me why the study should be carried out on swallows in North America. In Europe, and even in Poland, there are swallows from the Hirundinidae family with very similar biology to Purple Martins. With the plan of 15 populations of one species, it may be useful to reconsider the plan and have less population of one species and take more species into account.

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 results of the project may potentially be of international impact on the scientific community as well as conservation organizations. However, as it is planned now, the study is corelative and not all aims may be possible to achieve (see next section).

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 very ambitious and requires very careful planning and extensive logistics. Even taking into account that the study has to be conducted in America (that I am not convinced about it), it is not known if the authors have collaborators there, and without

any collaborators, the project seems to be not feasible. In my opinion, it can be simplified without losing its impact, and at the same time it can be improved in terms of sample size and data collected. The methodology is only broadly elaborated on and it lacks sufficient level of detail in places.
The location of the study is not clearly described, especially as the authors will be using existing nest boxes used for breeding. It is not known how far apart the sites will be. What other abiotic and biotic factors might be different at each site? Will the study sites be independent or paired? How will the authors ensure that the difference between the populations is only due to ALAN? I wonder why the authors will install temperature and humidity loggers (HOBO Temperature/RH Data Logger MX20301A) in the nest and how this will contribute to the project aims. This HOBO measurement seems to be less important than GPS tracking, and the plan is to buy only 20 GPS trackers and 40 HOBOs per study site. Do we know what the return rate is; how many of these 20 will reach the wintering site and return? Given that there is no budget limit, why not use the 200 birds designed for the survival check for GPS tracking? Are you sure that the birds will return to exactly the same breeding site each year? I know it is stated that "as long as conditions are suitable, Purple Martins return to the exact same breeding site each year (Bridge et al, 2016)" but did Bridge et al. take light pollution into account in their study? If the ALAN-exposed birds do not return to the same site, does this mean that they have not survived or that they are choosing the site without ALAN exposure? Moreover, to check survival, 200 birds from each study area will be randomly selected for ringing (lines 176-177). However, chicks from 40 selected nests will already be ringed (lines 182-183). This gives an average of 240 chicks per site with information on parents, stress hormones and light pollution. Why randomly select another 200 birds about which we know nothing?
Reproduction and development rates will be checked every three days by manual inspection and weight. There will be 15 study sites, each with 40 nests and up to 6 eggs per nest. A total of 3600 chicks will need to be manually checked and weighed every three days. There may be some shifts between nests and study sites, but they will overlap anyhow, and make the study not feasible with a team consisting of 6 people (but I am not sure if there will be 6 people as the travel cost are only for 3). In my opinion, it would not be feasible to do this within a single study site, let alone across 15 different study sites, which would require travelling by car.
The authors describe in detail how CORT is extracted, but there is no information on how it is measured. This is usually done with radioimmunoassays. Do the authors have access to such equipment and experience? I cannot see this from the method description or the budget.
The statistical description lacks sufficient detail. It is written that GLM will be used, but it is not stated which factors and co-factors will be included such as season, nest, light pollution etc. The authors want to use mean values for each box, but the better approach will be to use nested models that allow to take into account all the variance of the studied traits.
The study is planned on a very large scale and will be carried out over 3 seasons = 3 years. The project itself is planned for 36 months. There are only a few months left to finalise the data and write the manuscripts. It would be good to show the timeline not only with the work done but also with milestones such as obtaining the development data, survival data, CORT data and most importantly the submission of the manuscripts. In was

mentioned in the proposal that there will be some measurements done on small scale- I do not know when it will be fitted in the schedule.

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

No.
It is planned to fund 3 scholarships and 3 salaries. It is not clear which of these people will go to the field, as only 3 people have field travel costs included in the budget. It is not clear why the ornithologist should be paid over 36 months. And whether this will be a full-time position. If yes, than 3500 PLN is too little to meet the minimum salary required by low in Poland (at the moment the cost to the employer is around 4300 PLN, which gives 3500 gros, and around 2700 net for the employee).
The cost of the trip does not match the sum, for example, for accommodation it is planned 157860 PLN, while when calculating 6535 PLN*8 months*3 seasons for 3 persons it gives 156840.
It would be better to give more details on how the diets were calculated. According to the rules of the Ministry, the cost of diet in the USA is 49 USD per day. This gives 250pLN per day*30days*8months*3people*3seasons = 540000PLN in total, not 99 840 PLN. The petrol cost did not fit into the explanations as well and I could not understand how the insurance cost was calculated. There is no explanation of what the "maintenance and rental" costs are.
I am missing some laboratory materials such as plastic, gloves, etc. needed for CORT extraction. The cost of radioimmunoassays is not included.
It is worth considering the purchase of laptops to be used in the field and later for data analysis and manuscripts writing. It may also be useful to plan for some office supplies and some small field materials. For example, there is a plan to buy nets, but they need to be stretched between something like polls after the bird has been caught, he/she need to be put in a bag, etc. As the authors of the project are new in the ornithology field it would be good to consult the budget with an ornithologist.

5. **Strengths of the proposal**
- An interesting and timely topic.
- Large scale of the project.
- Modern methods included – GPS tracking.
- Well written, given a very short time period and the carrier stage of the authors.
- Planned collaboration with experts in this research.

6. **Weaknesses of the proposal**
- Correlative study.
- Conducting in the USA while it can be done in Poland.
- Huge amount of work that seems to be not feasible for 3 people in the field.
- The area of expert collaboration is not well defined.
- Lack of important details in methodology (mainly in study site chose, statistic methods).

Anastasiia Mykhailenko

The topic of the study is very relevant and covers one of the urgent and concerning factors of urbanization and its effect on wildlife. As far as I can judge, the knowledge gap addressed in the study is necessary for the complete picture on how light pollution affect bird populations.

Aims and methodology, mentioned in the proposal allow for a detailed describing the effects of light pollution, but I raise doubt that it is possible to make assessments as to evolutionary consequences. Even though there are there seasons of observation, in each of
the studied sites, its hard to say if any of the effects on the individual's fitness are permanent and whether levels of stress or fecundity won't normalise if birds are placed in better conditions. I think to speak about the evolutionary consequences there should be an additional study on the reversibility of deleterious effects caused by artificial light.
Also, as long as I understood the description, levels of CORT measured along the proposed three seasons are assumed to assess the effects on long-lasting health of the birds. If it is coming from a fact, that prolonged in time high levels of CAMPS have deleterious effects the question would be: for how long should this exposure continue? Are there established thresholds for purple martins? Maybe, in this study, there should be another part of the research that evaluates those thresholds to make the part about the stress response more conclusive.

I think that the results of this study will be extremely relevant for two main categories of researchers: ornithologists, as the study will provide extensive data on the migratory behaviour of purple martins and some insights into their physiology; urban ecologists and ecologists in general because there will be a good description to what levels of light pollution are acceptable for birds and even some thresholds of the tolerance can be assessed. The results of such work will for sure be accepted for a good publication and I could predict a sufficient impact on the field. As a specific potential outcome of the publication, I can also see this data being used practically and being included in the justification for recommendations on urban planning.
However, I was a little confused while reading about the influence of the study. I am not sure if the last statement in described significance is about those 3 years this study is planned for or if is it a premise for prolongation of the research and how beneficial it could be? I believe the idea about the prolongation of the study is good and relevant but I am not sure if this is what the authors meant.

I am expressing doubts about the feasibility of the project. I am not a specialist in the field, but am curious: are six people enough to collect and ring 3600 birds in one season? It is especially difficult to imagine taking into account that there is also additional preparation and sampling from nesting pairs. The populations of purple martin are sufficient to sample in such quantities, but there should be some risk management described as to how sampling and analysis are synchronised as long as sampling is happening in the USA and further analysis in Poland. The further processing of data I think could be easily implemented taking that all samples are collected on time.
Also, in the methods, there is no mention of sampling from the areas with light pollution below 1.6 Luxes. Does it mean that there won't be a control? It is either not clear from the proposal, or the control should most likely be included.
All costs are justified, as far as I am concerned, the only doubt I express is about the external hiring of ortithologists. As long as they are hired in the USA, is the salary enough? Maybe it could be better not to pay them throughout the year, but per season in bulk, and then still the salary should be higher. I can imagine collecting 3600 birds in a season is considered to be a full-time job.

The strength of the proposal is its relevance for urban ecology and potential applicability in the future. It is an original study that is clearly directed to filling the existing gap in knowledge.

The weaknesses of the study are that one of the main questions of the study cannot be fully addressed via proposed methods and that there is a potential problem with managing sampling on time.

Martyna Marzec

The project is focused on a highly relevant topic in the present time as light pollution is considered to be a serious risk factor of disrupting natural biological cycles regulated by light. Consequences of such a disruption might be potentially harmful for both humans and wild animals. In the project authors focused on influence of artificial lightning at night (ALAN) on the migration and its evolutionary consequences on Purple Martin.
In the first paragraph of Scientific goal of the project authors briefly describe the scientific background of the project and ask the research questions concerning the topic. Questions are formulated correctly and easily to understand.
To answer the research questions authors formulate 4 hypothesis. The hypotheses have been formulated accurately, however, the hypothesis number 2: "More artificial light at night causes migratory pattern shifted in time and migration distance is shortened for adults and nestlings." can be misleading for non-specialist reader. The world "nestlings" refers to a young bird individual that stays in the nest and is not able to fly. The usage of this term in the context of birds' migration can be interpreted incorrectly. I would suggest to use the term "offspring" which doesn't necessarily indicate the age of bird but the generation.
Authors will not be able to verify the hypothesis number 3 with suggested methodology and work plan. By monitoring the migration success rate of offspring kept in the light polluted area during nestling period the hypothesis number 1,2 and 4 can be evaluated but not the permanence of the ALAN effect on future generation. One way to verify this effect is to expose the parents to ALAN while not exposing their young in the nest.
In the last paragraph of *Scientific goal of the project* authors present short description of the planned experiments and methods that will be used to verify proposed hypothesis. I would suggest to put this fragment into *General work plan* part of the project rather than in the goal part.
In the Significance of the project part the authors describe the species they will use in their research. Based on the description reader can deduce why the authors decide to pick this specific species of bird but it is not directly explained by the authors.
In general work plan and research methodology the reader can find the information about the number of the research areas but there is no information about the region where the research will be performed and about the distribution of areas in relation to each other.
Additionally, authors mention that the range of light intensity will start from 1.6 Lux. The information about the intensity of night light in natural conditions (moon light: about 0,27 lx) would be helpful for reader to compare and understand the level of light pollution.

The project results will fill the knowledge gap in the research field. Authors describe the current knowledge based on many publications and indicate the missing data. The research have a chance to help in better understanding effects of ALAN in wild life and predict its possible consequences. Although authors present some already published data concerning the topic, more research has to be done to confirm proposed hypothesis.

The feasibility of the proposed project is high. Authors plan to collaborate with few specialists in the research field which makes their project more feasible and lower the risk of not succeeding. However, some aspects were not included in risk assessment. Firstly, the CORT analysis can be disrupted by handling nestlings and birds. It can cause additional stress and elevate the level of CORT resulting in incorrect interpretation of obtained results. Secondly, the level of light intensity should be measured more often during breeding season due to a fluctuation in light amount near urbanized areas.

The costs of the project are well justified with some minor mistakes and they may be slightly overrated. The prices of plane ticket will be probably lower than predicted by authors (around 7000 PLN instead of 8000 PLN). The project is also costly due to the accommodation and living costs in foreign country. Authors should consider reducing the duration of time spend abroad especially when they have collaborators on site. They should consider online contact as well .

The project is written in a reader-friendly form. Proposed hypothesis and research goals are clear. The graph attached in the work plan part make the text easier to follow and help to understand the authors' idea. The study is well-designed and the chosen methodology is accurate. The project is long-term, which is a significant factor in observation of evolutionary consequences of the studied phenomena.

The authors do not take into consideration the risk of obtaining false results due to the intervention of experimentalists into natural habitat of the studied birds. Additionally, the description of exact reasons for experimental groups division is not clear. The reader can be confused about the difference between experiments performed on group 1vs 3.

Farzeen Saeed

The proposed project provides potential knowledge gaps in our understanding of how ALAN can impact migration and evolution in Purple Martins. Even though multiple studies have already been conducted to assess how artificial light has the potential to disturb the migratory patterns, reduce the reproductive fitness, and induce stress to the birds of other species (i.e. great tits), it is interesting to learn how this research will fill the gaps of how artificial light could impact species of songbirds that are yet to be studied and whether negative impacts of artificial light to birds is a continuous phenomenon that spans across other species of Martins, and ultimately all birds.
This research holds valuable importance since it will provide the data on reduced fitness of birds (Purple Martins) as a result of stress and since birds are a crucial part of all ecosystems, they need to be protected for the sustenance of life. Along with that, the findings of this research will help us to come up with solutions to help these species and many others species of birds that show migratory behaviors and are negatively impacted by man-made structures, every single year.
This research will not be novel and is not completely original since similar studies have been conducted on other bird species for the same parameters. And since inference is one of the most commonly used tools in animal related studies, we can already infer that Purple Martins will show similar behaviors to ALAN as other bird species have done so.

The quality of the research project is very basic and can give a fundamental understanding of the effect of artificial light on migration and evolution in Purple

Martins. However, it would have been nice to see the development of this research on a higher scale to bring about uniqueness and newness in the findings of this research.

This research will bring about a further understanding of already researched areas however, it will deal with new parameters in Purple martins that have never been studied in this context. And since it is a research that is going to be conducted on birds that are not limited to just one country, there is the potential to receive international attention and quite possibly have an impact on worldwide research fields. Since other researches conducted on studying the effects of light pollution and their impacts on birds have found their place in high-end journals such as Nature and Frontiers in-Veterinary Science, it is highly likely that the findings of this research will also be welcomed in high quality research publication journals.

According to the methodology proposed in the project, this research is highly feasible and can be conducted with the help of experts in this field. However, one of the parameters that have to be measured is the level of stress in birds as a result of artificial light. The hormone that they proposed to measure for this purpose is "cortisol". Upon research from the internet, it has been found that cortisol is a stress hormone that is found in fish and mammals whereas corticosterone is the main hormone produced by birds in response to stress. They also mentioned a previously conducted similar study in this field where the levels of corticosterone have been measured to deduce the stress levels in birds. In this manuscript the authors are mentioning the words corticosterone and cortisol interchangeably in various parts (i.e lines 19, 142). It would be nice if the authors could resolve this conflict between the two terms to reduce the confusion that may arise for the readers.
Another issue is that in this research the abbreviation "CORT" was used to represent the term "corticosterone". Since this abbreviation is so broad in terms of its inclusion of three hormones i.e. cortisol, cortisone, and corticosterone, it needs to be mentioned whether the authors are using this abbreviation for cortisol or corticosterone since both hormones were mentioned in the text. If these issues are resolved as well as the methodology related to the hormone in question, then it is highly likely that this research can be conducted without any problems.
In risk analysis, the authors mentioned the purchase of good quality equipment, collaborations from experts in this field, and testing all stages of this experiment in a smaller sample size, they didn't mention how they will counter the effects of natural environmental hazards such as unpredicted rainfalls, hurricanes, tornadoes, and wildfires (North America is home to these unexpected natural phenomenons).
The equipment mentioned in the manuscript is of very good quality and can no doubt give accurate results, strengthening the impact that this research will have across the globe.

Yes, the costs are fairly justified with regards to the research conducted and its scope.

The proposal is very well written and the methodology that is mentioned in order to measure all the parameters of this research is thoroughly studied and its similarity with the previous studies conducted in this field is on point, considering that this proposal was written by non-specialists in this field.

The authors have mentioned the equipment that is of very good quality and will definitely help in the collection of relevant data.
The hypotheses are clearly mentioned in the project proposal and they highly resonate with the work plan of this research.

Although this research is new in the case of purple martin, prior researches have been done to test the impact of artificial light on similar parameters in other songbirds. Strong inferences from those researches can be made for all species.
There is space for improving the factors/parameters to be studied and at a research of this scale with collaborations from foreign experts and huge budget, it would be expected that something new was to be tested to fill to the overflowing well of knowledge that already exists in this field.
The authors need to decide if they are measuring cortisol or corticosterone since it will highly impact the quality of their proposal, their hypotheses, methodology, and the findings of their research.
The authors didn't mention how they chose the size of their research specimen since it plays a huge role when it comes to migration in birds.
The authors mentioned "stress" as one of their parameters to be tested but they didn't mention if this stress would be short termed or long termed since that could also improve the understanding of the reader and better the quality of the research that they are conducting.

FINAL VERSION

Migratory and ontological consequences of artificial night lighting on Purple Martin (*Progne subis*) - behavioral observations.

Martyna Gorkowska, Patrycja Dziurowicz, Michał Strączyński

Summary:

Artificial lightning at night (ALAN) is an alarming and growing problem for wildlife including birds. It negatively influences their biological rhythms, reproduction, and migration. Even though there are many studies concerning ALAN impact on birds there are still many gaps in the knowledge in this field. Our research project aims to answer several questions concerning the subject. What is the influence of ALAN on the reproductive success of Purple Martins and the offspring development? How strongly ALAN disturbs the photoperiod and changes the timing and pattern of bird migration? Are individuals exposed to more ALAN more stressed in comparison to the ones living in the less light polluted places? Does the level of stress hormones correlate with the survival rate of Purple Martin population? Does living in more ALAN and an elevated level of stress potentially lead to the weakness of birds and shortening of migration distance? To answer these questions, we will conduct several field experiments on Purple Martins populations in 15 research areas with differing intensity of ALAN. Thanks to the ringing of big groups of birds and GPS tracking, we will observe their migration patterns and survival rates. Observations of brood size and nestling development will help answer the question about reproduction success. Furthermore, measurements of corticosterone levels in both offspring and adults will enable the corelation between stress levels induced by ALAN and survival as well as migration patterns.
We believe that the extensive analysis of the obtained results will allow researchers to fill the gap in the current understanding of evolutionary consequences of ALAN's negative influence on birds' migration patterns and development. Additionally, because of the large spread of light pollution worldwide and its known effects on birds, it is crucial to focus on this subject to determine the threat to biodiversity posed by light pollution.

1) Scientific goal of the project

Artificial lighting at night (ALAN) is an alarming and growing problem for animal lives, as it can negatively alter individual behaviour, biological rhythms, and reproduction (Da Silva et al., 2015; Dominoni, 2015; Grunst et al., 2020). There is also some evidence that it may negatively influence bird migration patterns. Nestlings exposed to artificial light at night were shown to grow slower which delay colony departure (Smith et al. 2021). It is not known whether such disturbed development of offspring may influence their migration patterns and what the evolutionary consequences of artificial night lightning. The gap in the knowledge raises several questions concerning these issues. What is the influence of ALAN on the reproductive success of Purple Martins and the offspring development? How strongly ALAN disturbs photoperiod and changes the timing and pattern of their migration? Are individuals exposed to more ALAN more stressed in comparison to the ones living in the less light polluted places? Does living in more ALAN and elevated level of long-term stress potentially lead to the weakness of birds and shortening of migration distance? In this research project, we plan to answer those questions and based on the current knowledge and observations we state the following hypotheses:

Hypothesis 1: Exposure to artificial lighting at night increases the level of stress hormone in offspring and adults.
Hypothesis 2: The nestlings exposed to (more) artificial light at night, are smaller as their ontological development becomes altered.
Hypothesis 3: More artificial light at night causes migratory pattern shifted in time and migration distance is shortened for adults and their offspring.
Hypothesis 4: The exposure to artificial lighting at night creates long term migration shifts that persist from generation to generation.
To test these hypotheses, we will study Purple Martin populations in 15 research areas with differing levels of light pollution, understood as the intensity of artificial light expressed in Luxes (lx). We will introduce different observations and measurements of eggs amount, nestlings' development, birds' behaviour and survival rate. Moreover, thanks to birds tracking system we will be able to observe the migration pattern and route of individuals. We believe that the extensive analysis of the obtained results will allow to fill the gap in the current understanding of evolutionary consequences of the ALAN negative influence on birds' development and migration patterns.

2) Significance of the project: Light pollution caused by artificial light at night is rising worldwide and is increasingly seen as a problem for wildlife (Da Silva et al., 2015; Grunst et al., 2020). Artificial light at night (ALAN) is one of the anthropogenic disruptors of natural environmental conditions and, therefore, can disrupt biological systems and cause physiological stress (Grunst et al., 2020). In birds, in addition to collisions with artificially lit structures, one of the most commonly reported effects of light pollution is a change in temporal behaviour (Dominoni, 2015). As shown in previous studies, free ranging birds can be negatively influenced by light pollution regardless of sex, with 1.6 Luxes (lx) being enough to postpone the time the birds fall asleep. Higher intensities may also possibly worsen the effects, although there is not enough evidence to show this conclusively (Raap et al., 2017). Such disturbed sleeping may lead to chronic consequences for the birds (Johnson et al., 2022). The problem becomes worse during the nesting period, when the mother bird and her young are especially vulnerable to light pollution, causing them to lack night-time sleep and increased begging behavior in the offspring. The additional energy costs of this begging behavior, combined with a lack of sleep may lead to reduced fitness of the offspring and the parent (Raap et al., 2016). Migration timing can be crucial for migratory birds - if arrival and departure times are mismatched with optimal breeding conditions, this can result in reduced fitness and ultimately population decline (Both et al., 2006; Neufeld et al., 2021). According to Smith et al. (2021) adult Purple Martins (*Progne subis*) who experienced ALAN for more than 10 nights, initiated spring migration 8 days earlier than others who experienced natural darkness. This advance in timing was not compensated for during migration and birds experiencing ALAN that had left earlier also arrived at their breeding grounds eight days earlier, suggesting the potential for mismatch between bird migration timing and the availability of resources in early spring. Purple Martin is one of the largest migratory birds belonging to the family Hirundinidae, measuring 19-20 cm and weight 45-60 g. This species is found in North America during the breeding period from January to August, while during the non-breeding period it is found in South America, mainly in Brazil (Brown, 1997; Santos et al., 2021). The pre-migratory period is characterized by the formation of flocks numbering thousands of individuals that congregate at specific sites used as roosts. Importantly as long as conditions are suitable, Purple Martins will return to the exact same breeding site each year (Bridge et al.,2016). According to information from the Connecticut state government, this species is characterized by sexual dimorphism and a monogamous lifestyle. The female lays an average of four to six eggs for 15 to 18 days. After hatching, the

young remain in the nest for 24 to 28 days and are fed insects by both adults (portal.ct.gov., 2016). Two years after hatching, they reach sexual maturity (Stutchbury, 1991). In our project, we decided to choose the Purple Martin species for several reasons: (1) Earlier research has experimentally shown that artificial light at night negatively affects this species. (2) This species is one of the largest members of the swallow (Hirundinidae). (3) Females only lay eggs once during their singular breeding season each year.

Another aspect is that exposure to ALAN can disrupt hormone production, activity, sleep, and recovery cycles and the health of wildlife (Grunts et al., 2020; Romero, 2004). One of the hormones that birds produce is corticosterone (CORT), which is responsible for adaptations to stressful situations (Blas, 2015). Elevated CORT concentrations have complex and adaptive functions in glucose metabolism and energy balance, flight responses, and the immune system. In turn, long-term elevation of CORT can result in chronic stress, impaired health, and decreased performance (Grunts et al., 2020; Romero, 2004; Sapolsky et al., 2000). There is a critical level of knowledge about whether exposure to ALAN results in elevated CORT concentrations in developing chicks and adults. Based on the fact that stress lowers the whole organism's condition, we posit that this diminished condition might lead to shortened distance of migration in both the parents and the offspring.

Although there are various publications concerning the influence of ALAN on birds functioning in different aspects, our research project is the first that will study the effect on migration survival rate, distance and timing. The study is pioneering, because it will be done in the field while previous studies in this issue were performed in the laboratory. Moreover, we plan to study the evolutionary consequences of ALAN on the population through reproduction and migration success, survival rates, and offspring development. Through the study of CORT levels in the birds, this will also be the first study to outline the effects of ALAN on the long-lasting health of free ranging migratory birds. We believe that such a study is worth conducting over a longer period of time in order to more accurately determine the effects of artificial light intensity at night on the migration and evolution of the species studied. This, in turn, will make it possible to determine the threat to biodiversity posed by light pollution.

3) Concept and work plan

General work plan: The experiment will be conducted for 36 months (3 breeding seasons), starting in the spring, after the return of Purple Martins to the breeding area. The 15 chosen areas will differ in the intensity of the light. Three areas with the lowest intensity will be used as a control group. As the moon light is about 0,27 Luxes (Kyba et al., 2014)) the lowest intensity that we expect will be around 0,3. Due to the fact, that even 1.6 Luxes can be enough to cause sleep interference in nesting females and behavioral changes of their nestlings (Raap et al., 2016), areas with 1.6 Luxes will be considered as the least light polluted. The gradient of intensity measured in the research experimental areas will be in range from 1,6 Luxes to 10 Luxes (a common intensity experienced in cities (Wigan Council FAQ)). In the 15 chosen areas, we will prepare the experimental setup which will include installation of temperature loggers in 40 nests (for each experimental area) inhabited by Purple Martins pairs. The change in nest temperature will identify when the offspring departs from the nest. In each of 15 areas, we will distinguish three experimental groups. All individuals studied will be ringed. The first group will consist of 200 Purple Martins that will provide information about migratory success. The second group will consist of 40 pairs (generation I) living in the prepared nests and their reproductive success and nestling (offspring I) development will be observed. The breeding season of birds will be observed to precisely determine the number of eggs laid in each nest. After hatching of the eggs, the nestlings will be counted, observed and

weighted every week. The time of their nest leaving will be noted. The last group will consist of 40 individuals (per each experimental area) that emerged from pairs from the second group. The ones will be chosen based on their condition and will be equipped with GPS trackers, which will allow following of their migration distance and route. During the whole experiment the survival rate of the population will be monitored. The whole experiment will be repeated for the three following breeding seasons (Fig. 1).

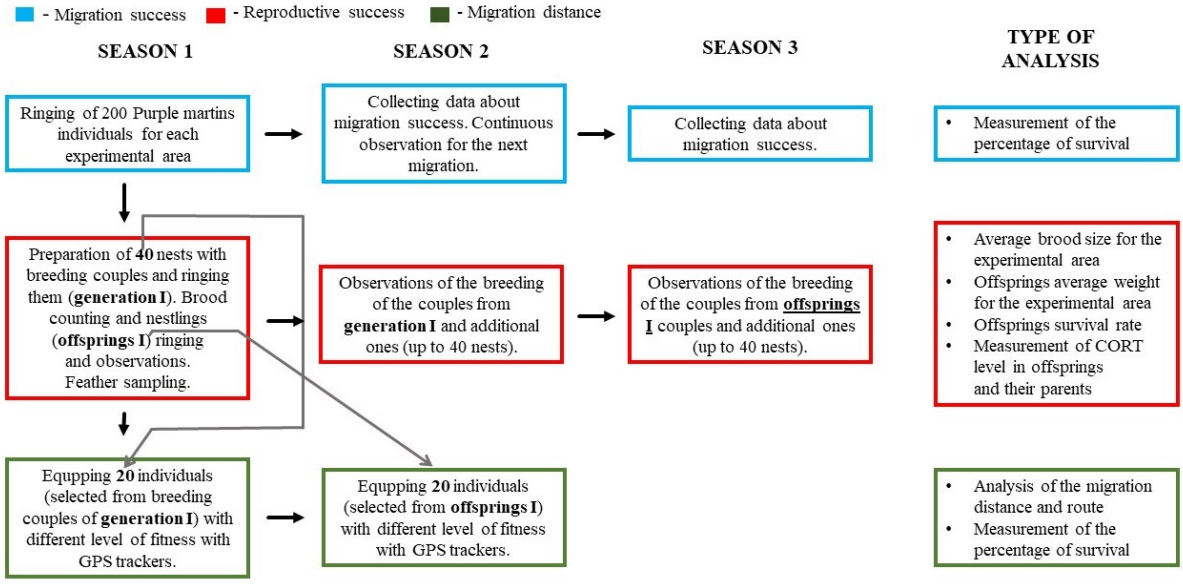


Figure 1: Diagram of the general work plan.

Specific research goals: Our research project aims to examine the following specific goals:

1. Monitoring of the reproduction success of different populations of Purple Martins in the chosen areas characterized with different intensity of ALAN.
2. Determination of the survival rate of nestlings and adult individuals during migration.
3. Observation and precise tracking of Purple Martin migration time, pattern, and route.
4. Monitoring of corticosterone level in the studied individuals.

Due to the fact that experiments will be carried out in multiple areas that vary in intensity of ALAN, the results will allow us to compare the groups with each other and to correlate the influence of ALAN on the studied aspects of Purple Martin's life.

Risk analysis: Due to the fact that in the course of experiment we are going to install temperature logs in the nests and a group of birds will be equipped with GPS trackers, there is a risk of the equipment damage. To avoid this, and the loss of data, we will purchase devices of good quality and set in on animals in a very precise way. As all principal investigators are not experienced in bird studies, collaboration is planned with experts in this research. Moreover, laboratory tests of CORT levels will be performed by a qualified laboratory technician. The people who will take care of birds handling and ringing of birds will be trained and experienced in it and possess the necessary licences. We will acquire the ethical permissions needed for the installation of GPS tracking devices and feather collection. To minimize project risk, all stages will be tested in a smaller sample size. To test the GPS trackers, at the beginning of the experiment we will equip a small number of birds to verify whether they work properly. We will also check if the chosen methods for CORT extraction from feathers and immunoassay to analyse its levels works well-functioning.

4) Research methodology

Study area: We will study Purple Martin (*Progne subis*) populations in 15 independent study sites. The locations will be chosen from existing bird box colonies used for breeding and grouped according to the intensity of light pollution. All research locations we plan to use are located in the Ontario territory, between Ottawa in the south-east, Lake Huron in the south-west and Lake Abitibi in the north. This will ensure as little variation between study sites as possible, leaving light as the main factor. Principal Investigators will be provided with a rented car to travel between research sites. The light intensity will be measured in Luxes (lx), using Benetech GM1020 before each breeding season and every week after it starts. The light intensity will be calculated from mean value of records taken recorded in 10 spots located at least 20 meters apart, within the general area of the bird boxes. Each location will have a total of 40 birds with GPS trackers installed on them. The bird boxes inhabited by the chosen 40 pairs will have temperature and moisture loggers (HOBO Temperature/RH data logger MX20301A) installed.

Staff: Five professional ornithologists will be paid to supervise and conduct the field research in the most remote areas, they will cover three locations each. We will also collaborate with the University of Torto Faculty of Arts & Science and use the help of their researchers and students in gathering data at the sites.

Offspring development: The offspring and their parents inhabiting the 40 designated boxes will be counted and weighed using electronic scales for birds. Every three days, the boxes will be manually controlled by specialized ornithologists.

Migration: We will use a mini-GPS tracker "PinPoint 10", manufactured by the company Lotek Wireless which weighs 1.1 g. The trackers will be strapped to the bird using leg-loop made of Teflon ribbon (Thaxter et al. 2014). The birds used for GPS tracking will be taken from the 40 breeding pairs and separated into four categories (cat 4 to 1) with five birds in each, based on their physical condition measured by the amount of fat built around the chest muscles responsible for flight (the more fat, the higher the category). From each research area 200 birds will be randomly selected for ringing. The birds will be caught using an ornithological net. In a situation where birds with a GPS transmitter do not return to their breeding site, we will be able to determine where they will be. This can show us if the bird has decided to leave its nest because of unfavorable environmental conditions.

CORT analysis: Feather collection for corticosterone analysis will take place after two weeks of chick life, then after a month and every month thereafter until the birds migrate. The young will have five contour feathers (breast) removed, and one wing feather will be extracted from the adults. At three weeks of age, the young birds will also be ringed. CORT will be extracted from feathers using methanol, with samples placed in a sonicating water bath at room temperature for 30 min and then incubated overnight in a shaking 50°C water bath. After methanol from feather using vacuum filtration, the methanol extracts will be dried under nitrogen gas and reconstituted in a saline buffer. After CORT extraction its level will be analyzed according to the enzyme immunoassay described by Berkvens (Berkvens, 2012).

Survival rates: Each season when the birds return, they will be caught using an ornithological net from 5 am in the morning. The number of birds returning with rings will be counted and compared to the initial 200 to calculate their death rates when migrating. Birds ringed each season will be breeding pairs and their offspring, with additional randomly chosen birds until the number of birds ringed reaches 200 again.

Repetition: The migration survival rates, weighing of birds and CORT analysis steps will be repeated for each of three seasons, while GPS marking will be carried out for two years during the project. In the third season, we will only monitor the departure of the birds and the distance of their migration because we will not have enough time to wait for their

return. Schematical diagram presenting experiments to carry out during the project is presented in Figure 1.

Data analysis: The collected data will be statistically analyzed (for example redundancy analysis (RDA), Generalized linear model (GLM), General Unified Threshold for Survival (GUTS Framework)). The numbers of eggs, and the nestlings as well as weight of the birds and corticosterone levels from nest boxes will be used to calculate mean values for each box. Migration survival rates for each study site will be noted in the following season. We will compare these results between the study sites to determine the influence of ALAN exposure on the measured aspects. Between seasons, it is planned to carry out analyses from each breeding season. To analyze the data collected from this experiment, we could use a variety of statistical methods, including descriptive statistics, regression analysis, and ANOVA. Descriptive statistics would be used to summarize the data and calculate measures of central tendency and variability for each variable. Regression analysis could be used to examine the relationship between ALAN intensity and the dependent variables, while controlling for confounding variables. ANOVA could be used to compare the means of the dependent variables across the different levels of the independent variable. Once the data has been obtained, a mathematical model will be created to show the evolutionary changes caused by ALAN.

5) Literature

Berkvens, C. N. (2012). Keratin Glucocorticoid Analysis by Enzyme Immunoassay in Mammals , Birds and Reptiles by presented to The University of Guelph In partial fulfilment of requirements for the degree of Doctor of Veterinary Science in Pathobiology Guelph , Ontario , Canada (p. 89-90).

Blas, J. (2015). Stress in birds. In Sturkie's avian physiology (pp. 769-810). *Academic Press*.

Both, C., Bouwhuis, S., Lessells, C. M., and Visser, M. E. (2006). Climate change and population declines in a long-distance migratory bird. *Nature,* 441(7089), 81-83. https://doi.org/10.1038/nature04539

Bridge, E.S., Pletschet, S.M., Fagin, T., Chilson, P.B., Horton, K.G., Broadfoot K.R. and Kelly J.K. (2016). Persistence and habitat associations of Purple Martin roosts quantified via weather surveillance radar. *Landscape Ecol* 31, 43–53. https://doi.org/10.1007/s10980-015-0279-0

Brown, C R. (1997). Purple Martin (Progne subis), The Birds of North America Online (A. Poole, Ed.). Ithaca: *Cornell Lab of Ornithology*; Retrieved from the Birds of North America Online: http://bna.birds.cornell.edu/bna/species/2

Da Silva, A., Valcu, M., and Kempenaers, B. (2015). Light pollution alters the phenology of dawn and dusk singing in common European songbirds. Philosophical Transactions of the Royal Society B: *Biological Sciences*, 370(1667), 20140126. https://doi.org/10.1098/rstb.2014.0126

Dominoni, D.M. (2015). The effects of light pollution on biological rhythms of birds: an integrated, mechanistic perspective. *J Ornithol* 156 (Suppl 1), 409–418. https://doi.org/10.1007/s10336-015-1196-3

Grunst, M. L., Raap, T., Grunst, A. S., Pinxten, R., Parenteau, C., Angelier, F., and Eens, M. (2020). Early-life exposure to artificial light at night elevates physiological stress in free-living songbirds. *Environmental Pollution*, 259. https://doi.org/10.1016/j.envpol.2019.113895

Johnsson, R.D., Connelly, F., Gaviraghi Mussoi, J., Vyssotski, A., Cain, E., Roth II, T. and Lesku, J. (2022). Sleep loss impairs cognitive performance and alters song output in Australian magpies. *Sci Rep* 12, 6645. https://doi.org/10.1038/s41598-022-10162-7

LED Street Lights Frequently Asked Questions, Wigan Council https://www.wigan.gov.uk/Docs/PDF/Resident/Parking-Roads-Travel/LED-street-lights-FAQ.pdf (accessed on 22.04.2023)

Neufeld, L.R., Muthukumarana, S., Fischer, J.D., Ray, J.D., Siegrist, J. and Fraser, K.C. (2021). Breeding latitude is associated with the timing of nesting and migration around the annual calendar among Purple Martin (Progne subis) populations. *J Ornithol* 162, 1009–1024 https://doi.org/10.1007/s10336-021-01894-w

Purple Martin, Department of Energy and Environmental Protection https://portal.ct.gov/DEEP/Wildlife/Fact-Sheets/Purple-Martin (accessed on 22.04.2023)

Raap, T., Pinxten, R., and Eens, M. (2016). Artificial light at night disrupts sleep in female great tits (Parus major) during the nestling period and is followed by a sleep rebound. *Environmental Pollution*, 215, 125-134. https://doi.org/10.1016/j.envpol.2016.04.100

Raap, T., Sun, J., Pinxten, R., and Eens, M. (2017). Disruptive effects of light pollution on sleep in free-living birds: Season and/or light intensity-dependent?. *Behavioural processes*, 144, 13-19. https://doi.org/10.1016/j.beproc.2017.08.011

Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in ecology and evolution*, 19(5), 249-255. https://doi.org/10.1016/j.tree.2004.03.008

Santos, C. O., Branco, J. M., Belotti, M. C., Abilleira, P., Siegrist, J., Fischer, J., Lima, L.M., Cohn-Haft, M. and Hingst-Zaher, E. (2021). Distribution and migration phenology of Purple Martins (Progne subis) in Brazil. *Ornithology Research*, 29(4), 213-222. https://doi.org/10.1007/s43388-021-00071-0

Sapolsky, R. M., Romero, L. M., and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine reviews*, 21(1), 55-89. https://doi.org/10.1210/edrv.21.1.0389

Smith, R. A., Gagné, M., and Fraser, K. C. (2021). Pre-migration artificial light at night advances the spring migration timing of a trans-hemispheric migratory songbird. *Environ. Pollut*. 269:116136. doi: 10.1016/j.envpol.2020.116136

Stutchbury, B. J. (1991). Floater behaviour and territory acquisition in male purple martins. *Animal Behaviour*, 42(3), 435-443.

Thaxter, C. B., Ross-Smith, V. H., Clark, J. A., Clark, N. A., Conway, G. J., Marsh, M., Leat, E. and Burton, N. H. (2014). A trial of three harness attachment methods and their suitability for long-term use on Lesser Black-backed Gulls and Great Skuas. *Ringing & Migration*, 29(2), 65-76. https://doi.org/10.1080/03078698.2014.995546

Kyba C. C. M., Mohar A., Posch T., (2014) How bright is moonlight? *Astronomy & Geophysics*, 58(1), 1.31–1.32

6) Table with budget of the project

	Amount in PLN
Direct costs, including	**4 052 839,76**
- personnel costs and scholarships	1 326 000,00
- research equipment/device/software cost	1 394 434,00
- other direct costs	1 332 405,76
Indirect costs, including:	**891 624,75**
- indirect costs of OA	81 056,80
- other indirect costs	810 567,95
Total costs	**4 944 464,51**

7) Breakdown of project costs and their relevance in the project

Category	**Description**	**Amount in PLN**	
Salaries and benefits	Scholarships	3 persons*2500 PLN*36 months	270 000

	Ornitologists	5 persons*8200 PLN*24 months (3 seasons)	984 000
	Laboratory technician	2 person*12000 PLN*3 season	72 000
Equipment	GPS-PinPoint 10	40 individuals*15 research stands*2 season*630 PLN	756 000
	Loggers HOBO MX20301A	40 nests*15 research stands*950 PLN	570 000
	Leg-lopp harness	40 individuals*15 research stands*2 season*8 PLN	9 600
	Ornithological net	2 nets*15 research stans*400 PLN	
	Ecotone MEDIUM bag	10 bags*3 research stans *140 PLN	12 000 420
	Bird wire stainless steel posts	4posts*15 research stans *150 PLN	9 000
	Personal hygiene products		10 000
	Plastic bags	1000 pieces	14,00
	Luxometr Benetech GM1020	2*150 PLN	300
	Laptop	3*5000 PLN	15 000
	Avian scales	15 research stands*450 PLN	6 700
	Rings for adults	200 birds*15 research stans*3 seasons* 0,3 PLN	2 700
	Rings for offspring	40 pair*5 eggs*15 research stands*3 seasons*0,3 PLN	2 700
Chemical reagents	Methanol	60 l per 3 seasons	1 800
	Nitrogen gas	60 l per 3 seasons	3 700

	Saline buffer	30 l*530 PLN	15 900
	Enzyme Immunoassay	9 x 250 ml Coating Buffer (Advansta, R-03730-D25) – 4500 pln 8 x Corticosterone Antiserum Kit (Sigma Aldrich, Product Number: C8784) – 8 000 8 x 500 ml EIA Buffer (Neogen, No. 301276) – 2 500	15 000
Trip	Plane tickets	3 persons*7000 PLN (two ways)*3 seasons	63 000
	Accomodations	6535 PLN*8 months*3 seasons for 3 persons	157 860
	Diet	250PLN *per day* 30days *8months* 3people* 3seasons	540 000
	Car rental	1344 PLN*8 months*3 seasons	32 256
	Petrol	40l*5,76 PLN*8 months*4 weeks*3 seasons	21 749,76
	Visa	3 persons*380 PLN	1 140
	Conferences	2*3 persons*5000 PLN	30 000
	Maintenance and rental bird houses	15 research stands*10000 PLN*3 seasons	450 000
		Total	**4 052 839,76**

The Mobilization of the Glymphatic System in a state of Torpor in an Avian Model and its Association with the Oxidative Stress

Farzeen Saeed, Patar Sinaga, Szymon Kantor

Abstract
Torpor is a state in which animals reduce their body temperature, metabolic rate, and organ function to conserve energy and survive periods of low food availability or extreme environmental conditions. Torpor is associated with physiological restoration and energy conservation, but also with the disposal of metabolic waste products such as reactive oxygen species (ROS) that are related to oxidative stress, which can decrease fecundity and survival. Birds are known to spend a lot of time sleeping during torpor or hibernation, and a glymphatic system (GS), which is responsible for disposing of wastes and metabolites in the brain, has been found to play a crucial role in maintaining brain homeostasis. However, the function of GS in birds during torpor has not been thoroughly investigated. The study will focus on the Zebra Finch (*Taeniopygia guttata*), which is known to spend a significant amount of time sleeping during torpor. This study will explore whether torpor serves as a mechanism to mobilize the glymphatic system in birds and reduce oxidative stress in the brain tissue. This research could potentially provide a broader understanding of torpor and metabolic waste transport from the brain in birds and other species.

1. Scientific goal of the project

In the proposed research project, the main scientific goal is to determine whether birds, in a state of daily torpor, mobilize the brain's glymphatic system and as a result, reduce oxidative stress in the brain tissues. In the earliest and the most simplistic models, during torpor, the animal's body functions slow down to decrease energy expenditure, allowing it to survive periods of low food availability (Schleucher 2004) or excessive environmental conditions (Wang 1989). However, it is correct to assume that torpor may have other crucial functions beyond just enrgy conservation. Moreover, it is highly possible that torpor and the nervous system share several important interactions, yet to the best knowledge of the authors of this project, research has been conducted focusing on the role of daily torpor in the functioning of the glymphatic system, especially in relation to oxidative stress.

Our hypotheses

1. The glymphatic system is activated in the brains of birds entering daily torpor
2. There is a continuous decrease in oxidative stress along the torpor bout due to the activation of glymphatic system
3. Dysfunction of the glymphatic system is reflected in an increase in levels of oxidative stress.

2. Significance of the project

To maintain euthermic body temperature, birds utilize a large energy expenditure for optimizing organ function, food digestion, and mobility – processes that are critical for survival, especially in cold conditions (Ruf and Geiser 2015). Such excessive circumstances are related to the availability of less food. One of the strategies developed by birds and some mammals to deal with this is to adjust body temperature by reducing

metabolic rate (hypometabolism) and the function of their internal organs (Lyman *et al.* 1982, McKechnie and Mzilikazi 2011). This state of sleep-like lethargy is called torpor, which can be divided into two types - daily torpor and seasonal torpor (hibernation). Small animals usually perform daily torpor and manage to lower their body temperature a few degrees above the ambient environmental temperature (Barnes 1989, Ambler *et al.* 2022) while hibernating animals lower their body temperature in a range far greater than during daily torpor. The characteristics possessed by animals that carry out daily torpor are; the duration of temperature reduction which includes several hours and variations in body temperature can reach 17°C, metabolic rate can decrease by about 30% of basal metabolic rate (BMR) (Geiser 1998).

The need for sleep in birds, such as during torpor, is associated with physiological restoration and energy conservation (Schmidt 2014, Ferretti *et al.* 2020). However, the benefits of sleep are also associated with the disposal of metabolic products, such as reactive oxygen species (ROS) - molecules that are related to the occurrence of oxidative stress (OS) (Reimund 1994). OS is considered the reason behind decreased fecundity and survival (Beckman and Ames 1998). ROS are highly reactive with molecules such as proteins, lipids, and DNA (Cooper-Mullin and McWilliams 2016). Because of that they can interfere with gene expression, tissue function, and affect the reproductive system (Cadenas and Davies 2000, Barja 2004, Bize *et al.* 2008). Disposal of ROS is also important for maintaining brain homeostasis (Jessen *et al.* 2015). That is possible thanks to the presence of a glymphatic system (GS) (Hablitz and Nedergaard 2021).

GS is the excretory system of the brain that is a nexus of lymphatic network, cranial nerves tracts and large vessels exiting the skull (Benveniste *et al.* 2019). The functions of GS include disposal of wastes and metabolites where this process occurs when sleeping (Hablitz and Nedergaard 2021). This action can be summarized in 3 stages: (i) the entry of cerebrospinal fluid (CSF) into the subarachnoid space and then into the periarterial spaces, (ii) the pumping of CSF from the periarterial to the interstitial fluid (ISF) space and then mixing CSF and ISF with metabolites, and (iii) draining the mixture of CSF, ISF and waste out of the brain to the perivenous compartment of central veins and, finally, to the circulation system (Benveniste *et al.* 2019). The function and whether GS is a protection mechanism from toxic metabolite waste in birds has rarely been discussed before, where birds are known to spend a lot of time sleeping through torpor or hibernation.

What is important, several studies have suggested that torpor may modulate neuronal survival and plasticity in certain brain regions and this adaptive function is hypothesized to be a protective mechanism against reperfusion injury during arousal, wherein the loss of synapses and subsequent neural damage may be mitigated by torpor-induced changes in brain activity (Popov *et al.* 2007; Von Der Ohe *et al.* 2006). Daily torpor can also trigger neuroprotective processes (Squarcio *et al.* 2023) and may have a beneficial effect on memory performance (de Veij Mestdagh *et al.* 2021).

A new discovery regarding the activity of the GS during daily torpor could be considered a breakthrough, especially because it would be highly interdisciplinary, combining the latest scientific achievements in the fields of neurobiology, immunology, and physiology. Additionally, there has been no research on whether torpor is a mechanism for sweeping ROS and alleviating oxidative stress in the brain of birds, which is an especially important knowledge gap yet to be answered. It is also projected that this question will later lead to further research and provide a broader picture of the torpor and metabolic waste disposal from the brain of other animal species.

3. Concept and work plan

We sought to discover if the glymphatic system is the neurobiological cause of reducing oxidative stress upon arousal from daily torpor, in birds (Figure 1). To test our hypotheses, we obtained 150 adult male and female zebra finches (*Taeniopygia guttata*) from Magnolia Farms Avian Breeder (Anaheim, CA). To conduct the experiment, we divided 126 birds into subgroups (i.e. control and experiment, based on the torpor phases) with seven males and seven females in each subgroup, respectively.

General work plan

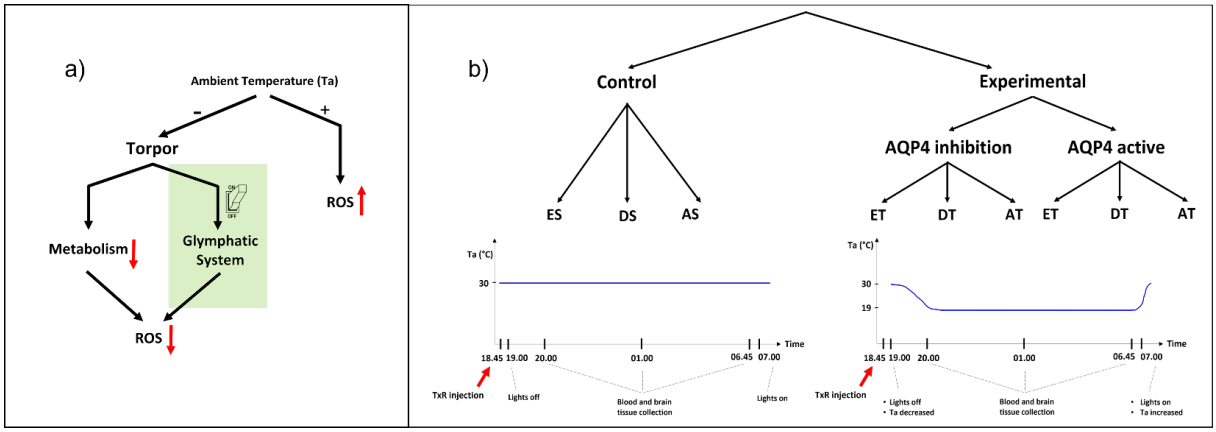


Figure 1: a) Torpor induced by ambient temperature as a mechanism to reduce oxidative stress. Notes: black arrows indicate the direction of the process, (+) indicates an increase in temperature, (-) indicates a decrease in temperature, an upward red arrow indicates an increase, and a downward red arrow indicates a decrease and b) Experiment design (time points - ES: early sleep, DS: during sleep, AS: awakening from sleep, ET: early torpor, DT: during torpor, and AW:
awakening from torpor; Ta: ambient temperature; TxR: Texas red with dextran and polysorbate-80 coating).

Specific research goals

1. We will weigh all the zebra finches to make sure that their mass is constant and does not influence the results of our experiments
2. Subcutaneous implantation of thermosensitive transponders followed by two-week continuous measurements of all birds' body temperature
3. Measurements of metabolic rate of all birds through open flow respirometry
4. Administration of fluorescent nanoparticles to all birds to determine activation profile of GS
   4.1) Induction of daily torpor state in animals from all experimental groups, while controls are allowed to naturally sleep in standard housing conditions
   4.2) Blocking the activity of the glymphatic system of animals from chosen experimental groups
5) Performance of perfusion and collection of peripheral blood and brains from our experimental birds over the course of the night
   5.1) Measurement of oxidative stress levels in tissue samples
   5.2) Immersion fixation of the brains for the purpose of confocal microscopy observations and preparation of microscope slides
6) Analysis of images acquired during microscopy observations
7) Detailed analysis of results using statistical tests and description of the results for publication.

Results of preliminary research

In our pilot studies, we have shown that during daily torpor in Zebra finches, the glymphatic system of the brain is activated and fluids effectively penetrate the tissue. What is more, the level of this activation is comparable to that during natural sleep (unpublished data).

Risk analysis

The proposed research is associated with minimal risk of failure. All planned procedures performed on live animals will be done with the knowledge and consent of the appropriate bioethical commission by competent and trained persons with appropriate permissions. The procedures presented by us, including the thermosensitive transponders implantation, induction of daily torpor, administration of protein inhibitors is a standard developed in our laboratory, and their effectiveness is reflected in numerous scientific publications from peer-reviewed journals. Laboratory equipment allows for the correct performance of all operations. Pilot measurements of the penetration of brain tissue by Texas red dye with dextran and polysorbate-80 coating via GS confirmed the correctness of the procedures proposed to obtain satisfactory results.

4. Research methodology

Ad 1. and 2. Birds of all groups will be weighted to make sure that they have constant weight (± 16 grams), then they will be implanted with thermosensitive transponders (Biomark Pit tags) two weeks before the start of experiment, giving them enough time to recover. In the meantime, they will be exposed to a wide range of ambient temperatures (30-19°C) to determine their thermoneutral range. They will, then, be exposed to sliding cold temperatures to determine the torpor curve and its properties. Continuous body temperature measurements during procedures are planned.

Ad 3. Birds will undergo a sliding cold exposure to measure summit metabolic rate, the maximum physiologically achievable energy expenditure. Metabolic rate is determined through open flow respirometry.

Ad 4. Texas red (TxR) fluorescent dye covalently linked to poly(n-butyl cyanoacrylate) dextran polymers (Dx) coated with polysorbate 80 nanoparticles (PBCA NP) is used to test the activity of the GS and the penetration of brain tissue by fluids. TxR with Dx alone is not able to cross the blood-brain barrier (BBB). Nanoparticulate integration of TxR with Dx and polysorbate-80 coating, however, allows it to cross the BBB and diffuse into the brain tissue.

Ad 4.1. Males and female zebra finches were kept separately in similar aviaries (L × W × H: 175 × 140 × 240 cm) in a single climatic chamber under thermoneutrality of 30°C (Calder 1964) and photoperiodic cycle of 12:12 L:D. Torpor will be induced in the birds in laboratory conditions by exposing the birds to sliding cold ambient temperatures and nighttime conditions, within the experimental chamber. Temperature manipulations will consist of 36 hours of acclimation followed by exposure to one of three temperatures: 30°C (thermoneutral zone), 23°C (7 degrees below the thermoneutral zone for acclimation), and 19°C (the torpor induction temperature for our experiment).

Ad 4.2. At 30 min before ET time point (and before DT and AT in subgroups with longer survival times during torpor) animals from the inhibition group receive a single i.p. injection of aquaporin-4 (AQP4) inhibitor - TGN-020 chloride salt (N-(1,3,4-thiadiazol-2-yl)pyridine-3-carboxamide dihydrochloride) (Ukrorgsyntez Ltd), at dosage 100 mg/kg.

TGN-020 is dissolved in sterile water for injection and titrated with 1 M NaOH to a pH of 8.0. Untreated animals and controls receive the same volume of isotonic saline.

Ad 5. Inhibited and non-inhibited experimental animals at time points ET, DT and AT, and controls at time points ES, DS, and AS are i.p. injected with lethal doses of Morbital (Biowet) and rapidly (to prevent the flushing of fluorophores from the tissue) transcardially perfused with 0.1 M phosphate-buffered saline (pH 7.4). Birds are then decapitated, and the brains are extracted from skulls by carefully cutting through the cranial bones and meninges and cut in half along *fissura longitudinalis cerebri*. Right
before starting the perfusion, blood samples will be collected from the heart in capillary tubes for biochemical research purposes.

Ad 5.1. After transferring the blood and brain tissue homogenates into the Eppendorf tubes, we will centrifuge them to test them for the markers of oxidative stress. d-Rom tests will be performed on the blood serum to measure hydroperoxides in serum via Fenton's reaction. Oxi tests will be performed on the plasma to detect the activity of the catalase enzyme. GSH Assay will be performed to measure the activity of glutathione in brain tissue homogenates, thereby understanding the level of oxidative stress accumulated in the bodies of our research specimen.

Ad 5.2. The left hemispheres of brain are placed individually in small containers (50 mL conical tubes) with 4% paraformaldehyde (PFA) solution in 0.1 M phosphate-buffered saline (pH 7.4) to fully immerse the tissue. Tissues are always stored in sealed, light-tight boxes to avoid photo-bleaching of the fluorophores. Brains are allowed to immersive fix for at least 3 days at 4°C. After this time brains are sliced by vibratome (Leica VT1000S) into 30 µm thick sections. Sections are stored in 24-well plates, in Tris-buffered saline enriched with 0.1% sodium azide (to inhibit bacteria formation) at 4°C. The sections are applied to gelatin-coated microscope slides and mounted in medium.

Ad 6. Inverted confocal microscope system (Zeiss LSM 410 with Zeiss Plan-Neofluar objectives) is used to observe the preparations. For visualization of Texas Red an Ar/Kr 488/568 laser is used with 590-nm longpass emission filter. The slices are imaged with a Zeiss Plan-Apochromat ×63/1.25 NA oil immersion objective. Switching of illumination is computerized. A confocal aperture is set digitally, and this setting is maintained throughout the study. All imaging parameters (contrast and brightness; Zeiss Imaging Software) are set in order to yield high-resolution images for both bright and dim sections. All settings must be kept constant for recording data within an experiment so that images from control and treated slices can be compared. Acquired microscopic images are quantitatively evaluated with the help of specialized computer software (e.g., Corel packages and ImageJ).

Ad 7. Statistical analysis of the obtained data will be carried out with the use of specialized computer programs, for example, Statgraphics Centurion, STATISTICA, and the R language environment. During statistical analysis, depending on the data distribution, parametric or non-parametric procedures will be applied. Relationships between telemetry, biochemical, and histological data will be assessed. Internal functions of the applied statistical programs and graphic packages will be used for data visualization. results obtained under this project will be published in the form of scientific articles in reputable English-language scientific journals with an international reach (i.e., *Journal of Neuroscience* and *Biological Reviews)*. Members of the research team will present the results and conclusions in an international forum during scientific conferences devoted to the topics addressed by this project (e.g., FENS Forum 2024 and 18th World Sleep Congress 2025). The results obtained as part of the project can play a preliminary and application role; the issues and efforts undertaken will be successively continued and developed by the team in future scientific projects.

5. Project literature

Ambler M., Hitrec T., Pickering A. 2022. Turn it off and on again: characteristics and control of torpor. Wellcome Open Research, 6:313

Barja, G. 2004. Free radicals and aging. Trends in Neurosciences 27:595–600

Barnes B. M. 1989. Freeze avoidance in a mammal: body temperatures below 0 degree C in an Arctic hibernator. Science, 244(4912): 1593–1595.

Beckman, K. B., and Ames B. N. 1998. The free radical theory of aging matures. Physiological Reviews 78:547–581

Beneveniste H., Liu X., Koundal S., Sanggaard S., Lee H., Wardlaw J. 2019. The glymphatic system and waste clearance with brain aging: a review. Gerontology, 65: 106–119

Bize P., Devevey G., Monaghan P., Doligez B., Christe P. 2008. Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. Ecology, 89(8), pp. 2584–2593

Cadenas, E., and K. J. A. Davies. 2000. Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biology and Medicine 29:222–230

Calder, W. A. (1964). Gaseous Metabolism and Water Relations of the Zebra Finch. *Taeniopygia castanotis.* Physiol. Zool. 37, 400–413.

Cooper-Mullin C, McWilliams SR. 2016. The role of the antioxidant system during intense endurance exercise: lessons from migrating birds. J Exp Biol 219:3684–95.

Ferratti A., McWilliams S. R., Rattenborg N. C., Maggini I., Cardinale M., Fusani L. 2020. Energy stores, oxidative balance, and sleep in migratory Garden Warblers (*Sylvia borin*) and Whitethroats (*Sylvia communis*) at a spring stopover site. Integrative organismal biology, pp, 1–15

Geiser F. 1998. Evolution of daily torpor and hibernation in birds and mammals: importance of body size. Clinical and experimental pharmacology and physiology 25, 736–740.

Hablitz L. M., and Nedergaard M. 2021. The glymphatic system: a novel component of fundamental neurobiology. The Journal of Neuroscience, 41(37): 7698–7711

Jessen N. A., Munk A. S. F., Lundgaard I., Nedergaard M. 2015. The glymphatic system – a beginner's guide. Neurochem Res. 40 (12): 2583–2599

Lyman C. P., Willis J. S., Malan A., & Wang L. C. H. 1982. Hibernation and Torpor in Mammals and Birds. Academic Press, New York, San Diego

McKechnie, A. E. and Mzilikazi, N. Heterothermy in afrotropical mammals and birds: a review. Integrative and comparative biology, vol. 51, no 3, pp. 349-363 doi:10.1093/icb/icr035

Reimund E. 1994. The free radical flux theory of sleep. Med Hypotheses 43:231–3

Ruf T., and Geiser F. 2015. Daily torpor and hibernation in birds and mammals. Biol. Rev. 90, pp. 891–926

Schmidt M. H. 2014. The energy allocation function of sleep: a unifying theory of sleep, torpor, and continuous wakefulness. Neuroscience and Biobehavioral Reviews, 47: 122–153

de Veij Mestdagh, C. F., Timmerman, J. A., Koopmans, F., Paliukhovich, I., Miedema, S. S. M., Goris, M., van der Loo, R. J., Krenning, G., Li, K. W., Mansvelder, H. D., Smit, A. B., Henning, R. H., & van Kesteren, R. E. (2021). Torpor enhances synaptic strength and restores memory performance in a mouse model of Alzheimer's disease. Scientific Reports, 11(1). https://doi.org/10.1038/s41598-021-94992-x

Geiser, F., & Brigham, R. M. (2012). The Other Functions of Torpor. In Living in a Seasonal World (pp. 109–121). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-28678-0_10

Nowack, J., Stawski, C., & Geiser, F. (2017). More functions of torpor and their roles in a changing world. Journal of Comparative Physiology B: Biochemical, Systemic, and

Environmental Physiology, 187(5–6), 889–897. https://doi.org/10.1007/s00360-017-1100-y

Popov, V. I., Medvedev, N. I., Patrushev, I. V., Ignat'ev, D. A., Morenkov, E. D., & Stewart, M. G. (2007). Reversible reduction in dendritic spines in CA1 of rat and ground squirrel subjected to hypothermia-normothermia in vivo: A three-dimensional electron microscope study. Neuroscience, 149(3), 549–560. https://doi.org/10.1016/j.neuroscience.2007.07.059

Schleucher, E. (2004). Torpor in birds: Taxonomy, energetics, and ecology. Physiological and Biochemical Zoology, 77(6), 942–949. https://doi.org/10.1086/423744

Squarcio, F., Hitrec, T., Piscitiello, E., Cerri, M., Giovannini, C., Martelli, D., Occhinegro, A., Taddei, L., Tupone, D., Amici, R., & Luppi, M. (2023). Synthetic torpor triggers a regulated mechanism in the rat brain, favoring the reversibility of Tau protein hyperphosphorylation. Frontiers in Physiology, 14, 400. https://doi.org/10.3389/fphys.2023.1129278

Von Der Ohe, C. G., Darian-Smith, C., Garner, C. C., & Heller, H. C. (2006). Ubiquitous and temperature-dependent neural plasticity in hibernators. Journal of Neuroscience, 26(41), 10590–10598. https://doi.org/10.1523/JNEUROSCI.2874-06.2006

Wang, L. C. H. (1989). Ecological, Physiological, and Biochemical Aspects of Torpor in Mammals and Birds (pp. 361–401). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-74078-7_10

6. **Table with budget of the project**

Direct costs	**Direct costs in total: 944 050 PLN**
Personnel costs and scholarships	**PI salaries:** **Farzeen Saeed (participation in the project: 1/3)** **Patar Sinaga (participation in the project: 1/3)** **Szymon Kantor (participation in the project: 1/3)** **5 000 PLN x 3 PI x 36 months of duration of a project = 180 000 PLN** **Lab technician** (for a duration of 36 months) **2000 x 36 = 72 000 PLN** **Scholarships for two master's students** (one during the first year of project duration, second during the second year): **1 500 PLN x 2 students x 2 x 12 months = 72 000 PLN**
Research equipment/device/software cost	**Experimental animals** Zebra finches (Magnolia Farms Avian Breeder (Anaheim, CA); **100 PLN x 150 = 15 000 PLN** **Aviaries** (necessary for housing birds in accordance with the requirements of the bioethics committee and the

	recommendations of the veterinarian): **2 500 PLN x 4 = 10 000 PLN** **Experimental cages** (necessary for housing birds in accordance with the requirements of the bioethics committee and the recommendations of the veterinarian): **1 200 PLN x 50 = 60 000 PLN** **Food and bedding for birds:** **15 000 PLN** **Equipment to measure temperature in birds:** **Biomark Pit tags (x150)** **70 PLN x 150 = 10 500 PLN** **Biomark sterile syringes for the implantation of pit tags (x 10)** **180 PLN x 10 = 1 800 PLN** **Test tag Fish key chain (x5)** **130 PLN x 5 = 650 PLN** **Pit tag detecting antennas (x28)** **4 300 PLN x 28 = 120 400 PLN** **Biomark temperature monitoring system (x4)** **15 000 PLN x 3 = 45 000 PLN** **Equipment for measurement of the metabolic rate (e.g.,** CA2ACO2 analyser SableSystems Inc.,LasVegas,NV, USA = 17 000 PLN; S-3A/IIO2 analyser (AMETEK, Pittsburgh,PA,USA) = 15 000 PLN) **17 000 PLN + 15 000 PL = 32 000 PLN** **Centrifuge** (equipment needed for oxidative stress measurement) **36 000 PLN** **Spectrophotometer** (equipment needed for oxidative stress measurement) **41 000 PLN** **Prefabricated kits for the oxidative stress measurements** (e.g., Innovation d-Roms *fast* kit [x4]

	6 000 PLN x 4 = 24 000 PLN OxiSelect™ Catalase Activity Assay Kit, Colorimetric [x4] 3 200 PLN x 4 = 12 800 PLN Invitrogen™ Glutathione (GSH) Detection Assay Kit [x4] 3 500 PLN x 4 = 14 000 PLN) **24 000 PLN + 12 800 PLN + 14 000 PLN = 50 800 PLN** **High-performance laptops** (minimum specification: 3.3 GHz processor, 16 GB RAM, 1 TB disk, 15.6-inch screen, 1920x1080 pixels, dedicated graphics card) - taking into account the specificity of the research methods used to carry out the research proposed in this application, a laptop with sufficiently high computing power and high technical parameters will be needed to collect, save, analyze and archive data from observations, measurement data and microscopic images. The laptop will also be necessary at the stage of advanced statistical analysis of the obtained data and the development and visualization of results, as well as for keeping project documentation: **5 000 PLN x 3 = 15 000 PLN** **Perfusion materials** (including Morbital®, formalin, sodium dihydrogen phosphate, sodium hydrogen phosphate): **4 000 PLN** **Spare and consumable parts for the peristaltic pump** (including rubber hoses, connectors, needles): **1 000 PLN** **TGN-020** (AQP_4 inhibitor) **40 000 PLN** **Spare and consumable parts for the vibratome:** **2 000 PLN** **Materials for microscopy** (including histological adhesives, media, solvents, buffers, slides and coverslips): **5 000 PLN**

	Spare and consumable parts for the confocal microscope: **15 000 PLN** **Disposable laboratory plastics** (e.g., syringes, eppendorfs, pipette tips, test tubes, containers, multi-well plates): **5 000 PLN** **Personal protective equipment** (including disposable gloves, caps, aprons, masks, shoe covers, protective glasses): **5 000 PLN** **Office and stationery supplies** (to keep project documentation): **1 000 PLN**
Other direct costs	**Postal, courier and transport services** (costs resulting from the need to bring materials, reagents, and apparatus to the laboratory and to conduct paper administrative and consulting correspondence): **8000 PLN** **Issuance of a qualified electronic signature** (necessary for the PIs to keep project documentation): **300 PLN x 3 = 900 PLN** **Ordering the Texas red fluorescent dye dextran polymers coated with polysorbate 80 nanoparticles synthesis by a specialized external laboratory specialized in this type of work: 20 000 PLN** **Domestic and foreign trips** (participation in two international conferences is planned in order to present this research project and the results obtained as part of it on an international forum: 1. 18th World Sleep Congress 2025 2. FENS Forum 2024 (Federation of European Neuroscience Societies), June 25-29, 2024, Vienna, Austria; The suggested amount has been calculated with the assumption of covering the costs of participation (e.g., conference fees, travel costs, hotel costs, daily allowances) of the

	three PIs in the above-mentioned conferences.) **60 000 PLN**
Indirect costs, including:	
- indirect costs of OA	**18 881 PLN**
- other indirect costs	**188 810 PLN**
TOTAL COST OF PROJECT	**1 151 741 PLN**

REVIEWS

Prof dr. hab. Mariusz Cichoń

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 am not an expert in the field, but from the information provided by the authors one can judge the field is important and the proposed study is sound and promising. In birds torpor is not often studied, particularly physiological benefits behind this process except the energy savings are to large extend unknown. The idea of the authors is that torpor serves as a way of mediating reduction of oxidative stress. This sounds as a very good idea with high scientific potential.

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 study is a basic one intended to reveal the potential role of torpor beyond the typical idea of energy savings during adverse environmental conditions with shortage of food and cold conditions. If this is true that torpor serves as a mechanism of reducing oxidative stress, one should expect this physiological behaviour to be commonly observed also under favourable conditions with food ad libitum and neutral thermal conditions. So, I wonder of whether it is so common under favourable conditions. The proposed research, if successful, may have strong impact internationally. The research outcome may be very likely to be accepted for publications in well recognized scientific journals.

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 research sounds like being far more advanced for preludium grant proposals. To me it is more like Opus grant devoted to experienced researchers. The research if feasible, but I am not sure the PIs are already experienced enough to deal with the proposed research. At least, I do not find good confirmation in the proposal that this is the case. It is worrying that the authors are not aware of potential risks by saying "The proposed research is associated with minimal risk of failure". This is just not true for any study. The authors provide an information of some preliminary results, but it is unclear if this preliminary research is of their own or some other researchers. The research requires collaboration with some researchers to perform microscopic observations. There is no information on this issue in the proposal. I have good reasons to think that all parts of planned research can be performed exclusively by the research group created by the PIs. At least there is no information in the proposal that the PIs possess the required skills.

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

The research budget is very large as for a Preludium grant. As I already mentioned Opus grant is more suitable choice for this research project. The costs are justified in my opinion, however I do not understand the role of master students in this research.

5. **Strengths of the proposal**

Sound research idea with strong potential of publications in recognized scientific journals.

6. **Weaknesses of the proposal**

Inadequate risk management. Many potential risks are not recognized and managed. Low experience of the research team to conduct rather demanding research skills. Inadequate choice of type of research grant. Should be rather Opus not Preludium.

Patrycja Dziurowicz

The proposed research project aims to investigate whether birds in daily torpor mobilise the brain's glymphatic system and reduce oxidative stress in brain tissues. The authors hypothesise that the glymphatic system is activated during torpor, leading to a continuous decrease in oxidative stress. Additionally, dysfunction of the glymphatic system would lead to increased levels of oxidative stress. The authors have described the background information in a very interesting and careful manner. In the description given, all the information forms a coherent whole, which affects the understanding of the topic even by people who are not specialists in the field. The authors have shown that there is a lack of knowledge on the established topic and that the results they obtained in the project will allow a better understanding of torpor and brain metabolic waste transport in birds, which may also be reflected in other species.

The proposed project is interdisciplinary in nature, combining fields such as neurobiology, physiology, and immunology. In my opinion, the results obtained could have an international character and will allow knowing whether torpor is a mechanism for sweeping ROS and alleviating oxidative stress in the brain of birds. In general, the proposed research project is well-thought out and has the potential to yield significant results in the field of neurobiology and physiology. An additional advantage is that the authors of the project see the need to continue research and develop it.

The project is very ambitious and requires very careful planning and extensive logistics. In my opinion, the authors have planned and thought carefully about their experiment in a very good way. However, the authors in the subsection on concept and work plan did not clearly describe how many subgroups they will have, the mere information about the division into subgroups according to the torpor phases does not give clear information about the number of subgroups. In my opinion there is no information on how long the project will run, it is not clear how long the different phases of the project are expected to run. Also, there is no indication of the duration of the entire project, which in my opinion should be found earlier than in the cost estimate.

The use of Zebra finches as a model organism is justified and the preliminary research results are promising. Risk analysis is thorough, and the procedures proposed are standard in the laboratory.
However, one potential concern is the small sample size of each subgroup (seven males and seven females). This may limit the statistical power of the study and the results may not be generalisable to the broader population. It would be beneficial to justify this sample size and discuss any potential limitations that may arise from it.
The research methodology is very detailed about the experimental procedures used. It includes information about the preparation of birds for experiments, tests and equipment used. The methodology is well organised and provides sufficient detail to replicate the experiments. However, in my opinion, before starting with the methods described, the authors should add information that the points e.g. Ad 4.2. refer to the points included in the fragment of the **Specific research goals.**
In the **results of preliminary research** the authors described their previously conducted research that has not been published, which raises the possibility of questioning the validity of the research results obtained. In my opinion, the authors should also include the results of unpublished data to validate their pilot study.
The authors will use 126 birds for the experiment, however they are purchasing 150, therefore do the authors assume the possibility that some specimens may die during the experiment and be replaced by another? If so, how could this potentially affect the final results of the experiment?

The authors have described the planned costs in a very precise way, the explanations of the different parts show the necessity of purchasing the equipment proposed by the authors. However, the costs presented as the cost of research equipment, device, and software should not exceed more than 30 % of the total costs. The authors should consider reducing the costs in the stated point. Also missing is a table summarising all expenses.

The project is clearly justified from the point of view of its importance in basic and applied sciences, and the research issues are clearly set out. The objective is clearly stated and the language is well formulated and written fluently. The design is reasonable for a three-year project. The objectives and importance of the project are very well structured.

The weak points of the proposal are the small sample size in the subgroups and the high risk of not obtaining enough data.

Alaa Hseiky

The proposed research project aims to investigate whether birds in daily torpor mobilise the brain's glymphatic system and reduce oxidative stress in brain tissues. The authors hypothesise that the glymphatic system is activated during torpor, leading to a continuous decrease in oxidative stress. Additionally, dysfunction of the glymphatic system would lead to increased levels of oxidative stress. The authors have described the background information in a very interesting and careful manner. In the description given, all the information forms a coherent whole, which affects the understanding of the topic even by people who are not specialists in the field. The authors have shown that there is a lack of knowledge on the established topic and that the results they obtained in

the project will allow a better understanding of torpor and brain metabolic waste transport in birds, which may also be reflected in other species.

The proposed project is interdisciplinary in nature, combining fields such as neurobiology, physiology, and immunology. In my opinion, the results obtained could have an international character and will allow knowing whether torpor is a mechanism for sweeping ROS and alleviating oxidative stress in the brain of birds. In general, the proposed research project is well-thought out and has the potential to yield significant results in the field of neurobiology and physiology. An additional advantage is that the authors of the project see the need to continue research and develop it.

The project is very ambitious and requires very careful planning and extensive logistics. In my opinion, the authors have planned and thought carefully about their experiment in a very good way. However, the authors in the subsection on concept and work plan did not clearly describe how many subgroups they will have, the mere information about the division into subgroups according to the torpor phases does not give clear information about the number of subgroups. In my opinion there is no information on how long the project will run, it is not clear how long the different phases of the project are expected to run. Also, there is no indication of the duration of the entire project, which in my opinion should be found earlier than in the cost estimate.
The use of Zebra finches as a model organism is justified and the preliminary research results are promising. Risk analysis is thorough, and the procedures proposed are standard in the laboratory.
However, one potential concern is the small sample size of each subgroup (seven males and seven females). This may limit the statistical power of the study and the results may not be generalisable to the broader population. It would be beneficial to justify this sample size and discuss any potential limitations that may arise from it.
The research methodology is very detailed about the experimental procedures used. It includes information about the preparation of birds for experiments, tests and equipment used. The methodology is well organised and provides sufficient detail to replicate the experiments. However, in my opinion, before starting with the methods described, the authors should add information that the points e.g. Ad 4.2. refer to the points included in the fragment of the **Specific research goals.**
In the **results of preliminary research** the authors described their previously conducted research that has not been published, which raises the possibility of questioning the validity of the research results obtained. In my opinion, the authors should also include the results of unpublished data to validate their pilot study.
The authors will use 126 birds for the experiment, however they are purchasing 150, therefore do the authors assume the possibility that some specimens may die during the experiment and be replaced by another? If so, how could this potentially affect the final results of the experiment?

The authors have described the planned costs in a very precise way, the explanations of the different parts show the necessity of purchasing the equipment proposed by the authors. However, the costs presented as the cost of research equipment, device, and software should not exceed more than 30 % of the total costs. The authors should consider reducing the costs in the stated point. Also missing is a table summarising all expenses.

The project is clearly justified from the point of view of its importance in basic and applied sciences, and the research issues are clearly set out. The objective is clearly stated

and the language is well formulated and written fluently. The design is reasonable for a three-year project. The objectives and importance of the project are very well structured.

The weak points of the proposal are the small sample size in the subgroups and the high risk of not obtaining enough data.

Anbarieh Saadat

The proposed research project aims to investigate whether birds in daily torpor mobilise the brain's glymphatic system and reduce oxidative stress in brain tissues. The authors hypothesise that the glymphatic system is activated during torpor, leading to a continuous decrease in oxidative stress. Additionally, dysfunction of the glymphatic system would lead to increased levels of oxidative stress. The authors have described the background information in a very interesting and careful manner. In the description given, all the information forms a coherent whole, which affects the understanding of the topic even by people who are not specialists in the field. The authors have shown that there is a lack of knowledge on the established topic and that the results they obtained in the project will allow a better understanding of torpor and brain metabolic waste transport in birds, which may also be reflected in other species.

The proposed project is interdisciplinary in nature, combining fields such as neurobiology, physiology, and immunology. In my opinion, the results obtained could have an international character and will allow knowing whether torpor is a mechanism for sweeping ROS and alleviating oxidative stress in the brain of birds. In general, the proposed research project is well-thought out and has the potential to yield significant results in the field of neurobiology and physiology. An additional advantage is that the authors of the project see the need to continue research and develop it.

The project is very ambitious and requires very careful planning and extensive logistics. In my opinion, the authors have planned and thought carefully about their experiment in a very good way. However, the authors in the subsection on concept and work plan did not clearly describe how many subgroups they will have, the mere information about the division into subgroups according to the torpor phases does not give clear information about the number of subgroups. In my opinion there is no information on how long the project will run, it is not clear how long the different phases of the project are expected to run. Also, there is no indication of the duration of the entire project, which in my opinion should be found earlier than in the cost estimate.
The use of Zebra finches as a model organism is justified and the preliminary research results are promising. Risk analysis is thorough, and the procedures proposed are standard in the laboratory.
However, one potential concern is the small sample size of each subgroup (seven males and seven females). This may limit the statistical power of the study and the results may not be generalisable to the broader population. It would be beneficial to justify this sample size and discuss any potential limitations that may arise from it.
The research methodology is very detailed about the experimental procedures used. It includes information about the preparation of birds for experiments, tests and equipment used. The methodology is well organised and provides sufficient detail to replicate the experiments. However, in my opinion, before starting with the methods described, the

authors should add information that the points e.g. Ad 4.2. refer to the points included in the fragment of the **Specific research goals.**
In the **results of preliminary research** the authors described their previously conducted research that has not been published, which raises the possibility of questioning the validity of the research results obtained. In my opinion, the authors should also include the results of unpublished data to validate their pilot study.
The authors will use 126 birds for the experiment, however they are purchasing 150, therefore do the authors assume the possibility that some specimens may die during the experiment and be replaced by another? If so, how could this potentially affect the final results of the experiment?

The authors have described the planned costs in a very precise way, the explanations of the different parts show the necessity of purchasing the equipment proposed by the authors. However, the costs presented as the cost of research equipment, device, and software should not exceed more than 30 % of the total costs. The authors should consider reducing the costs in the stated point. Also missing is a table summarising all expenses.

The project is clearly justified from the point of view of its importance in basic and applied sciences, and the research issues are clearly set out. The objective is clearly stated and the language is well formulated and written fluently. The design is reasonable for a three-year project. The objectives and importance of the project are very well structured.

The weak points of the proposal are the small sample size in the subgroups and the high risk of not obtaining enough data.

Mateusz Chechetkin

The proposed research is original due to its interdisciplinary nature (combining animal physiology and neurobiology and using both to propose a potential explanation of the studied phenomenon) and substantially novel, as no similar studies have ever been performed. The project is very relevant for its specific subfield (neurophysiology), and it is of clear importance to fundamental research. The relevance to broader fields (e.g. ecology and evolution) is mentioned but not elaborated upon beyond the project summary.

There is an evident impact of the research on fundamental neurobiology and animal physiology. There is some potential for impact on broader fields, such as ecology and evolution (and on physiological ecology in particular), however, authors do not mention the specifics. In my opinion, authors should have stated if the potential discoveries have values for e.g. understanding the tradeoffs between activity and energy preservation in birds, particularly during migration, or on the evolution of torpor and sleep in animals in general. Additionally, it is unclear to me whether there is any potential for impact in applied sciences or whether the research is purely fundamental.

From my point of view, the connection between the proposed experiments and the hypothesis is not completely explained. In particular, based on the text I am not sure whether the experimental groups of birds are expected to only go into torpor or whether the effect of sleep and torpor is combined. The cited literature (and other available studies) suggest that torpor and sleep can co-occur, but that at certain temperatures only

torpor occurs and normal sleep is disrupted. The first hypothesis suggests that authors mean to assess the effect of torpor alone, however methodology (e.g. photoperiod and lighting condition descriptions) suggest that birds in the experimental group experience both sleep and torpor. The statistics part of methodology also does not describe in what way the control and experimental groups are compared and what observations would confirm or falsify the hypothesis.
Furthermore, while the methodology is very well described with justification and citations for every protocol and manipulation, it is not entirely explained why each manipulation is performed. For example, it is not very clear from the text why the aquaporin blocking is done. From the general description, I understand that it is done to block the glymphatic system, however, I think the flow of the proposal would be better if it focused on the connection between hypothesis and methodology instead of describing the specific protocols in such detail. The main element missing for me is predictions for each hypothesis and a description of what kind of results of each experiment would confirm or falsify the outlined hypotheses.
In terms of risk management and equipment, the authors appear to have a thorough, detailed plan of work, and the planned experiments have a low risk of failure. The methods they are using are supported in the literature and the team has access to all necessary equipment.

The budget is extensively described and well-justified. The scope of the project and its cost are appropriate to its goals and planned experiments.

The strengths of this project are its novelty, its interdisciplinary nature, and the obvious importance for its specific field. Based on both the research proposal and a search of the literature, the topic is new and not very well studied, and the hypotheses that the authors plan to test make sense in the context of existing knowledge on the topic. Additionally, the thorough preparation of the research plan (such as the detailed methodology and well-calculated costs) suggest that the authors are qualified to perform every task and are prepared for every possibility.

The main weakness of the proposal is the apparent discrepancy between the hypotheses and the experiments planned. As I outlined previously, it is not described in the text whether the experimental group is expected to only have effects of torpor, or effects of sleep and torpor combined. It is also not described why the aquaporin blocking is performed. Neither the research plan or statistics describe which groups are going to be compared, which factors will be considered, and how these experiments would test the outlined hypotheses. Though the hypotheses themselves make sense and are falsifiable, I would like to see an improved research plan which clearly outlines grouping, predictions, and potential outcomes.

FINAL VERSION

The Mobilization of the Glymphatic System in a State of Torpor in an Avian Model and its Association with the Oxidative Stress

Farzeen Saeed, Patar Sinaga, Szymon Kantor

Abstract

Torpor is a state in which animals reduce their body temperature, metabolic rate, and organ functions to conserve energy and survive periods of low food availability or extreme environmental conditions. Daily torpor is associated with physiological restoration and energy conservation. It also corresponds to the disposal of metabolic waste products such as reactive oxygen species (ROS) that can build up and cause oxidative stress thereby leading to decreased fecundity and survival. The glymphatic system (GS) has been studied to remove metabolites from the brain, playing a crucial role in maintaining brain homeostasis. However, the function of the GS in birds during daily torpor has not yet been investigated. The study will focus on the zebra finch (*Taeniopygia guttata*), to study the relationship between torpor and the activation of glymphatic system in birds to reduce oxidative stress in the brain tissue upon awakening. This research will analyze torpor from a neuroscientific perspective and answer the gaps in fundamental knowledge of daily torpor in zebra finches and other bird species.

1. Scientific goal of the project

In the proposed research project, the main scientific goal is to determine whether birds, in a state of daily torpor, mobilize the brain's glymphatic system and as a result, reduce oxidative stress. In the earliest and the most simplistic models, during torpor, the animal's body functions slowed down to decrease energy expenditure, allowing it to survive periods of low food availability (Schleucher 2004) or harsh environmental conditions (Wang 1989). However, it is correct to assume that torpor may have other crucial functions beyond just energy conservation. Moreover, it is highly possible that torpor and the nervous system share several important interactions, yet to the best knowledge of the authors of this project, no research has been conducted focusing on the role of daily torpor in the functioning of the glymphatic system, especially in relation to oxidative stress.

Our hypotheses

1. The glymphatic system is activated in the brains of birds entering daily torpor, just like in a natural sleep.
2. There is a continuous decrease in oxidative stress along the torpor bout due to the activation of glymphatic system.
3. Dysfunction of the glymphatic system is reflected in an increase in levels of oxidative stress.

2. Significance of the project

In times of low ambient temperatures and reduced food availability, birds conserve their energy by entering a state of torpor characterized by a period of controlled reduction of metabolism and body temperature. The timing and duration of daily torpor is controlled by the internal clock that ensures that bouts of torpor alternate with the resumption of euthermic functions such as activity, foraging, and sleep within the 24 h daily cycle (Ruff and Geiser 2015).

Torpor can be divided into two types - daily torpor and seasonal torpor (hibernation). Small animals usually perform daily torpor and manage to lower their body temperature a few degrees above the ambient environmental temperature (Barnes 1989, Ambler *et al.* 2022) while hibernating animals lower their body temperature in a range far greater than during daily torpor. The characteristics possessed by animals that carry out daily torpor are the duration of temperature reduction which includes several hours and variations in body temperature can reach 17°C, metabolic rate can decrease by about 30% of basal metabolic rate (BMR) (Geiser 1998).

The need for sleep in birds, such as during torpor, is associated with physiological restoration and energy conservation (Schmidt 2014, Ferretti *et al.* 2020). However, the benefits of sleep are also associated with the disposal of metabolic products, such as reactive oxygen species (ROS) - molecules that are related to the occurrence of oxidative stress (OS) (Reimund 1994). OS is considered the reason behind decreased fecundity and survival (Beckman and Ames 1998). ROS is highly reactive with molecules such as proteins, lipids, and DNA (Cooper-Mullin and McWilliams 2016). Because of that they can interfere with gene expression, tissue function, and affect the reproductive system (Cadenas and Davies 2000, Barja 2004, Bize *et al.* 2008). Disposal of ROS is also important for maintaining brain homeostasis (Jessen *et al.* 2015). That is possible thanks to the presence of a glymphatic system (GS) (Hablitz and Nedergaard 2021).

GS is the excretory system of the brain that is a nexus of lymphatic network, cranial nerves tracts and large vessels exiting the skull (Benveniste *et al.* 2019). The functions of GS include disposal of wastes and metabolites where this process occurs when sleeping (Hablitz and Nedergaard 2021). This action can be summarized in 3 stages: (i) the entry of cerebrospinal fluid (CSF) into the subarachnoid space and then into the periarterial spaces, (ii) the pumping of CSF from the periarterial to the interstitial fluid (ISF) space and then mixing CSF and ISF with metabolites, and (iii) draining the mixture of CSF, ISF and waste out of the brain to the perivenous compartment of central veins and, finally, to the circulation system (Benveniste et al. 2019). In the GS, aquaporin 4 (AQP4) - a water channel protein that is abundantly expressed in the brain - plays a crucial role in the clearance of waste products and a growing body of research suggests that dysfunction of AQP4 channels may contribute to the pathogenesis of neurodegenerative diseases (Silva *et al.* 2021). The function and whether GS is a protection mechanism from toxic waste metabolites in birds has rarely been discussed before, even though some species of birds spend a lot of time sleeping or in sleep-like state of torpor.

Previous studies have suggested that torpor modulates neuronal survival and plasticity in certain brain regions and this adaptive function is thought to be a protective mechanism against reperfusion injury during arousal, wherein the loss of synapses and subsequent neural damage may be mitigated by torpor-induced changes in brain activity (Popov *et al.* 2007; Von Der Ohe *et al.* 2006). Daily torpor can also trigger neuroprotective processes (Squarcio *et al.* 2023) and may have a beneficial effect on memory performance (de Veij Mestdagh *et al.* 2021).

A new discovery regarding the activity of the GS during daily torpor could be considered a breakthrough, especially because it would be highly interdisciplinary, combining the latest scientific achievements in the fields of neurobiology, immunology, and physiology. Additionally, there has been no research on whether torpor is a mechanism for sweeping ROS and reducing oxidative stress in the brain of birds, which is an especially important knowledge gap yet to be answered. It is also projected that this exploratory research has the potential to help future studies that will be conducted in this field to take reference from and provide a broader picture of the torpor and metabolic waste disposal from the brain of other animal species.

3. Concept and work plan

We are trying to discover if the GS is the neurobiological cause of reducing oxidative stress upon arousal from daily torpor, in birds (Figure 1). To test our hypotheses, we will obtain 150 adult male and female zebra finches (*Taeniopygia guttata*) from Max Plank Institute of Ornithology, Germany. To conduct the experiment, we will divide the experimentation birds into subgroups (i.e., control and experiment, based on the torpor phases) with seven males and seven females in each subgroup (3 subgroups for control: ES, DS, AS and 6 subgroups for experimental: ET, DT, AT).

General work plan

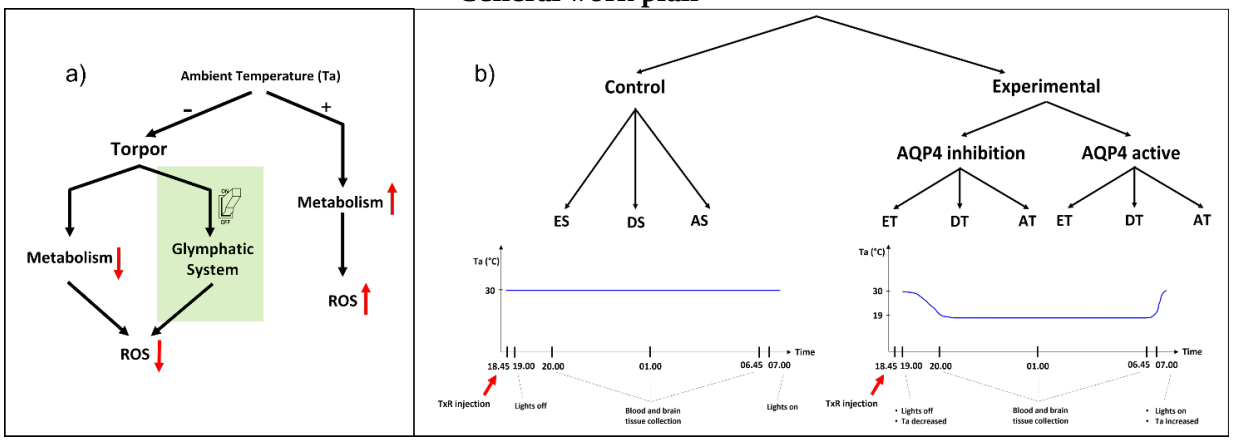


Figure 1: a) Torpor induced by ambient temperature as a mechanism to reduce oxidative stress. Notes: black arrows indicate the direction of the process, (+) indicates an increase in temperature, (-) indicates a decrease in temperature, an upward red arrow indicates an increase, and a downward red arrow indicates a decrease and b) Experiment design (time points - ES: early sleep, DS: during sleep, AS: awakening from sleep, ET: early torpor, DT: during torpor, and AW: awakening from torpor; Ta: ambient temperature; TxR: Texas red with dextran and polysorbate-80 coating).

Specific research goals

1. Weighting all the zebra finches to make sure that their mass is constant and does not influence the results of our experiments
2. Subcutaneous implantation of thermosensitive transponders followed by two-week continuous measurements of all birds' body temperature
3. Measurements of metabolic rate of all birds through open flow respirometry
4. Administration of fluorescent nanoparticles to all birds to determine activation profile of GS
   - 4.1) Induction of daily torpor state in animals from all experimental groups, while controls are allowed to naturally sleep in standard housing conditions
   - 4.2) Blocking the activity of the glymphatic system of animals from chosen experimental groups
5) Performance of perfusion and collection of peripheral blood and brains from our experimental birds over the course of the night
   - 5.1) Measurement of oxidative stress levels in tissue samples
   - 5.2) Immersion fixation of the brains for the purpose of confocal microscopy observations and preparation of microscope slides
6) Analysis of images acquired during microscopy observations
7) Detailed analysis of results using statistical tests and description of the results for publication.

Risk Analysis

The proposed research is associated with minimal risk of failure. The planned procedures will be performed with the knowledge and approval of the appropriate bioethics committee. The procedures presented by us, including the thermosensitive transponders implantation, induction of daily torpor, injections, administration of protein inhibitors, microscopic observations are standards well adapted in our laboratory, and their effectiveness is reflected in numerous scientific publications from peer-reviewed journals. In our laboratory, we have access to most of the necessary equipment (for example, an inverted confocal microscope system, professional aviaries, centrifuges, spectrometers, etc.).

However, even under these conditions, some things require special attention. The hot spot of our research is the implantation of animals, as a result of which they can sometimes develop infection and inflammation. For this purpose, body temperature and weight will be monitored after implantation. Unjustified changes, which may indicate the development of infection [e.g., pain (*dolor*) is one of the five local symptoms of inflammation], will be carefully considered. If an infection/inflammation is diagnosed, the animals will be given anti-inflammatory drugs (Tolfine 4%, Vetoquinol) and an antibiotic (Borgal 24%, Virbac S.A.) according to the veterinarian's prescription. However, due to the stress of implantation or the possible effects of infection, some animals may die or be euthanized before the start of the actual experiment. Therefore, we plan to purchase 150 animals, even though we will use 126 individuals of both sexes.

4. Research Methodology

Each of the following methods is related to specific research goals as indicated in the previous chapter.

Ad 1. and 2. Birds of all groups will be implanted with thermosensitive transponders (Biomark Pit tags) two weeks before the start of experiment, giving them enough time to recover. They will be also weighed fourteen, seven and one day before planned procedures to make sure that they have constant weight (average 16 g). Experiment cages will be prepared with pit tag detecting antennas set up around the cages to continuously measure the body temperature of the birds without having to touch them to reduce any discrepancies in our experiment results. During the experiment, the birds will be exposed to sliding cold temperatures to determine the torpor curve and its properties.

Ad 3. We will then measure the summit metabolic rate of the birds, the maximum physiologically achievable energy expenditure by exposing them to gradually decreasing temperatures. We need it to measure the maximal thermogenic capacity of our research specimen. Metabolic rate will be determined through open flow respirometry.

Ad 4. Texas red (TxR) fluorescent dye covalently linked to poly(n-butyl cyanoacrylate) dextran polymers (Dx) coated with polysorbate 80 nanoparticles (PBCA NP) will be used to test the activity of the GS and the penetration of brain tissue by fluids. TxR with Dx alone is usually not able to cross the blood-brain barrier (BBB). Nanoparticulate integration of TxR with Dx and polysorbate-80 coating, however, allows it to cross the BBB and diffuse into the brain tissue.

Ad 4.1. Males and female zebra finches will be kept separately in similar aviaries (L × W × H: 175 × 140 × 240 cm) in a single climatic chamber under thermoneutrality of 30°C (Calder 1964) and photoperiodic cycle of 12:12 L:D. Torpor will be induced in the birds in laboratory conditions by exposing the birds to nighttime conditions and sliding cold ambient temperatures from 30°C (thermoneutral zone) to 19°C (the lowest torpor induction temperature for our experiment), within the experimental chamber.

Ad 4.2. At 30 min before ET time point (and before DT and AT in subgroups with longer survival times during torpor) animals from the inhibition group will receive a single i.p. injection of aquaporin-4 (AQP4) inhibitor - TGN-020 chloride salt (N-(1,3,4-

thiadiazol-2-yl)pyridine-3-carboxamide dihydrochloride) (Ukrorgsyntez Ltd), at dosage 100 mg/kg. TGN-020 will be dissolved in sterile water for injection and titrated with 1 M NaOH to a pH of 8.0. Untreated animals and controls will receive the same volume of isotonic saline.

Ad 5. Inhibited and non-inhibited experimental animals at time points ET, DT and AT, and controls at time points ES, DS, and AS will be i.p. injected with lethal doses of Morbital (Biowet) and rapidly (to prevent the flushing of fluorophores from the tissue) transcardially perfused with 0.1 M phosphate-buffered saline (pH 7.4). Birds will then be decapitated, and the brains will be extracted from skulls by carefully cutting through the cranial bones and meninges and cut in half along *fissura longitudinalis cerebri*. Right before starting the perfusion, blood samples will be collected from the heart in capillary tubes for biochemical research purposes.

Ad 5.1. After transferring the blood and brain's right hemispheres homogenates into the Eppendorf tubes, we will centrifuge them to test them for the markers of oxidative stress. d-Rom tests will be performed on the blood serum to measure hydroperoxides in serum via Fenton's reaction. Oxi tests will be performed on the plasma to detect the activity of the catalase enzyme. GSH Assay will be performed to measure the activity of glutathione in brain tissue homogenates, thereby understanding the level of oxidative stress accumulated in the bodies of our research specimen.

Ad 5.2. The left hemispheres of brains will, individually, be placed in small containers (50 mL conical tubes) with 4% paraformaldehyde (PFA) solution in 0.1 M phosphate-buffered saline (pH 7.4) to fully immerse the tissue. Tissues will be stored in sealed, light-tight boxes to avoid photo-bleaching of the fluorophores. Brains will be allowed to immersive fix for at least 3 days at 4°C. After this time brains will be sliced by vibratome (Leica VT1000S) into 30 µm thick sections. Sections will be stored in 24-well plates, in Tris-buffered saline enriched with 0.1% sodium azide (to inhibit bacteria formation) at 4°C. The sections will be applied to gelatin-coated microscope slides and mounted in medium.

Ad 6. Inverted confocal microscope system (Zeiss LSM 410 with Zeiss Plan-Neofluar objectives) will be used to observe the preparations. For visualization of Texas Red an Ar/Kr 488/568 laser will be used with 590-nm longpass emission filter. The slices will be imaged with a Zeiss Plan-Apochromat ×63/1.25 NA oil immersion objective. Switching of illumination will be computerized. A confocal aperture will be set digitally, and this setting will be maintained throughout the study. All imaging parameters (e.g., contrast and brightness; Zeiss Imaging Software) will be set in order to yield high-resolution images for both bright and dim sections. All settings will be kept constant for recording data within an experiment so that images from control and treated slices will be compared. Acquired microscopic images will be quantitatively evaluated with the help of specialized computer software (e.g., Corel packages and ImageJ).

Ad 7. Statistical analysis of the obtained data will be carried out with the use of specialized computer programs, for example, Statgraphics Centurion, STATISTICA, and the R language environment. During statistical analysis, depending on the data distribution, parametric or non-parametric procedures will be applied. Relationships between telemetry, biochemical, and histological data will be assessed. Internal functions of the applied statistical programs and graphic packages will be used for data visualization. results obtained under this project will be published in the form of scientific articles in reputable English-language scientific journals with an international reach (i.e., *Journal of Neuroscience* and *Biological Reviews)*. Members of the research team will present the results and conclusions in an international forum during scientific conferences devoted to the topics addressed by this project (e.g., FENS Forum 2024 and 18th World Sleep Congress 2025). The results obtained as part of the project can play a preliminary and application role; the issues and efforts undertaken will be successively continued and developed by the team in future scientific projects.

5. Project literature

Ambler M., Hitrec T., Pickering A. 2022. Turn it off and on again: characteristics and control of torpor. Wellcome Open Research, 6:313

Barja G. 2004. Free radicals and aging. Trends in Neurosciences 27:595–600

Barnes B. M. 1989. Freeze avoidance in a mammal: body temperatures below 0 degree C in an Arctic hibernator. Science, 244(4912): 1593–1595.

Beckman K. B., and Ames B. N. 1998. The free radical theory of aging matures. Physiological Reviews 78:547–581

Beneveniste H., Liu X., Koundal S., Sanggaard S., Lee H., Wardlaw J. 2019. The glymphatic system and waste clearance with brain aging: a review. Gerontology, 65: 106–119

Bize P., Devevey G., Monaghan P., Doligez B., Christe P. 2008. Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. Ecology, 89(8), pp. 2584–2593

Cadenas E., and Davies K. J. A. 2000. Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biology and Medicine 29:222–230

Calder W. A. 1964. Gaseous Metabolism and Water Relations of the Zebra Finch. *Taeniopygia castanotis.* Physiol. Zool. 37, 400–413.

Cooper-Mullin C., McWilliams S. R. 2016. The role of the antioxidant system during intense endurance exercise: lessons from migrating birds. J Exp Biol 219:3684–95.

de Veij Mestdagh C. F., Timmerman J. A., Koopmans F., Paliukhovich I., Miedema S. S. M., Goris M., van der Loo R. J., Krenning G., Li K. W., Mansvelder H. D., Smit A. B., Henning R. H., & van Kesteren R. E. 2021. Torpor enhances synaptic strength and restores memory performance in a mouse model of Alzheimer's disease. Scientific Reports, 11(1). https://doi.org/10.1038/s41598-021-94992-x

Ferratti A., McWilliams S. R., Rattenborg N. C., Maggini I., Cardinale M., Fusani L. 2020. Energy stores, oxidative balance, and sleep in migratory Garden Warblers (*Sylvia borin*) and Whitethroats (*Sylvia communis*) at a spring stopover site. Integrative organismal biology, pp, 1–15

Geiser F. 1998. Evolution of daily torpor and hibernation in birds and mammals: importance of body size. Clinical and experimental pharmacology and physiology 25, 736–740.

Geiser F., & Brigham R. M. 2012. The Other Functions of Torpor. In Living in a Seasonal World (pp. 109–121). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-28678-0_10

Hablitz L. M., and Nedergaard M. 2021. The glymphatic system: a novel component of fundamental neurobiology. The Journal of Neuroscience, 41(37): 7698–7711

Jessen N. A., Munk A. S. F., Lundgaard I., Nedergaard M. 2015. The glymphatic system – a beginner's guide. Neurochem Res. 40 (12): 2583–2599

Nowack J., Stawski C., & Geiser F. 2017. More functions of torpor and their roles in a changing world. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 187(5–6), 889–897. https://doi.org/10.1007/s00360-017-1100-y

Reimund E. 1994. The free radical flux theory of sleep. Med Hypotheses 43:231–3

Ruf T., and Geiser F. 2015. Daily torpor and hibernation in birds and mammals. Biol. Rev. 90, pp. 891–926

Schmidt M. H. 2014. The energy allocation function of sleep: a unifying theory of sleep, torpor, and continuous wakefulness. Neuroscience and Biobehavioral Reviews, 47: 122–153

Popov V. I., Medvedev, N. I., Patrushev, I. V., Ignat'ev, D. A., Morenkov, E. D., & Stewart, M. G. 2007. Reversible reduction in dendritic spines in CA1 of rat and ground squirrel subjected to hypothermia-normothermia in vivo: A three-

dimensional electron microscope study. Neuroscience, 149(3), 549–560. https://doi.org/10.1016/j.neuroscience.2007.07.059
Schleucher E. 2004. Torpor in birds: Taxonomy, energetics, and ecology. Physiological and Biochemical Zoology, 77(6), 942–949. https://doi.org/10.1086/423744
Silva I., Silva J., Ferreira R. et al. Glymphatic system, AQP4, and their implications in Alzheimer's disease. Neurol. Res. Pract. 3, 5. 2021. https://doi.org/10.1186/s42466-021-00102-7
Squarcio F., Hitrec T., Piscitiello E., Cerri M., Giovannini C., Martelli D., Occhinegro A., Taddei L., Tupone D., Amici R., & Luppi M. 2023. Synthetic torpor triggers a regulated mechanism in the rat brain, favoring the reversibility of Tau protein hyperphosphorylation. Frontiers in Physiology, 14, 400. https://doi.org/10.3389/fphys.2023.1129278
Von Der Ohe C. G., Darian-Smith C., Garner C. C., & Heller H. C. 2006. Ubiquitous and temperature-dependent neural plasticity in hibernators. Journal of Neuroscience, 26(41), 10590–10598. https://doi.org/10.1523/JNEUROSCI.2874-06.2006
Wang L. C. H. 1989. Ecological, Physiological, and Biochemical Aspects of Torpor in Mammals and Birds (pp. 361–401). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-74078-7_10

6. Breakdown of project costs and their relevance in the project

Direct costs	Each of the following costs is related to **specific research goals** (see above, from 1 to 7).
Personnel costs and scholarships	**PI salaries:** Farzeen Saeed (participation in the project: 1/3) Patar Sinaga (participation in the project: 1/3) Szymon Kantor (participation in the project: 1/3) **5 000 PLN x 3 PI x 36 months of duration of a project =** **= 540 000 PLN (ad 1. – 7.)** **Lab technician** (for a duration of 36 months): 2000 PLN x 36 months of duration of a project **= 72 000 PLN (ad 1. – 7.)** **Scholarships for two master's students** [one during the first year of project duration, second during the third year]: **1 500 PLN x 2 students x 2 x 12 months =** **= 72 000 PLN (ad 1. - 6.)**
Research equipment/device/software cost	**Experimental animals** - Zebra finches *Taeniopygia guttata* [it is necessary to purchase animals from a reputable breeder (Max Planck Institute for Ornithology, Seewiesen, Germany), because it is not planned to breed them on your own]; **100 PLN x 150 = 15 000 PLN (ad 1. - 5.)** **Food and bedding for birds** (necessary for housing birds in accordance with the requirements of the bioethics committee and the recommendations of the veterinarian): **15 000 PLN (ad 1. - 5.)** **Equipment to measure temperature in birds:**

	Biomark Pit tags (x126) **70 PLN x 126 = 8 820 PLN** **Biomark sterile syringes for the implantation of pit tags (x5)** **180 PLN x 5 = 900 PLN** **Test tag Fish key chain (x5)** **130 PLN x 5 = 650 PLN** **Pit tag detecting antennas (x14)** **4 300 PLN x 14 = 60 200 PLN** **Biomark temperature monitoring system (x2)** **15 000 PLN x 2 = 30 000 PLN]** **8 820 PLN + 900 PLN + 650 PLN + 60 200 PLN + + 30 000 PLN = 100 570 PLN (ad 1. - 2.)** **Equipment for measurement of the metabolic rate** (e.g., CA2ACO2 analyser Sable Systems Inc., LasVegas, NV, USA = 17 000 PLN S-3A/IIO2 analyser AMETEK, Pittsburgh, PA, USA = 15 000 PLN) **17 000 PLN + 15 000 PL = 32 000 PLN (ad 3.)** **Prefabricated kits for the oxidative stress measurements** (e.g., Innovation d-Roms *fast* kit [x4] 6 000 PLN x 4 = 24 000 PLN OxiSelect™ Catalase Activity Assay Kit, Colorimetric [x4] 3 200 PLN x 4 = 12 800 PLN Invitrogen Glutathione (GSH) Detection Assay Kit [x4] 3 500 PLN x 4 = 14 000 PLN) **24 000 PLN + 12 800 PLN + 14 000 PLN =** **= 50 800 PLN (ad 5.1.)** **Perfusion materials** (including Morbital®, formalin, sodium dihydrogen phosphate, sodium hydrogen phosphate): **= 4 000 PLN (ad 5.)** **Consumable parts for the peristaltic pump** (including rubber hoses, connectors, needles): **= 1 000 PLN (ad 5.)** **TGN-020** (AQP4 inhibitor, Ukrorgsyntez Ltd, Kyiv, Ukraine) **= 40 000 PLN (ad 4.2.)** **Consumable parts for the vibratome** (including razor blades, scalpels, scalpels): **= 2 000 PLN (ad 5.2.)**

	Disposable materials for microscopy (including histological adhesives, media, solvents, buffers, slides and coverslips): **= 5 000 PLN (ad 6.)** **Spare and consumable parts for the confocal microscope:** **= 15 000 PLN (ad 6.)** **Disposable laboratory plastics** (e.g., syringes, eppendorfs, pipette tips, test tubes, containers, multi-well plates): **= 5 000 PLN (ad 3. - 6.)** **Personal protective equipment** (including disposable gloves, caps, aprons, masks, shoe covers, protective glasses): **= 5 000 PLN (ad 1. - 6.)** **Office and stationery supplies** (to keep project documentation): **= 1 000 PLN (ad 1. - 7.)**
Other direct costs	**Postal, courier and transport services** (costs resulting from the need to bring materials, reagents, and apparatus to the laboratory and to conduct paper administrative and consulting correspondence): **= 8 000 PLN (ad 1. - 6.)** **Issuance of a qualified electronic signature** (necessary for the PIs to keep project documentation): **400 PLN x 3 = 1 200 PLN (ad 1. - 7.)** **Ordering the Texas red fluorescent dye dextran polymers coated with polysorbate 80 nanoparticles synthesis by a specialized external laboratory specialized in this type of work** (HCS Pharma, Loos, France): **= 20 000 PLN (ad 4.)** **Domestic and foreign trips** (participation in two international conferences is planned in order to present this research project and the results obtained as part of it on an international forum): 1. 18th World Sleep Congress 2025, the date and location has not yet been announced (participants: Farzeen Saeed, Patar Sinaga) 2. FENS Forum 2024 (Federation of European Neuroscience Societies), June 25-29, 2024, Vienna, Austria (participants: Szymon Kantor, Farzeen Saeed) The suggested amount has been calculated with the assumption of complete covering the costs of

	participation (e.g., conference fees, travel costs, hotel costs, daily allowances) of the PIs in the above-mentioned conferences. **= 60 000 PLN (ad 7.)**

7. **Table with budget of the project**

	Amount in PLN
Direct costs, including:	1 064 570,00
- personnel costs and scholarships	684 000,00
- research equipment/device/software cost	147 570,00
- other direct costs	233 000,00
Indirect costs, including:	
- indirect costs of OA	21 291,40
- other indirect costs	212 914,00
Total costs:	1 298 775,40

Some pictures taken curtosy of the tired yet excited participants and the unusual things that they encountered on their advenutorus hike to the Observation Tower on Magurka (24.04.2023)