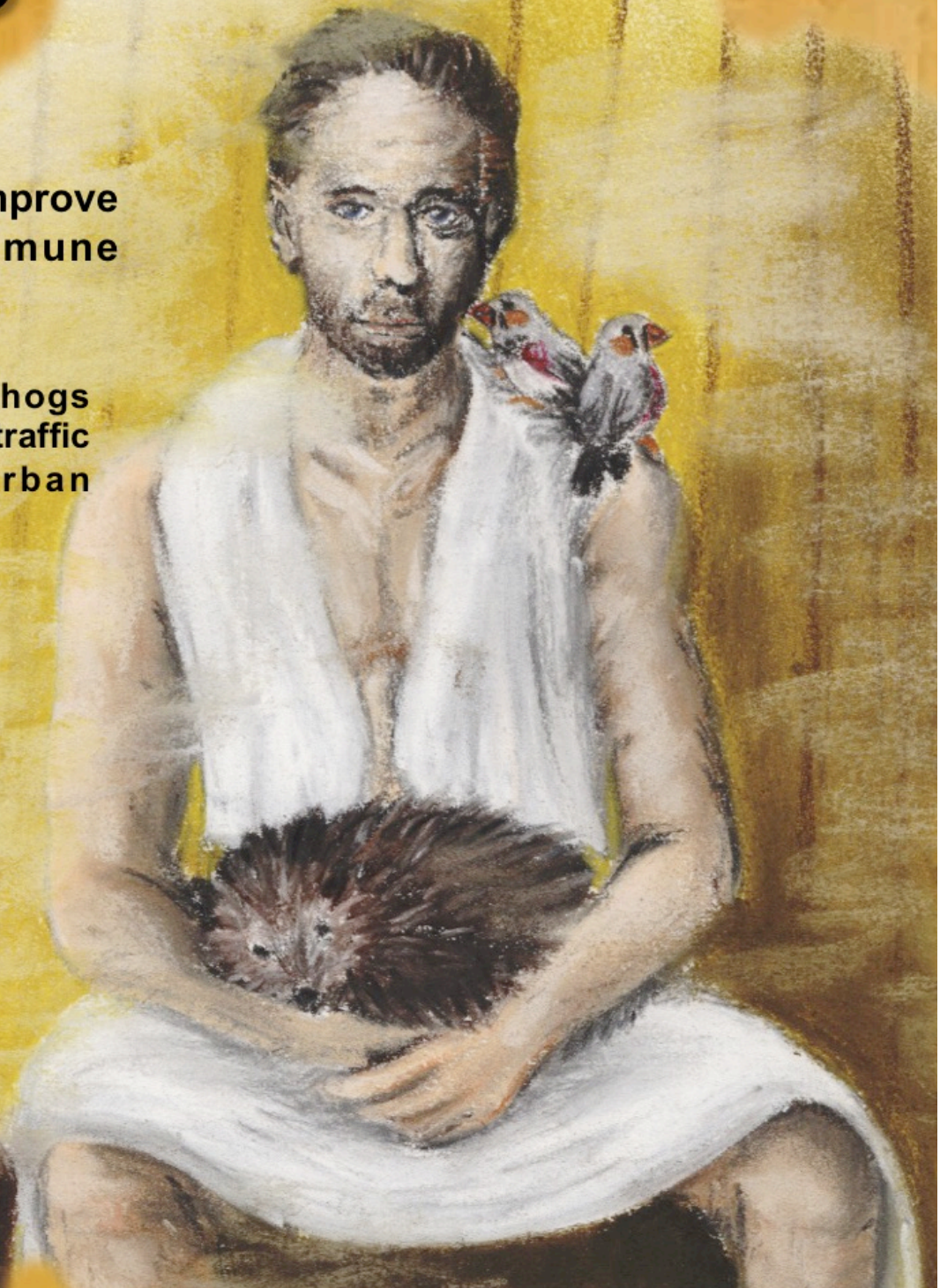


# nejczer.

**Does Sauna improve  
the innate immune  
response?**

**European hedgehogs  
response to car traffic  
in rural and urban  
areas.**

**Explanation for  
birds longevity!**



**METHODOLOGICAL WORKSHOP IN  
EVOLUTIONARY BIOLOGY FOR PhD  
STUDENTS – GRANT WRITING PART**

**OCHOTNICA GÓRNA 18-23.04.2018**

Cover prepared by Marta Grosiak  
Raport prepared by Amadusz Bryła

## TABLE OF CONTENTS

---

List of participants.....	3
Research topics proposed by participants .....	4
Does Finnish Sauna improve the innate immune response against pathogens of the respiratory system? .....	6
Reviews .....	15
Final version .....	23
Investigating the role of autophagy under caloric restriction and oxidative stress for longevity in birds .....	32
Reviews .....	38
Final version .....	45
European hedgehogs ( <i>Erinaceus europaeus</i> ) response to car traffic in rural and urban areas .....	52
Reviews .....	59
Final version .....	67

ORGANISER:

Dr hab. Joanna Rutkowska, Institute of Environmental Sciences, JU

REVIEWERS:

Dr hab. Łukasz Michalczyk Institute of Zoology and Biomedical Research, JU

Dr hab. Piotr Skórka Institute of Nature Conservation, PAS

Prof Wiesław Babik Institute of Environmental Sciences JU

Mgr Stanisław Bury Institute of Environmental Sciences, JU

PARTICIPANTS:

Evgenii Baiakhmetov Institute of Botany, JU

Michał Bełcik Institute of Nature Conservation, PAS

Amadeusz Bryła Institute of Environmental Sciences, JU

Miriam Gonzalez Gonzalez Institute of Zoology and Biomedical Research, JU

Marta Grosiak Institute of Environmental Sciences, JU

Jayasravanthi Mokkaapati Institute of Environmental Sciences, JU

## RESEARCH TOPICS PROPOSED BY PARTICIPANTS

---

Does climate change affect ectotherm diet? (Evgenii)

Does different food types affect reproductive success of bee drones? (Evgenii)

How epigenome of feather grass will be changed in different environmental conditions? (Evgenii)

Does the excess of iron affect human health status? (Miriam)

The importance of circadian cycles and thermoneutrality for lab mice welfare and stress response (Miriam)

**Does sauna improve immune response against pathogens of respiratory system? (Miriam)**

Can we recycle plastic waste with bees? (Jayasravanthi)

Do herbicides affect plant-pollinator interactions? (Jayasravanthi)

The impact of mixture toxicity of pesticides on beneficial organisms (Jayasravanthi)

Does lactation affect dental changes in females of small mammals with bigger reproductive output? (Marta)

Do differences in insect species composition induced by human activity can change composition in enamel in shrews? (Marta)

Does the intensity of Dehnel effect in shrews depend on chromosomal background? (Marta)

The role of algae in agile frog development (Amadeusz)

Why small dogs live longer than big ones? (Amadeusz)

**The role of autophagy and food restriction on longevity in birds (Amadeusz)**

Impact of dead wood and management strategies on amphibian dispersion in forests (Michał)

**Evolution of bravery in hedgehogs (Michał)**

Are animals choosy about the cellars? (Michał)

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

## DOES SAUNA IMPROVE IMMUNE RESPONSE AGAINST PATHOGENS OF RESPIRATORY SYSTEM?

Miriam Gonzalez Gonzalez, Evgenii Baiakhmetov



## THE ROLE OF AUTOPHAGY AND FOOD RESTRICTION ON LONGEVITY IN BIRDS

Amadeusz Bryła, Jayasravanthi Mokkaipati



## EUROPEAN HEDGEHOGS (ERINACEUS EUROPEAUS) RESPONSE TO CAR TRAFFIC IN RURAL AND URBAN AREAS

Michał Bełcik, Marta Grosiak



## **DOES FINNISH SAUNA IMPROVE THE INNATE IMMUNE RESPONSE AGAINST PATHOGENS OF THE RESPIRATORY SYSTEM?**

---

Authors: Evgenii Baiakhmetov, Miriam Gonzalez Gonzalez

### Abstract

#### 1. Research project objectives/Research hypothesis

Infections of the respiratory tract are very common and affect a lot of people from different range of age throughout the year. In some cases, transitory diseases are especially the ones related with the upper respiratory system, but in the case of affections of the lower respiratory system they are correlated with a high mortality rate. The innate immune system is the first one to act in case of a potential infection and there is high presence of different cells from the innate immune system in the respiratory ways, underlying the function of the macrophages and neutrophils. Recent studies have shown that there is a potential therapeutic role of Finnish Sauna as an inductor of short term hyperthermia, increasing the immune response. We hypothesize that Finnish Sauna sessions improve the immunological response against pathogens of the respiratory tract. There is a high necessity of developing prevention techniques for common diseases as respiratory tract infections, not only due to economical implications for health care systems and governments, but also for improving the quality of life.

#### 2. Research project methodology

The study will be firstly conducted in healthy volunteers and secondly, in patients with a medical background of respiratory tract infections. Groups of volunteers will be divided into sex (men and women) and if they are regular users of Finnish Sauna or not. Blood sample tests will be taken before and after sauna sessions, as well as the respiratory capacity. Neutrophils and monocytes /macrophages will be obtained from the samples and basic antimicrobial functions will be studied.

#### 3. Expected impact of the research project on the development of science, civilization and society

Issues related to the development of prevention against respiratory infections appeal interest not only for the economical point of view, but also for society as a whole because it is directly related to human health. Our results will provide a very easy-to-use routine for preventing the infections of the respiratory tract, impacting directly on the quality of life of the people.

## 1. Research objective

Respiratory Tract Infections (RTIs) are one of the most common infections in the human body. A general division could be done depending on the organs that comprise the upper respiratory tract (mouth, nose, throat, larynx, and trachea) and the lower (bronchial tubes and lungs). Some of these affections are seasonal, and the others affect more due to environmental conditions as the air pollution (e.g. smog).

Lower respiratory tract infections (LRTIs) are the sixth most important cause of mortality (2.7% cases) and are responsible for 9.9 thousand deaths annually in Poland (World Health Organization 2012). The RTIs differ with regard to the etiological agent. In particular, the innate immune system has a critical role of fighting against the pathogens that affect the lower and upper respiratory tracts, inasmuch as they are the first immunological cells to detect the exogenous pathogens. **The proposed research understands that specially resident macrophages and neutrophils play a critical role in this response, thus, this two cell types have higher presence in lungs and bronchioles.** Consequently, we propose to study these two cell types in particular, and their mainly microbial activities.

Hyperthermal conditions can be defined as increasing of the basal temperature of an organism, in some cases caused by an infection (fever) and in other because of environmental conditions (e.g. sauna). It has been demonstrated that a short hyperthermal phase can be beneficial for humans, since it increases the expression of Heat-Shock Proteins (Torigoe et al. 2009). A traditional way of increasing body temperature is the traditional “Finnish Sauna” that has a special room with an average temperature of 80-100°C and around 10-20% of humidity. Despite the body temperature do not raise more than one degree during sauna bath, it trigger interesting physiological responses. In the proposed research project **we will analyze innate immunological responses a) in the peripheral blood of healthy volunteers before and after sauna sessions (experiment I) and b) in the peripheral blood of patients with medical background of RTIs (experiment II).**

**Therefore, we hypothesize that Finnish Sauna sessions improve the immunological response against the pathogens of the respiratory tract.** We presume that our project have a very interesting social outcome, being such prevention is not only very important for the economic point of view, but also for the quality of life of the people.

### **Following hypothesis will be tested:**

#### In experiment I- healthy volunteers:

1. The effect of sauna sessions is positive for the innate immune response in healthy volunteers
2. The immune response in sauna experienced volunteers is higher than in naive ones

#### In experiment II- patients of RTIs

3. Sauna sessions help patients with relevant medical history to suffer less episodes of RTIs
4. Sauna sessions accelerate the recuperation of patients with RTIs

Understanding the relation between sauna sessions and the innate immune response may determinate a new paradigm of simple and inexpensive approaches for preventing infectious diseases. Furthermore, understanding how hyperthermia could have a positive role in increasing the immune response would allow people to use this very simple and traditional routine for reducing the risk of suffering from potentially lethal diseases.

## 2. Significance of the project

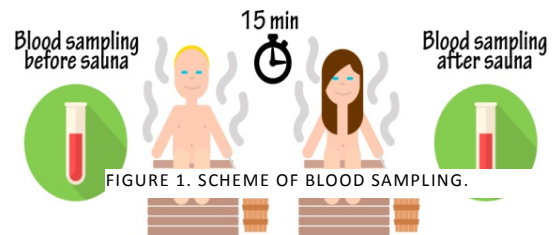
The respiratory truck infections (RTIs) are a group of diseases with different etiology. We can divide RTIs between microbiological and viral. Infections as pneumonia are caused by Streptococcus pneumonia, and are considered as a very problematic due to the high mortality (van Heijil et al. 2018; Guzek et al. 2015). The abusive use of antibiotics is creating an incontrollable and increasing resistant bacterium that cannot be treated so easily. For this reason is highly needed to find out preventing habits that can help patients to overcome this infections, improving their outcome. But we should not



forget that viral infections as the common flu, cause seasonal epidemics that can end in several complications in immunocompromised patients.

The innate immune system is the first weapon that our body has to fight against the potential pathogens that try to get in to it. The respiratory tract is one of the main ways where potential pathogens can enter and colonize the human body. This is why there is high presence of different leukocytes. In concrete, we can underline the function of two cell types: macrophages and neutrophils. The macrophages are cells specialized in phagocytose pathogens that are tissue-resident mainly, but when we find them in blood they are called monocytes. In contraposition, neutrophils are cells mainly present in blood that are patrolling the vessels in search of any potential harm, being the first ones recruited in case of infection. In the lung, there are an important number of resident macrophages, and neutrophils are constantly patrolling the bronchioles. **This two cell types will be study in our project. By this way, different functions will be analyze as the reactive oxygen species (ROS) production, nitric oxide (NO) production, phagocytic activity, formation of Extracellular Traps (ETs) and Heat-Shock protein expression.** All these parameters well establish defensive mechanisms in both cells.

Finnish Sauna can be considered as one of the most ancient traditions in the human being. The benefits of sauna have been widely studied and several epidemiological experiments have been done in respect. The results are very promising especially in cardiovascular field where it has been demonstrated that there is a link between sauna sessions and reduced risk of cardiovascular outcomes and improved arterial compliance (Laukkanen et al. 2018). In addition, very promising studies have been done in the field of immunology and inflammation where sauna sessions improved the level of white blood cell profile (Guzek et al. 2015), reduced the risk of pneumonia (Kunutsor et al. 2017), and the induction of the Heat Shock proteins (Torigoe at al. 2009). **Hence the effect of sauna sessions may be positive for improving the innate immune response against pathogens of the upper and lower respiratory truck, but yet today is unclear the molecular mechanisms and also, the vast majority of the studies have been done in men.** In addition, the studies use different patterns of exposition that makes the extrapolation of the results very difficult. An established and general protocol would be very interesting for potential patients and further studies, as well as their possible implementation in the medical prevention.



### 3. Work plan

#### Healthy volunteers and patients

We have established a protocol for Sauna session that consists in one immersion between 10 up to 15 minutes of duration. Experimental individuals will need to go to a sauna once per week and then, each three months blood samples will be taken and respiratory capacity will be measured. In this way, a blood sample for each patient/subject will be taken before sauna which will serve as internal control and a sample after sauna. Importantly, we will have also control volunteers that will not come up to sauna sessions regularly.

#### Experiment I (non randomized approach)

This experiment is designed to estimate the effect of hyperthermia in healthy volunteers. Healthy volunteers will be considered as individuals with less than six upper respiratory truck infections or/and one low respiratory infection in the last year. Potential volunteers will be asked to answer a questionnaire about these aspects before the experiment, and during each blood sample another questionnaire will be given with some monitoring questions as if they have had any episode of respiratory infection. Besides, it will be taken in consideration if these patients are regular users of sauna (experienced group) or not (inexperienced group). Experienced group will be formed for

subjects that during the last year went to sauna at least monthly, if not will be consider inexperienced. We will monitor the direct effect of sauna after one session (acute response) and before (long-term response). In addition, we assume that is very important to consider if the subjects had any experience with sauna, inasmuch as we want to take into consideration the adaptation to sauna sessions.

**We consider that is necessary to make a separation between females and males inasmuch as their different physiological responses to treatments.**

From control group (formed only by healthy volunteers without sauna treatment) one single blood sample will be obtained from them each three months and the same immunological responses will be analyzed.

From experimental individuals, two blood samples will be obtained from each patient in each sauna session, one before sauna that will be our internal control and the other one after sauna session. Moreover, we will measure the respiratory capacity (aspiration test) in the same pattern. The respiratory capacity will give us very valuable information about the fast affectivity of the session and relief sensation that is very important for the psychological perception of the patients.

**By obtaining these values, we will estimate if sauna sessions have any beneficial effect on innate immune response.** According to the literature, the higher expression of Heat-Shock proteins is a positive indicator, so that these proteins play an important role as “endogenous danger signals”, and when they are realized from damage cells promote the cross presentation of this danger signals to the dendritic cells (antigen-presenting cells; Torigeo et al. 2009) which may unchain in a optimal immunological response. **We hypothesize that sauna sessions will have a positive effect improving the response against inflammatory stimuli in the neutrophils and monocytes/macrophages.** As explained before, different immunological phenomena will be analyzed, and depending on how successful they are we will consider the role of these cells in the adaptation to sauna sessions.

The potential risk of this experimental part can be divided in different points;

1. Volunteers decide to decline in the middle of the experimental process. For this porpoise, we will provide free entrance to the sauna installation during the days the sessions that will take place. In the case of the control group, if they complete all the duration of the experiment will have free access to the sauna resort during two days per week.
2. Monocyte/macrophage and neutrophils samples from the patients do not show any improved response against different inflammatory stimuli. In this case, the analysis will be carry out in cells of the adaptive immune response.

### **Experiment II (non-randomized approach)**

Our ultimate goal is to establish if Finnish Sauna session during a week pattern will be useful for preventing and treating (as an adjuvant) RIs, for this porpoise subjects with RTIs will be recruited. As previously, the before and after blood sample as well as measurement of respiratory capacity will be taken. From this study will be excluded patients with additional chronic diseases as diabetes, arthrosclerosis or any life-threatening conditions. In addition, patients will be required to inform any RTIs episodes or any drug treatment. In case of upper respiratory truck infections with minor importance, patients will be allowed to conduct the experiments.

**We predict that the sauna sessions will be helpful as coadjutant to treat RIs and also, will help patients that usually suffer from these diseases to reduce the frequency of their episodes. In addition, we expect higher antimicrobial activity of their monocytes/macrophages and neutrophils from blood samples after sauna session but in addition an accumulative positive effect as much as the patients will be exposed to sauna during time.**

The potential risks of failure of this experiment are that:

1. Patients decide to decline, for this reason, we will carry out the same approach as before

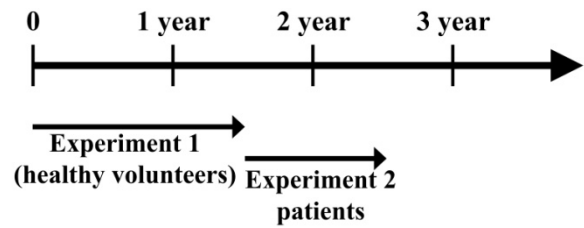


FIGURE 2. TIME FIELD, CONTROLLED AND CLINICAL TRIALS

2. Direct relation between the specific RIs and the outcome, for this porpoise, we will consider the etymology of the disease
3. Finnish sauna sessions do not reduce the probability of having fewer episodes of respiratory infections. Despite this, we think that the data and the approach would be useful for analyzing other adjuvant treatments for the respiratory diseases.

**4. Methods of research** (manner of conducting research, methods, techniques and tools, data reduction and treatment schemes, equipment and devices used in the research);

### Volunteers

For experimental group will be used at least 200 participants (100 women and 100 men, see Figure 3), but for control group only 100 subjects will be used (50 women and 50 men). The research will be performed first in healthy volunteers of both sexes, between 18 and 60 years old and will be classified also, depending on the experience of sauna. In this way, volunteers will be classified as none experienced with less than 12 sessions of sauna during the last year and experienced with 12 or more sessions of sauna during the last year. In brief a) gender classification and b) sauna experience.

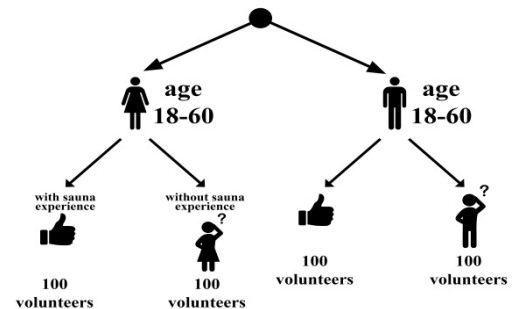


FIGURE 3. GENERAL SCHEME OF THE EXPERIMENT

### Patients

The second experimental approach will be done in patients that have medical history of RIs, following the same pattern as specified before for experimental patients (no control, in this case only experimental patients will be analyzed) been a final sample of 200 patients. In brief a classification between a) gender and b) sauna experience will be done. Subjects with a medical history of more than six episodes of upper respiratory truck infections and/or more than two lower respiratory truck infections in the last year will be consider as potential patients. For this porpoise they will be asked in the initial questionnaire about this particular data. The project activities will be conducted following the protocol ICH GCP (Pharmaceuticals for Human Use Good Clinical Practice 2015).

### Sauna protocol and blood sampling

Sauna sessions will be provided in a sauna that will accommodate up to 20 people at once by external agreement with a local business. All analyses with blood samples will be conducted in the Institute of Zoology and Biomedical Research of the Jagiellonian University, due to it has all the basic devices for carrying out the cell culture experiments. In brief, a basic cell culture laboratory with an incubator, bath, flow laminar hood, and centrifuge will be used. For analyzing absorbance and fluorescence a Tecan Plate Reader will be used and for analyzing images a confocal microscopy will be used (Gonzalez Gonzalez et al., 2018). Sauna's conditions will have dry air (humidity 10%±5) with temperature 80°C±5 and will be monitored constantly. Each sauna session will take for participants 10-15 minutes. The healthy volunteers will take sauna once per week during 1.5 year, but 10 seasons can be skipped. In case of more than 10 absences in the whole period, the subject will be removed from the study and will not have further access to the sauna free entrance.

### Blood sampling and manipulation

Peripheral blood sampling will be performed using hypodermic needles and Vacutainers with anticoagulants by an experienced nurse at the first day of the experiment and then accidentally around each three months for 1.5 year. Collected samples will be placed to storage containers with 2-6 °C to

maintain the optimal viability. Further monocytes and neutrophils will be extracted according modified protocols for monocytes (Menck et al. 2014) and neutrophils (Oh et al. 2008). In case of patient from control group, only one blood sample will be taken each three months.

### **Determination of spirometric measurements and immune response**

1. Spirometric measures will include the forced expiratory volume and forced vital capacity (Moore et al., 2012). We are planning to buy a portative compact Spirometer 6600 (Vitalograph, the USA) that allows us to measure lung capacity
2. Reactive oxygen species (ROS) (Hoer-Yue et al., 2016) will be tested using a commercial kit.
3. Nitric Oxide Production (NO) (Schmölz et al., 2017) will be tested using a commercial kit.
4. Phagocytic Activity (Oben et al., 1988) using Zymosan beads stained with a fluorophor.
5. Extracellular Traps Quantification (Farrera et al., 2013).
6. Heat-Shock 90 protein determination by immunocytochemistry (own laboratory protocol).

### **Statistical Analyses**

Statistical significance will be tested with two- way ANOVA using Bonferroni's multiple comparison test (GraphPad Software version 6, San Diego, CA). Differences were considered statistically significant at \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .

### **5. Literature**

1. Farrera C., Fadeel B. 2013 “*Macrophage clearance of neutrophil extracellular traps is a silent process*” Journal of Immunology 191
2. Gonzalez Gonzalez M., Cichon I., Scislowska-Czarnecka A., Kolaczowska E. 2018 “*Challenges in 3D culturing of neutrophils: Assessment of cell viability*” The Journal of Immunological Methods
3. Guzek A., Rybicki Z., Korzeniewski K., Mackiewicz K., Saks E., Chciałowski A., Zwolińska E. 2015 “*Etiological factors causing lower respiratory tract infections isolated from hospitalized patients*” Advances in Experimental Medicine and Biology 835
4. Hor-Yue Tan, Ning Wang, Sha Li, Ming Hong, Xuanbin Wang, Yibin Feng “*The Reactive Oxygen Species in Macrophage Polarization: Reflecting Its Dual Role in Progression and Treatment of Human Diseases*” Oxidative Medicine and Cellular Longevity 2016
5. Kunutsor S. K., Laukkanen T., Laukkanen J. A. 2017. “*Sauna bathing reduces the risk of respiratory diseases: a long-term prospective cohort study*” European Journal of Epidemiology 32 (12)
6. Laukkanen J. A, Kunutsor S. K, Niemelä M., Kervinen K., Thuesen L., Mäkikallio T. H. 2017 “*All-cause mortality and major cardiovascular outcomes comparing percutaneous coronary angioplasty versus coronary artery bypass grafting in the treatment of unprotected left main stenosis: a meta-analysis of short-term and long-term randomised trials*” British Medical Journal 4 (2)
7. Menck K., Behme D., Pantke M., Reiling N., Binder C., Pukrop T., Klemm F. 2014 “*Isolation of Human Monocytes by Double Gradient Centrifugation and Their Differentiation to Macrophages in Teflon-coated Cell Culture Bags*”. Journal of Visualized Experiments 91
8. Moore V.C. 2012 “*Spirometry: step by step*” Breathe 8
9. Oben J. A., Foreman J. C. 1988 “*A simple quantitative fluorimetric assay of in vitro phagocytosis in human neutrophils*” Journal of Immunological Methods 112
10. Oh H., Siano B., Diamond S. 2008 “*Neutrophil Isolation Protocol*” Journal of Visualized Experiments 17
11. Lisa Schmölz, Maria Wallert, Stefan Lorkowski. 2017 “*Optimized incubation regime for nitric oxide measurements in murine macrophages using the Griess assay*”, The Journal of Immunology Methods 449
12. Torigoe T., Tamura Y., Sato N. 2009 “*Heat shock proteins and immunity: application of hyperthermia for immunomodulation*” International Journal of Hyperthermia 25
13. van Heijl I., Schweitzer V. A., Zhang L., van der Linden P. D., van Werkhoven C. H., Postma D. F. 2018 “*Inappropriate Use of Antimicrobials for Lower Respiratory Tract Infections in Elderly Patients: Patient- and Community-Related Implications and Possible Interventions*” Drugs Aging

## 6. Budget Table

1. Table with budget of the project.

Item	Funds for each budget year (PLN)				Total
	2019	2020	2021	2022	
<b>1. Direct costs, including:</b>	47600	72800	72800	15500	208700
(a) Salaries and benefits	9600	12800	12800	3200	38400
(b) Equipment	11000	0	0	0	11000
(c) Other direct costs	27000	60000	60000	12300	134700
<b>2. Indirect costs</b>	14640	29120	29120	6200	79080
<b>Total costs</b>	62240	101920	101920	21700	287780

Direct costs include materials (chemicals, food), costs of conferences, daily allowance (delegacje) etc.

Equipment includes computers, microscopes, etc.

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

### Investigator / Staff Costs

a) Number of Investigators: 2

b) Investigators: nature of their contribution in the project and a justification of investigator costs

No	Full name / Nature of contribution in the project / Type and character of position	Employing entity	Project-related remuneration period (months)	Contracted working time as a % of full time equivalent (FTE)	Rate of salary pool/banding	Total salary cost on grant (PLN)	Type of employment relationship
1	Mgr Evgenii Baiakhmetov	The Jagiellonian University, Krakow, the Faculty of Biology and Earth Sciences	36	48	500 / month	18000,00	Contract of specified task
	Scope of work within individual project tasks:	Planning, preparing and conducting experiments (measurements of ROS, NO); statistical analyses of obtained results; compilation of results and conclusions; presentations at scientific conferences; articles writing.					

2	Mgr Miriam González González1	The Jagiellonia n University, Krakow, the Faculty of Biology and Earth Sciences	36	48	500 / month	18000,00	Contract of specified task
	Scope of work within individual project tasks:	Planning, preparing and conducting experiments (measurements of phagocytic activity; quantification of neutrophil extracellular traps; determination of intracellular heat shock protein); statistical analyses of obtained results; compilation of results and conclusions; presentations at scientific conferences; articles writing.					
3	A nurse	The Hospital on Siemiradzkiego	1	4	66.67 / month	2400, 00	Contract of specified task
	Scope of work within individual project tasks:	Peripheral blood sampling, and spirometric measures once per each 3 months in the sauna during the project (in total 12 working days).					
						Total	38400,00

#### List of equipment to be purchased and/or built

No	Equipment name	Purchasing entity	Year of purchase	Amount	Unit cost (PLN)	Contribution of the NCN (PLN)
1	Spirometer 6600	The Jagiellonian University, Krakow, the Faculty of Biology and Earth Sciences	2019	1	11000,00	11000,00
	<b>Justification of purchase</b>	Spirometer 6600 (Vitalograph, the USA) will allow us to provide spirometric measurements in the sauna. The purchase of this item will do our study more flexible (patients will not need to go for measurements to a special medical office).				
					Total	11000,00

#### Other direct costs

Materials (expendable goods for direct use in the project) Cost – **104300** PLN.

1. BD Vacutainers 367835 – 4700 PLN (in total 4300 Vacutainers; It will be enough to get blood samples from 4200 patients).

2. BD Vacutainer Eclipse Blood Collection Needles 368650 – 11500 PLN (in total 4300 Needles; It will be enough to get blood samples from 4200 patients).

3. Gloves Nitrilex PF – 1500 PLN (8600 gloves for working with patients; 4300 gloves for lab working).
4. Premier Hard Surface Wipes – 500 PLN (4800 wipes for rapid disinfection).
5. Griess Reagent System G2930 – 4000 PLN (for Nitric Oxide Production).
6. Neutrophil/Monocyte Respiratory Burst Assay Kit 601130 – 3000 PLN (for analysis of reactive oxygen species).
7. Zymosan A (*S. cerevisiae*) BioParticles™, Alexa Fluor™ 488 conjugate Z23373 – 3700 PLN (for analyzing phagocytic activity).
8. SYTOX™ Green Nucleic Acid Stain S7020 – 3000 PLN (for extracellular traps quantification).
9. Paraformaldehyde P6148-1KG – 600 PLN (for extracellular traps quantification).
10. Phosphate-Buffered Saline (10X) pH 7.4 AM9625 – 5300 PLN (for extracellular traps quantification).
11. Anti-HSP90 antibody MBS245471 – 7000 PLN (for Heat-Shock protein 90 determination).
12. Penicillin-Streptomycin (10,000 U/mL) 15140122 – 4200 PLN (for Heat-Shock protein 90 determination).
13. RPMI-1640 Medium R7509-1L – 300 PLN (for Heat-Shock protein 90 determination).
14. Fetal Bovine Serum F0679-500ML – 55000 PLN (for Heat-Shock protein 90 determination).

**Outsourced services** (services ordered from entities external to the Host Institution) – **10400** PLN.

1. Renting a sauna – 10400 PLN (130 days, each day – 80 PLN).

**Conferences and business trips** (by members of the research team) – **5000** PLN.

1. Participation in national and international scientific conferences (including nutrition, accommodation, printing of posters, etc.) – 5000 PLN.

**Other costs** (such as do not fit into other categories, including the cost of disseminating the results) – **15000** PLN.

1. Materials/services for manuscript preparation and publication; materials for data storage – 1000 PLN.
2. Publications costs – 14000 PLN.

## REVIEWS

---

### **Prof. Wiesław Babik**

#### **Quality of Science**

The project is interesting, well described and planned. The hypotheses are clearly stated and testable, however I am not sure whether as planned, the project would really be able to address them – most of the assays test various aspects of immune cells' activity, but do not provide a direct link to the performance of the subject's immune system in fighting pathogens. For example, is it possible that increased activity of these cells could actually cause health problems? This should be better explained in the proposal. Additionally, it looks like hypotheses are somewhat mixed with predictions. The most serious criticism however concerns statistical methods. The experiments are well described and it is clear from the description that their design is complex, including multiple explanatory variables, repeated measures of the same subjects and multiple response variables. Analysis would thus require complex mixed modeling, while the description of statistical methods takes two lines of text and mentions two-way ANOVA and Bonferroni correction...

#### **Significance: novelty and impact on the field**

There are certainly novel aspects in the project and it should meet with interest in its field. The project has both basic and applied aspects. If the results are to be communicated to lay public, it is important to emphasize, in addition to statistical significance, also the effect size, which is important to assess the practical potential of the approach.

#### **Budget**

Budget is prepared carefully and costs are presented in detail. It would be helpful to include in the budget description the number of samples on which calculations are based.

#### **Feasibility**

The project is feasible, the investigators have most required skills as well as access to appropriate facilities. Risk analysis has been performed and the solutions to potential problems have been proposed. When considering risks for Experiment I, the authors say that if they see no improvement in innate immune response after the sauna treatment, they will analyse adaptive immunity. What is the rationale for that? Isn't no improvement an important result in itself? I see two additional points that may affect feasibility: i) it is not clear how the subjects will be recruited, ii) the investigators do not appear to have enough experience in experimental design and statistical analysis (see above).

#### **Strengths**

- Clearly defined aims and testable hypotheses
- Well written project containing novel aspects
- Clear links between tested hypotheses and planned experiments
- Good description of the work plan and methodology

#### **Weaknesses**

- The relevance of in vitro test to assessing the performance of the innate immune system should be better justified
- Poorly described statistical analysis, a major weakness, as the planned experiments require quite complex modeling



- There is no information about dissemination of results
- English could be improved, it is not bad, but grammatical and spelling mistakes (truck->tract, porpoise->purpose, etymology->etiology) are quite common

## **Dr hab. Piotr Skórka**

First and foremost I must admit that I am not a specialist in human health (my life style is also far from healthy) nor immunology and I am not familiar with molecular techniques, thus some of my criticisms stem from the fact I work in different research area and my some comments may be naive. However, in case of submitting the proposal to National Science Centre it will be evaluated by experts in the field of human health or medicine.

Despite I liked the idea of the project I think there are several weak points that should be carefully considered and addressed.

My major criticism is following. There is no description of mechanism how high temperatures could positively affect activity and number of leucocytes. Is not that possible that high temperature negatively affect reproduction and spread of pathogens directly first rather than immunological system? For example, one may imagine that with increasing temperatures pathogens are becoming impaired and the immune system (leucocytes) is more efficient in removing them without increasing overall number of leucocytes or their activity. In that case you may find no differences between experimental groups and thus (falsely) conclude that “Finnish Sauna” has no impact. Please, note that even if authors find increased activity of the leucocytes in experiment 1, they can not exclude above explanation. Thus, what is needed in this project is a procedure that estimates the activity and reproduction of pathogens when exposed to “Sauna” conditions. Of course, this may complicate the entire project far beyond formal and financial framework permissible in the Preludium scheme.

I have also several other critical points that should be considered during preparation of a new version of the project. I listed them below:

### Point 1.

Description of the project (especially method section) is vague. I am not quite sure what is the purpose of the experiment 2. What new findings will be achieved by performing it and why it is necessary.

### Point 2.

Different indicators of immune system functioning are to be used in the project (e.g reactive oxygen species (ROS) production, nitric oxide (NO) production, formation of Extracellular Traps (ETs) and Heat-Shock protein expression) but it should be better explained which leucocytes are associated with these consecutive indicators.

### Point 3.

The experiment 1 is designed to estimate the effect of hyperthermia in healthy volunteers. Healthy individuals will be considered as these with less than six upper respiratory tract infections or/and one low respiratory infection in the last year. Is this an arbitrary threshold or is there a rationale for this specific criterion?(this question applies also to experiment 2).

### Point 4.

Age may be very tricky variable in this study. It may strongly (non-linearly) correlate with immune system activity. Is not that possible the infection frequency increases with age? How are authors going to include this variable in their analyses?

Point 5.

Statistical analysis is described poorly. Personally, I always pay special attention on this part (as many NCN panel experts do) because it undoubtedly indicates if authors have deep understanding what they want to achieve. Following information should given: what are dependent variables, what are explanatory variables, what is structure of the statistical model, how authors will cope with confounding variables (e.g. age of studied objects). It is also desirable to write which results will support stated hypotheses. Last but not least, I insist to use freely available R in analyses rather than any commercial software.

Point 6

There are many linguistic mistakes (some of them are fairly funny, e.g. using word “affection” instead “infection”).

Point 7.

Cost of the project seems to be reasonable. However, I am unable to assess the justification of “other costs”. The publication cost is high (14 000 PLN) and it would be desirable to directly write journal names which authors plan to submit their manuscripts.

## **Mgr Stanisław Bury**

The project has a nice structure and is easy to follow. I have only few minor concerns. The first paragraph of the Objective ends with justification why you will focus on specific types of cells. This is a justification of the method, so not necessarily needed here. I would suggest to go immediately to hyperthermia and sauna, since this is the major point of your project. Similarly in the second paragraph. Here you end with information what you will not, but not what hypothesis you will test. First say what are your hypotheses and then how you will approach them.

Similar problem concerns the Significance section. Your project is about the possible impact of sauna on immunity, but sauna and hyperthermia comes only in third paragraph. I suggest you to be more to the point and go to you major issue sooner. You could do it easily, since in the first paragraph you clearly stated about the need of developing easy way to prevent circulatory infections. This statement can be easily linked to sauna and hyperthermia. Therefore I would reorder the text and put the third paragraph as the second one. After you state in this first paragraph the mechanism underlying the positive effect of sauna is not known. Now in the end you will have a paragraph on immune cells as a proposed mechanism which makes the whole section more fluent and easy to follow.

## **Michał Belcik**

The project has been written in due diligence, and it meets the criteria for a scientific proposal. It clearly meets the criteria for basic research. It is generally written in a good English, but there are some grammatical and spelling errors, as well as misuse of words.

Abstract is written in good way, and it generally makes for a good overview of a whole research proposal. Research generally describes what scientific questions authors are planning to answer with their research, although it makes it in somehow chaotic way. However, it is important to note that research hypotheses are well formulated and clearly stated. Figures included help to understand the authors statements, but sometimes they are untidy and should be put in text in a different way (Fig.1.,

line 34, Fig.2., line 12). Knowledge gap is well presented, leaving a little room for doubt. Work plan generally manages to present an outline of the study, but makes it in a rather messy way, similar to the research objective. Methods of research seem to be rather well described, although a feeling of general chaos is prevalent, in similarity to the previous parts. Budget seems to be very well described and justified.

That being said, I found some flaws in this proposal. The biggest and most obvious one is in my opinion a general organisation of research objectives, methods and so on, which makes for a rather messy research proposal. A better general overview of a work plan would be in order. Another issue that I had problems with is that I felt not informed enough by the authors about the link between macrophages and neutrophils and Finnish sauna. My next issue is connected to the patient selection method (regarding the patients with medical history). It seems to me that this method is not well rooted in contemporary research (or authors failed to convince me that it is), so what would help is a better overview of methods used in other studies from that field. My last remark is about that I found it rather unnecessary to cite about the statistical significance on that stage, especially given the fact that those levels are the ones that are commonly used.

Generally speaking, I find this proposal interesting, although a rather disorganized. The whole project is worth financing in my opinion, and authors are to be commended for undertaking such practical yet important topic. This could possibly be an important step in preventive medicine, as well as reducing the healthcare burden on state budget.

## **Amadeusz Bryła**

Project proposal without doubt meets basics requirement for scientific proposal, is written in easy to follow and clear way with only few spelling mistakes. However criteria outlined in the call for proposals require for up to 3 years projects this rule needs to be revisited in this proposal. Basic research proposed in this project could have future consequences on defining new way to prevent infections of the respiratory tract. This is a main point of this project that's why I would suggest start abstract with much stronger sentence, which immediately outlines how serious problem are infections of the respiratory tract. Research objective are written in clear way with strong arguments but authors do not include what is connection between sauna and innate immune, while this is main hypothesis tested in this study. Authors made a point about immunocompromised patients but it is not clear why. Do this sauna protocol could be used to help these patients? Work plan has very clear structure and is clearly related to main research question and hypothesis. However it is not clear why in first experiment authors want to estimate effect of hypothermia in healthy volunteers, and they consider an individuals with less than six upper respiratory track infections – while they should include in this part only people without any medical event in last year- as proper healthy group. Role of control group is not clear, these individuals will not went through sauna sessions but will be provided with free access to sauna after experiment? In description you mentioned only about experienced and inexperienced groups nothing about controls.

Strong point of this research is fact of possible contribution to improving human health, and proposal in convincing way shows feasibility of this research. Proposal consist two experiments that fully cover answer to main research question. However the weakest point is planning is budget that is planed for 4 years what is against rules for these proposals

## Marta Grosiak

I found the proposed project really innovative and interesting and also scientific significant. Planned experiments help to investigate very emerging problems directly connected with human health. The summary is divided into three well defined parts, the scientific and economic significance of proposed project is well explained. A scientific problem and proposed solution is written clear in well-organized way, but I think that formulation of more detailed predictions of your experiments for all of presented hypotheses will be helpful in this part. The part about significance of your project provide a lot of convinced outcomes of using sauna for respiratory and other type of diseases. I suggest to put more references in some places, and discuss a little other, negative physiological implications from induced heat shock proteins. I have objectives for two of presenting pictures – first is a little misleading, the second one is unnecessary. In work plan you explain well all proposed protocols and say exactly what will be proved applying them into your project. In the part of description the potential risk, you should add, that only after positive results of first part you will performed the second part of project. Methodology is planned correctly, you described everything very detailed but you do not explain where and how you will searching for volunteers. I am impressed how precisely you planned every detail for each part of needed laboratory staff. Generally, very professional job!

The strongest parts: abstract, significance of project, justification of costs

The weakest parts: I think that any part do not need such much improvement, so I don't put here any of it ☺

### Abstract

The summary is divided into three well defined parts, what makes it easier to follow. The significance and size of predicted outcome of project is well explained. I don't have any objections to this part.

### Research objective:

A scientific problem and proposed solution is written clear in well-organized way. I think that these two presented hypotheses should be more detailed, e.g.

45 – “accelerate the recuperation” – Is it means that they are became healthier in shorter time?

I think that you should formulated more detailed predictions of your experiments for all of these hypotheses.

### Significance of the project

Scientific background is written very detailed and the story about done research is presented in logical order and by understandable way. You have a good idea to show your main experiment in the picture, however, it provides a little misleading because it suggests that you will take blood samples before sauna only from men and blood sampling after sauna from woman.

2-3 – “Infections as pneumonia are caused by Streptococcus pneumonia, and are considered as a very problematic due to the high mortality” – Is it worldwide problem or only in these countries with higher pollution problem? It is important information here.

3-5 – “The abusive use of antibiotics is creating an incontrollable and increasing resistant bacterium that cannot be treated so easily.” – Do you have any references for this point?

23-24 – the same comment as above.

You provide a lot of convincing references and facts about positive impact of sauna for patient with diseases but are any other, negative physiological implications from induced heat shock proteins? Is sauna recommended in every age and can have any risk for health?

35 – “but yet today is unclear the molecular mechanisms” but you will not work on the molecular mechanisms?

#### Work plan

In this part you have a good explanation of proposed protocols and exactly what will be proved applying them into your project. I have one objective about this graph – I think that it isn't necessary because it means only that you divided 2 experiment into 2 years. I think that you can use a picture to show e.g. time of taking blood samples and performing aspiration test on each groups. It will have more benefits to clarify everything. In the part of potential risk description you should add that only after positive results you will performed the second part of project. What about case where sauna have negative effect on patients with respiratory diseases?

#### Materials and methods

Methodology is planned correctly, you described everything very detailed. You do not explain where and how you will searching for these volunteers? You do not say what variables you will take for your statistical analyses.

#### Budget and justification

I am impressed how precisely you planned every detail for each part of laboratory needed staff! But I am not sure that you need your own spirometer, there is no possibility of using this equipment in Kraków?

I really like your proposal, good job!

## Jayasravanthi Mokkapati

### EVALUATION OF PROPOSAL OF RESEARCH PROJECT –

- Has the proposal been written with all due diligence?  
-Yes
- Does the project meet the criteria of a scientific proposal?  
-Yes
- Does the project meet the criteria of basic research?  
-No
- Does the project meet other eligibility criteria outlined in the call for proposals?  
-Yes

### A. EVALUATION OF THE PROJECT (WEIGHTING 75%)

#### A.1. EVALUATION OF PLANNED RESEARCH OR PROJECT TASKS (WEIGHTING 60%)

3.Good. Upon its completion the project results are likely to be published in specialist academic press/ journals.

**Justification:** Good. Although this project proposal clearly benefits the society to some extent, the methodological approach need to be revised against the following queries

- What does it mean by the study of microbial activity of neutrophils or macrophages in the Research objective section (lines 18-21)?

- Objective of the study need to be justified with preliminary data, whether the treatment of Finnish sauna could be a prevention of RI.
- The proposed two hypothesis contradict the objective of the research; first hypothesis tests the treatment as prevention whereas second hypothesis as cure!

**A.2. ASSESSMENT OF THE PROJECT'S INNOVATIVE POTENTIAL AND ITS IMPACT FOR THE ADVANCEMENT OF THE SCIENTIFIC FIELD/DISCIPLINE (WEIGHTING 15%)**

- Innovative nature of the proposed research:
  1. The project has innovative elements.
- Impact of the research project on the advancement of the scientific field/discipline:
  0. The project will have no impact on the advancement of the scientific field/ discipline or the project has been submitted to a wrong review panel.

**Justification:**

- In terms of advancement of scientific research, the proposed project doesn't involve any new technological or scientific developments or not modifying the treatment methods to further improve already existing ones.

**B. EVALUATION OF THE QUALITY OF TEAM MEMBERS (WEIGHTING 20%)**

Not given, hence not reviewed.

**C. ASSESSMENT OF PROJECT FEASIBILITY (WEIGHTING 5%)**

- **Assessment of the feasibility of the proposed project, including the principal investigator's qualifications, the structure of the research team, research facilities etc.:**
  - ✓ 2 Good.

**Justification:**

- Proposed project is clearly feasible to conduct the experiment. However, patient/subject withdrawal agreement have to framed clearly.
  - Regarding test methodology, including sauna sessions at different temperature regimes could be beneficial.
- **Are the costs to be incurred well justified with regards to the subject and scope of the research?**  
-yes
  - **Does the proposal meet the criteria allowing for its re-submission in a subsequent edition of the PRELUDIUM and OPUS calls?**  
- yes

**JUSTIFICATION FOR EVALUATION**

**Strengths of the proposal:**

- The proposed research clearly justified the significance of the project.
- Immediate beneficial effects of the research to society give this research much attention.
- Strong justification to feasibility and budget management of this research clearly shows the passion of the investigators in conducting this work.

**Weaknesses of the proposal:**

- Proposed test methods are not sufficient enough to make a conclusion on the treatment effects.

- There are many specific biomarkers identified for particular respiratory diseases, or in general, CRP or PCT can be used to specifically address lower respiratory infections than cell counts.
- Many pharmaceutical or nutraceutical drugs and advanced treatment technologies were being developed against RIs since the last two decades. This can be justified by providing their pros and cons in this proposal.
- The approximate time period between blood collection and biological tests need to mentioned. Perhaps, it shouldn't be no longer than two weeks since collection due to natural cell death. However, strong literature support is necessary in these aspects.

## FINAL VERSION

---

Authors: Evgenii Baiakhmetov, Miriam Gonzalez Gonzalez

**Title: Does Finnish Sauna improve the innate immune response against pathogens of the respiratory system?**

Abstract

#### 4. Research project objectives/Research hypothesis

Infections of the respiratory tract are very common and affect a lot of people from a range of ages throughout the year. In some cases, transitory diseases are especially the ones related with the upper respiratory system, but in the case of infections of the lower respiratory system they are correlated with a high mortality rate. The innate immune system is the first one to act in case of a potential infection and there is high presence of different cells from the innate immune system in the respiratory tracks, underlying the function of the macrophages and neutrophils. Recent studies have shown that there is a potential therapeutic role of Finnish Sauna as an inductor of short term hyperthermia, increasing the immune response. However, a lack of knowledge about molecular mechanisms limits its potential. We hypothesize that Finnish Sauna sessions improve the immunological response against pathogens of the respiratory tract. There is a high necessity of developing prevention techniques for common diseases as respiratory tract infections, not only due to economical implications for health care systems and governments, but also for improving the quality of life.

#### 5. Research project methodology

The study will be firstly conducted in healthy volunteers and secondly, in patients with a medical background of respiratory tract infections. Groups of volunteers of both sexes will be divided depending on whether they are regular users of Finnish Sauna or not. Blood sample tests as well as the respiratory capacity will be taken before and after sauna sessions,. Neutrophils and monocytes /macrophages will be obtained from the samples and basic antimicrobial functions will be studied.

#### 6. Expected impact of the research project on the development of science, civilization and society

Issues related to the development of prevention against respiratory infections appeal interest not only for the economical point of view, but also for society as a whole because it is directly related to human health. Our results will provide a very easy-to-use routine for preventing the infections of the respiratory tract, impacting directly on the quality of life of the people.



## 1. Research objective

**In our grant proposal, we hypothesize that Finnish Sauna sessions improve the immunological response against the pathogens of the respiratory tract.** We presume that our project have a very interesting social outcome, being such prevention is not only very important for the economic point of view, but also for the quality of life of the people. The scientific problem aimed to be solved is that Finnish Sauna baths boost the immune system of humans against the respiratory tract infections (RTIs), and if so, this knowledge could be applied into preventing strategies for respiratory infections in the general public.

Hyperthermal conditions can be defined as increasing of the basal temperature of an organism, in some cases caused by an infection (e.g. fever) and in other because of environmental conditions (e.g. sauna). It has been demonstrated that a short hyperthermal phase can be beneficial for humans, since it increases the expression of Heat-Shock Proteins (Torigoe at al. 2009). A traditional way of increasing body temperature is the traditional “Finnish Sauna” that has a special room with an average temperature of 80-100°C and around 10-20% humidity. Despite the body temperature do not raise more than one degree during sauna bath, it trigger interesting physiological responses.

Respiratory Tract Infections are one of the most common infections in the human body. A general division could be done depending on the organs that comprise the upper respiratory tract (mouth, nose, throat, larynx, and trachea) and the lower (bronchial tubes and lungs). Some of these infections are seasonal, and the others affect more due to environmental conditions as the air pollution (e.g. smog). Lower respiratory tract infections (LRTIs) are the sixth most important cause of mortality (2.7% cases) and are responsible for 9.9 thousand deaths annually in Poland (World Health Organization 2012). The RTIs differ with regard to the etiological agent, but are mainly from bacterial or virial origin.

### **Following hypotheses will be tested:**

#### In experiment I- healthy volunteers:

5. The effect of sauna sessions is positive for the innate immune response in healthy volunteers
6. The immune response in sauna experienced volunteers is higher than in naive ones

#### In experiment II- RTIs patients

7. Sauna sessions help patients with relevant medical history to suffer less episodes of RTIs
8. Sauna sessions accelerate the recuperation of patients with RTIs

Understanding the relation between sauna sessions and the innate immune response may determinate a new paradigm of simple and inexpensive approaches for preventing infectious diseases. Furthermore, understanding how hyperthermia could have a positive role in increasing the immune response would allow people to use this very simple and traditional routine for reducing the risk of suffering from potentially lethal diseases.

## 2. Significance of the project

The respiratory tract infections (RTIs) are a group of diseases with different etiology. We can divide RTIs between microbiological and viral. Infections such as pneumonia are caused by *Streptococcus pneumoniae*, and are considered as a very problematic due to the high mortality (van Heijil et al. 2018; Guzek et al. 2015). The abusive use of antibiotics is creating an incontrollable and increasing resistant bacterium that cannot be treated so easily. For this reason it is highly needed to find out preventing habits that can help patients to overcome these infections, improving their outcome. But we should not forget that viral infections such as the common flu, cause seasonal epidemics that can end in several complications in immunocompromised patients.

In the proposed research project **we will analyze the innate immunological function a) in the peripheral blood of healthy volunteers before and after sauna sessions (experiment I) and b) in the peripheral blood of patients with medical background of RTIs (experiment II).** The innate

immune system is the first weapon that our body has to fight against the potential pathogens that try to get in to it. The respiratory tract is one of the main ways where potential pathogens can enter and colonize the human body. This is why there is high presence of different leukocytes. In concrete, we can underline the function of two cell types: monocytes/macrophages and neutrophils. The monocytes/macrophages are cells specialized in phagocytizing pathogens. When they are circulating into the blood are denominated monocytes and when they are tissue-resident are macrophages. On the other hand, neutrophils are cells mainly present in blood that are patrolling the vessels in search of any potential harm, being the first ones recruited in case of infection. In the lung, there are an important number of resident macrophages, and neutrophils are constantly patrolling the bronchioles. **This two cell types will be study in our project. By this way, different functions will be analyze as the reactive oxygen species (ROS) production, nitric oxide (NO) production, phagocytic activity, formation of Extracellular Traps (ETs) and Heat-Shock protein expression.** All these parameters are well establish defensive mechanisms in both cells. The phagocytic activity, ROS and NO production are boosted when these leukocytes find potential pathogens and can be easily measured. The formation of Extracellular Traps is a very new discovered mechanism, both neutrophils and monocytes/macrophages under some inflammatory stimuli can eject their DNA decorated with proteases for neutralizing and in some cases killing bacteria. Finally, Heat-Shock proteins are produced by the cells in stressful conditions and act as chaperones, special proteins that ensure the proper assembly of other proteins. The *in vitro* approach will be done using bacterium and viruses that cause normally infections in the respiratory tract, *Haemophilus influenza*, *Streptococcus pneumoniae* and rhinovirus.

Finnish Sauna can be considered as one of the most ancient traditions in the human being. The benefits of sauna have been widely studied and several epidemiological experiments have been done in respect. The results are very promising especially in cardiovascular field where it has been demonstrated that there is a link between sauna sessions and reduced risk of cardiovascular outcomes and improved arterial compliance (Laukkanen et al. 2018). In addition, very promising studies have been done in the field of immunology and inflammation where sauna sessions improved the level of white blood cell profile (Guzek et al. 2015), reduced the risk of pneumonia (Kunutsor et al. 2017), and the induction of the Heat Shock proteins (Torigoe at al. 2009). **Hence the effect of sauna sessions may be positive for boosting the innate immune response against pathogens of the upper and lower respiratory tract, but yet today is unclear the molecular mechanisms and also, the vast majority of the studies have been done in men no in women.** In addition, the studies use different patterns of exposition that makes the extrapolation of the results very difficult. An established and general protocol would be very interesting for potential patients and further studies, as well as their possible implementation in the medical prevention.

### 3. Work plan

#### Healthy volunteers and patients

We have established a protocol for Sauna session that consists in one immersion between 10 to 15 minutes. Experimental individuals will need to go to a sauna once per week and then, each three months blood samples will be taken and respiratory capacity will be measured. In this way, a blood sample for each patient/subject will be taken before sauna (approximately 30 min before), which will serve as internal control, and a sample after sauna (approximately 15 min after). Importantly, we will have also control volunteers that will not come up to sauna sessions regularly. The number of monocytes/macrophages and neutrophils will be estimated always before carrying out the experiments, so being adjusted for doing always the experiments with a concrete cell concentration per  $\text{mm}^2$ . It would be necessary to stress that we are mainly interested in the capacity of this two leukocytes in carrying out antimicrobial strategies against pathogens that attack the respiratory tract.

### **Experiment I (non-randomized approach)**

This experiment is designed to estimate the effect of hyperthermia (sauna sessions) in healthy volunteers in the antimicrobial response of the monocytes/macrophage and neutrophils. Healthy volunteers will be considered as individuals with less than six upper respiratory tract infections or/and one low respiratory infection in the last year. Potential volunteers will be asked to answer a questionnaire about these aspects before the experiment, and during each blood sample another questionnaire will be given with some monitoring questions as if they have had any episode of respiratory infection. Besides, it will be taken in consideration if these patients are regular users of sauna (experienced group) or not (inexperienced group). Experienced group will be formed for subjects that during the last year went to sauna at least monthly, if not will be consider inexperienced. We will monitor the direct effect of sauna after one session (acute response) and before (long-term response). In addition, we assume that is very important to consider if the subjects had any experience with sauna, inasmuch as we want to take into consideration the adaptation to sauna sessions.

**We consider that is necessary to make a separation between females and males inasmuch as their different physiological responses to treatments.**

From **control group** (formed only by healthy volunteers without sauna treatment) one single blood sample will be obtained from them each three months and one test about respiration capacity will be done.

From **experimental individuals**, two blood samples will be obtained from each patient in each sauna session, one up to 30 minutes before sauna that will be our internal control and the other up to 15 minutes after sauna session. Moreover, we will measure the respiratory capacity (aspiration test) in the same pattern. The respiratory capacity will give us very valuable information about the fast affectivity of the session and relief sensation that is very important for the psychological perception of the patients.

**By obtaining these values, we will estimate *in vitro* if sauna sessions have any beneficial effect on innate immune response against some concrete pathogens.** According to the literature, the higher expression of Heat-Shock proteins is a positive indicator, so that these proteins play an important role as “endogenous danger signals”, and when they are realized from damage cells promote the cross presentation of this danger signals to the dendritic cells (antigen-presenting cells; Torigeo et al. 2009) which may unchain in a optimal immunological response. **We hypothesize that sauna sessions have a positive effect improving the response against inflammatory stimuli in the neutrophils and monocytes/macrophages *in vitro*.** As explained before, different immunological phenomena will be analyzed, and depending on how successful they are we will consider the role of these cells in the adaptation to sauna sessions.

The potential risk of this experimental part can be divided in different points;

3. Volunteers decide to decline in the middle of the experimental process. For this purpose, we will provide free entrance to the sauna installation during the days the sessions that will take place. In the case of the control group, if they complete all the duration of the experiment will have free access to the sauna resort during two days per week.
4. Monocyte/macrophage and neutrophils samples from the patients do not show any improved response against different inductors. In this case, it would be very valuable the information obtained because despite the literature supports our approach other cells of the innate immune system would be involved. This could open a new actuation window and other cells like dendritic cells or eosinophils /basophils could be analyzed.

### **Experiment II (non-randomized approach)**

Our ultimate goal is to establish if Finnish Sauna sessions during a week pattern will be useful for preventing and treating if so (as an adjuvant) RTIs, for this purpose volunteers with RTIs will be recruited. As previously explained, the before and after blood sample as well as measurement of respiratory capacity will be taken. From this study will be excluded patients with additional chronic

diseases as diabetes, arthrosclerosis or any life-threatening conditions. In addition, patients will be required to inform any RTIs episodes or any drug treatment. In case of upper respiratory tract infections with minor importance, patients will be allowed to conduct the experiments.

**We predict that the sauna sessions will be helpful to prevent or treat as adjunct to RTIs and also, will help patients that usually suffer from these diseases to reduce the frequency of their episodes or recover sooner.** In addition, we expect higher antimicrobial activity of their monocytes/macrophages and neutrophils from blood sample after sauna session but in addition an accumulative positive effect as much as the patients will be exposed to sauna during time.

The potential risks of failure of this experiment are that:

4. Patients decide to decline, for this reason, we will carry out the same approach as before.
5. Direct relation between the specific RIs and the outcome, for this purpose, we will consider the etiology of the disease in the questionnaires.
6. Finnish sauna sessions do not reduce the probability of having fewer episodes of respiratory infections. Despite this, we think that the data and the approach would be useful for analyzing other preventing/adjutant treatments for the respiratory diseases.

**4. Methods of research** (manner of conducting research, methods, techniques and tools, data reduction and treatment schemes, equipment and devices used in the research)

#### Volunteers

All the volunteers will be recruited from Krakow through the local out-of hospital healthcare centers. Prior to the participation of the study, all volunteers will be informed about the research purposes and measurement procedures. All study participants will provide written informed consent.

For experimental group will be used at least 200 participants (100 women and 100 men) for control group we also planning to recruit 200 volunteers (100 women and 100 men). The research will be performed first in healthy volunteers of both sexes, between 18 and 60 years old and will be classified also, depending on the experience of sauna. In this way, volunteers will be classified as none experienced with less than 12 sessions of sauna during the last year and experienced with 12 or more sessions of sauna during the last year. In brief a) gender classification and b) sauna experience.

#### Patients

The second experimental approach will be done in patients that have medical history of RIs, following the same pattern as specified before for experimental patients (no control, in this case only experimental patients will be analyzed) been a final sample of 200 patients. In brief a classification between a) gender and b) sauna experience will be done. Subjects with a medical history of more than six episodes of upper respiratory tract infections and/or more than two lower respiratory tract infections in the last year will be consider as potential patients. For this purpose they will be asked in the initial questionnaire about this particular data. The project activities will be conducted following the protocol ICH GCP (Pharmaceuticals for Human Use Good Clinical Practice 2015).

#### Sauna protocol and blood manipulation

Sauna sessions will be provided in a sauna that will accommodate up to 20 people at once by external agreement with a local business. All analyses with blood samples will be conducted in the Institute of Zoology and Biomedical Research of the Jagiellonian University, due to it has all the basic devices for carrying out the cell culture experiments. In brief, a basic cell culture laboratory with an incubator, bath, flow laminar hood, and centrifuge will be used. For analyzing absorbance and fluorescence a Tecan Plate Reader will be used and for analyzing images a confocal microscopy will be used (Gonzalez Gonzalez et al., 2018). Sauna's conditions will have dry air (humidity 10%±5) with temperature 80°C±5 and will be monitored constantly. Each sauna session will take for participants 10-15 minutes. The healthy volunteers will take sauna once per week during 1.5 year, but 10 seasons

can be skipped. In case of more than 10 absences in the whole period, the subject will be removed from the study and will not have further access to the sauna free entrance.

### **Blood sampling**

Peripheral blood sampling will be performed using hypodermic needles and Vacutainers with anticoagulants by an experienced nurse at the first day of the experiment and then accidentally around each three months for 1.5 year. Collected samples will be placed to storage containers with 2-6 °C to maintain the optimal viability. Further monocytes and neutrophils will be extracted according modified protocols for monocytes (Menck et al. 2014) and neutrophils (Oh et al. 2008). In case of patient from control group, only one blood sample will be taken each three months.

### **Determination of spirometric measurements and immune response**

7. Spirometric measures will include the forced expiratory volume and forced vital capacity (Moore et al., 2012). We are planning to buy a portative compact Spirometer 6600 (Vitalograph, the USA) that allows us to measure lung capacity
8. Monocytes/macrophages and neutrophils will be stimulated with three different pathogens that normally cause infections;
  - O-deacylated lipooligosaccharide (LOS) from *Haemophilus influenza*
  - Purified capsule from *Streptococcus pneumoniae*
  - formalin inactivated single RhinoVirus serotype (RV13)
9. Reactive oxygen species (ROS) (Hoer-Yue et al., 2016) will be tested using a commercial kit.
10. Nitric Oxide Production (NO) (Schmölz et al., 2017) will be tested using a commercial kit.
11. Phagocytic Activity (Oben et al., 1988) using Zymosan beads stained with a fluorophor.
12. Extracellular Traps Quantification (Farrera et al., 2013).
13. Heat-Shock 90 protein determination by immunocytochemistry (own laboratory protocol).

### **Statistical Analyses**

Statistical significance will be tested using multivariate analysis of variance and the  $\chi^2$  test. Risk factors for main outcomes will be analyzed using the multivariable Cox model. Cox models enable identification of which independent variables act as intensifiers, when analyzed as a set, and takes into account their statistical significance within a confidence interval. For all three tested groups we will have two categorical variables: gender and the sauna experience. Cox multivariable models will be adjusted for response variables: age, parameters of the forced expiratory volume, forced vital capacity, ROS, NO, phagocytic activity, extracellular traps quantification, Heat-Shock 90 protein. Statistical analyses will be performed using Statistica ver. 10 (StatSoft, Inc. 2011).

## **5. Literature**

- Farrera C., Fadeel B. 2013 “Macrophage clearance of neutrophil extracellular traps is a silent process” *Journal of Immunology* 191(5): 2647–2656
- Gonzalez Gonzalez M., Cichon I., Scislowska-Czarnecka A., Kolaczowska E. 2018 “Challenges in 3D culturing of neutrophils: Assessment of cell viability” *The Journal of Immunological Methods* pii: S0022-1759(17)30512-4
- Guzek A., Rybicki Z., Korzeniewski K., Mackiewicz K., Saks E., Chciałowski A., Zwolińska E. 2015 “Etiological factors causing lower respiratory tract infections isolated from hospitalized patients” *Advances in Experimental Medicine and Biology* 835: 37–44
- Hor-Yue Tan, Ning Wang, Sha Li, Ming Hong, Xuanbin Wang, Yibin Feng “*The Reactive Oxygen Species in Macrophage Polarization: Reflecting Its Dual Role in Progression and Treatment of Human Diseases*” *Oxidative Medicine and Cellular Longevity* 2016: 2795090
- Kunutsor S. K., Laukkanen T., Laukkanen J. A. 2017. “Sauna bathing reduces the risk of respiratory diseases: a long-term prospective cohort study” *European Journal of Epidemiology* 32(12): 1107–1111
- Laukkanen J. A., Kunutsor S. K., Niemelä M., Kervinen K., Thuesen L., Mäkikallio T. H. 2017 “*All-cause mortality and major cardiovascular outcomes comparing percutaneous coronary angioplasty versus coronary artery bypass grafting in the treatment of unprotected left main stenosis: a meta-analysis of short-term and long-term randomised trials*” *British Medical Journal* 4(2): e000638

- Menck K., Behme D., Pantke M., Reiling N., Binder C., Pukrop T., Klemm F. 2014 “*Isolation of Human Monocytes by Double Gradient Centrifugation and Their Differentiation to Macrophages in Teflon-coated Cell Culture Bags*”. Journal of Visualized Experiments 9;(91): e51554
- Moore V.C. 2012 “*Spirometry: step by step*” Breathe 8: 232–240
- Oben J. A., Foreman J. C. 1988 “*A simple quantitative fluorimetric assay of in vitro phagocytosis in human neutrophils*” Journal of Immunological Methods 9;112(1): 99–103
- Oh H., Siano B., Diamond S. 2008 “*Neutrophil Isolation Protocol*” Journal of Visualized Experiments (17): 745
- Lisa Schmözl, Maria Wallert, Stefan Lorkowski. 2017 “*Optimized incubation regime for nitric oxide measurements in murine macrophages using the Griess assay*”, The Journal of Immunology Methods 449: 68–70
- Torigoe T., Tamura Y., Sato N. 2009 “*Heat shock proteins and immunity: application of hyperthermia for immunomodulation*” International Journal of Hyperthermia 25(8): 610–616
- van Heijl I., Schweitzer V. A., Zhang L., van der Linden P. D., van Werkhoven C. H., Postma D. F. 2018 “*Inappropriate Use of Antimicrobials for Lower Respiratory Tract Infections in Elderly Patients: Patient- and Community-Related Implications and Possible Interventions*” Drugs Aging 1–10

## 6. Budget Table

2. Table with budget of the project.

Item	Funds for each budget year (PLN)				Total
	2019	2020	2021	2022	
<b>1. Direct costs, including:</b>	47600	72800	72800	20500	213700
(a) Salaries and benefits	9600	12800	12800	3200	38400
(b) Equipment	11000	0	0	0	11000
(c) Other direct costs	27000	60000	60000	17300	164300
<b>2. Indirect costs</b>	14640	29120	29120	8200	81080
<b>Total costs</b>	62240	101920	101920	28700	294780

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

### Investigator / Staff Costs

a) Number of Investigators: 2

b) Investigators: nature of their contribution in the project and a justification of investigator costs

No	Full name / Nature of contribution in the project / Type and character of position	Employing entity	Project-related remuneration period (months)	Contracted working time as a % of full time equivalent (FTE)	Rate of salary pool/banding	Total salary cost on grant (PLN)	Type of employment relationship
1	Mgr Evgenii Baiakhmetov	The Jagiellonian University, Krakow, the Faculty of Biology	36	48	500 / month	18000,00	Contract of specified task
	Scope of work within individual project tasks:	Planning, preparing and conducting experiments (measurements of ROS, NO); statistical analyses of obtained results; compilation of results and conclusions; presentations at scientific conferences; articles writing.					
2	Mgr Miriam González González1	The Jagiellonian University, Krakow, the Faculty of	36	48	500 / month	18000,00	Contract of specified task

		Biology					
	Scope of work within individual project tasks:	Planning, preparing and conducting experiments (measurements of phagocytic activity; quantification of neutrophil extracellular traps; determination of intracellular heat shock protein); statistical analyses of obtained results; compilation of results and conclusions; presentations at scientific conferences; articles writing.					
3	A nurse	The Hospital on Siemiradzkie go	1	4	66.67 / month	2400,00	Contract of specified task
	Scope of work within individual project tasks:	Peripheral blood sampling, and spirometric measures once per each 3 months in the sauna during the project (in total 12 working days).					
Total							38400,00

#### List of equipment to be purchased and/or built

No	Equipment name	Purchasing entity	Year of purchase	Amount	Unit cost (PLN)	Contribution of the NCN (PLN)
1	Spirometer 6600	The Jagiellonian University, Krakow, the Faculty of Biology	2019	1	11000,00	11000,00
	<b>Justification of purchase</b>	Spirometer 6600 (Vitalograph, the USA) will allow us to provide spirometric measurements in the sauna. The purchase of this item will do our study more flexible (patients will not need to go for measurements to a special medical office).				
Total						11000,00

#### Other direct costs

Materials (expendable goods for direct use in the project) Cost – **104300** PLN.

1. BD Vacutainers 367835 (in total 4300 Vacutainers; It will be enough to get blood samples from 4200 patients) – 4700 PLN.
2. BD Vacutainer Eclipse Blood Collection Needles 368650 (in total 4300 Needles; It will be enough to get blood samples from 4200 patients) – 11500 PLN.
3. Gloves Nitrilex PF (8600 gloves for working with patients; 4300 gloves for lab working) – 1500 PLN.
4. Premier Hard Surface Wipes (4800 wipes for rapid disinfection) – 500 PLN.
5. Griess Reagent System G2930 (for Nitric Oxide Production) – 4000 PLN.
6. Neutrophil/Monocyte Respiratory Burst Assay Kit 601130 (for analysis of reactive oxygen species) – 3000 PLN.
7. Zymosan A (*S. cerevisiae*) BioParticles™, Alexa Fluor™ 488 conjugate Z23373 (for analyzing phagocytic activity) – 3700 PLN.
8. SYTOX™ Green Nucleic Acid Stain S7020 (for extracellular traps quantification) – 3000 PLN.
9. Paraformaldehyde P6148-1KG (for extracellular traps quantification) – 600 PLN.

10. Phosphate-Buffered Saline (10X) pH 7.4 AM9625 (for extracellular traps quantification) – 5300 PLN.

11. Anti-HSP90 antibody MBS245471 (for Heat-Shock protein 90 determination) – 7000 PLN.

12. Penicillin-Streptomycin (10,000 U/mL) 15140122 (for Heat-Shock protein 90 determination) – 4200 PLN.

13. RPMI-1640 Medium R7509-1L (for Heat-Shock protein 90 determination) – 300 PLN.

14. Fetal Bovine Serum F0679-500ML (for Heat-Shock protein 90 determination) – 55000 PLN.

**Outsourced services** (services ordered from entities external to the Host Institution) – **10400 PLN**.

1. Renting a sauna (130 days, each day – 80 PLN) – 10400 PLN.

**Conferences and business trips** (by members of the research team) – **10000 PLN**.

1. Participation in national and international scientific conferences (including nutrition, accommodation, printing of posters, etc.). We are planning to attend the XX International Sauna Congress 2021 as well as Preventive Medicine Conference 2022 – 10000 PLN.

**Other costs**– **15000 PLN**.

1. Materials/services for manuscript preparation and publication; materials for data storage – 1000 PLN.

2. Publications costs (Preventive Medicine – 10000 PLN, Environmental Health and Preventive Medicine – 4000 PLN) – 14000 PLN.



## INVESTIGATING THE ROLE OF AUTOPHAGY UNDER CALORIC RESTRICTION AND OXIDATIVE STRESS FOR LONGEVITY IN BIRDS

---

Authors: Amadeusz Bryła, Jayasravanthi Mokkaapati

### **Abstract**

In biology, ageing of birds is exceptional since albeit of their higher energy expenditure through flight than similar sized mammals, birds live longer. According to free radical theory of aging, organisms with higher metabolic rate should have higher oxidative stress, age faster and have lower life span, but surprisingly is not observed in birds. This lack of knowledge about mechanisms underlying slow ageing in birds irrespective of their high energy expenditure need to be addressed. Unlike mammals, birds (e.g., Zebra finch) fast comparatively long during each night suggesting the likely connection between caloric restriction and longevity. It is well explained in humans, rodents, and invertebrates that caloric restriction can induce autophagy, a conserved cellular process responsible for removing damage or non-functional cell components. Furthermore, induced process of autophagy can prevent oxidative damage substantially increasing the life span. Therefore, herewith we propose the development of a possible signalling pathway connecting the activation of autophagy under caloric restriction as a preventive mechanism of oxidative stress consequently resulting longevity in birds. Once the interlinks between activation of autophagy with caloric restriction and oxidative stress are established, their correlation with longevity in birds can be validated in terms of rate of telomeres shortening. The research findings of this project answer whether autophagy caused by caloric restriction is the underlying mechanism of longevity in birds, if yes, to what extent autophagy influence oxidative damage, and how these phenomena are interlinked within and ultimately to longevity in birds.

## 1. Research objective

The Nobel prize awarded research on molecular discoveries in autophagy by Yoshimori Ohsumi paved the way to our current understanding of autophagy mechanisms involving caloric restrictions and ageing associated diseases. In general, birds live much longer than mammals of the same size. It seems paradox, since many adaptations to flight should accelerate ageing giving short lifespan to birds. One of the theories explaining differences in life span is free radical theory of ageing; ageing is a process caused by accumulation of damage by free radicals (Montgomery et al., 2012). The overall high metabolic rate of birds should lead to higher production of free radicals consequently should accelerate ageing but surprisingly is not observed in birds (Hickey et al., 2012). The cellular mechanisms responsible for this slow ageing processes in birds are not yet been investigated. It is known in humans, rodents, and other invertebrates that caloric restriction can induce autophagy, a conservative cellular process responsible for removing damage or non-functional cell components as a mechanism that provided cells with amino acids and substrates for energy production when nutrients are depleted (Kon and Cuervo, 2011). Unlike rodents and other mammals which are active whole days, most of the birds (e.g., Zebra finch) eat only during day and comparatively fast long during each night. This innate calorie restriction in birds and subsequent autophagy activation can explain long lifespan of birds. On the other hand, autophagy is potentially a counteracting mechanism to oxidative stress in glutathione peroxidases (GPx) depleted organisms (Filomeni et al., 2015). However, it is still not clear to what extent the physiological/molecular processes of removing oxidative damage increase the longevity in calorie restricted birds. This uncertainty about the pertinence of the oxidative stress theory in explaining longevity among caloric restricted birds motivated us to fill this knowledge gap by examine whether the activation of autophagy is the underlying cellular mechanism of longevity in birds. This research project address three hypothesis, first, the increased overnight fasting can activate autophagy in birds. In the second part, we want to study the effect of increased oxidative stress on autophagy in calorie restricted birds, here we hypothesize that the increased oxidative damage in birds can be removed by activated autophagy caused by increased caloric restriction. Finally, the hypothesis we assume in the last part of our research is that induced autophagy in calorie-restricted birds prevent oxidative damage resulting improved longevity in terms of decreased shortening of telomeres.

## 2. Significance of the project

The proposed study will let to explain the interactions between autophagy activation under caloric restriction and oxidative stress with the longevity in birds. In particular, we aim at demonstrating separate interlinks among food deprivation, removal effects of oxidative stress and induced autophagy ultimately resulting long life span in birds. From purely scientific point of view, it will be a major breakthrough in Ornithology particularly in Birds Physiology as the possible interlinks between caloric restrictions, oxidative stress and autophagy with longevity will be well established as a cellular signalling pathway. Studies on cellular and molecular activities, and phenomenon like ageing always have applied aspects, as they are usually interrelated among living organisms. In this particular case such link is obvious, and potential impact of the study results on society cannot be overestimated: developing the link between oxidative, caloric restriction and induced autophagy to longevity will open up new gates to proposing specific solutions to improve longevity in general. Based on the project results, it is anticipated to develop a cellular signalling pathway linking stress induced autophagy to longevity.

## 3. Work plan

The ultimate goal of the project is to investigate the role of autophagy activation under caloric restriction and oxidative stress in increasing the longevity in birds. This general goal requires focusing on three specific tasks, determining the main specific goals and the work plan:

- (1) Study of autophagy activation against caloric restriction,
- (2) Role of autophagy under increased oxidative damage in caloric restricted birds, and

(3) Investigation of autophagy activation under caloric restriction and oxidative stress in increasing longevity in birds. It is clear from our pilot experiments that the circadian oscillation in oxidative damage (dROM) resulted overnight decrease in the hepatocytes size in zebra finch suggesting the involvement of overnight caloric restriction as a protective mechanism against oxidative damage (Bryła et al., 2018). However, before starting above said defined tasks, some preliminary experiments will be conducted to confirm the basal protein and gene expression levels of selected markers under food deprivation in birds. Below more detailed description of the specific tasks and the general work plan is given. Table 1 presents distribution of responsibilities of the project personnel and Table 2 – the timeline for major activities within the project. Certainly, before starting our experiments, we will apply to Local Institutional Animal Care and Use Committee for ethical permits.

Table 1. Distribution of responsibilities of the project personnel: black – task leader; dark grey – major responsibilities; light grey – participation.

Team member	Project coordination	Task 1	Task 2	Task 3
Project leader 1				
Project leader 2				
Technician 1				
Technician 2				

Table 2. Time schedule for major activities within the project.

	2019		2020				2021				
Purchasing equipment and reagents											
Literature search											
Preliminary experiments											
Task 1											
Task 2											
Task 3											
Presentations/conferences											
Publications											

The risk analysis of the outcomes of each tasks are executed by proposing alternative hypothesis given herewith

Task 1:

If the autophagy is not activated with caloric restriction in birds, it may suggest that there is sovereign cellular process involved which can further be identified with specific markers for other mechanisms per se mTOR signaling pathway (Blagosklonny, 2010).

Task 2:

From the literature it is known that GPx, a major antioxidant is involved in activation of autophagy (Filomeni et al., 2015), but this mechanism has never been confirmed in birds. If the link between suppressed GPx levels with autophagy activation was established, it can suggest that autophagy is the underlying mechanism for removing damaged cell components under oxidative stress. On the other hand, if no activation of autophagy is observed under suppressed GPx levels, it makes even more interesting inferences that other mechanisms are involved in preventing oxidative damage other than autophagy.

Task 3:

There are several in vitro studies reported that increased metabolism under stress can increase telomere loss consequently shortening life span (Boonekamp et al., 2017). However, this prediction is not conclusive enough in vivo. With our experiments under fully controlled conditions in this task, we can answer questions concerning autophagy under the influence of oxidative stress on telomere shortening in calorie restricted birds (either positive or negative correlation can be established).

For publications, all three tasks together allow us to answer main research question, but on the other hand each task independently give significant contribution to field of experimental physiology specifically in birds. The advantage of this planned independent tasks is that they can give several articles connecting caloric restriction, autophagy, oxidative stress and ageing in birds, and methodology papers separately.

#### **4.Methods of research**

Proposed research will be conducted using zebra finch, *Taeniopygia guttata*, the common avian model organisms. As described earlier, from our pilot experiments, the decreased hepatocytes size in zebra finch under oxidative damage (dROM) during night suggest the involvement of overnight calorie restriction as a protective mechanism against oxidative damage (Bryła et al., 2018). It is clear from literature that Beclin-1, autophagy related gene 5 (ATG-5), B-cell lymphoma 2 (Bcl-2), microtubule-associated protein light chain 3 (LC-3) and Dynein are the major marker proteins involved in the signaling pathway of autophagy activation. Beclin-1 and ATG-5 are required for the autophagosome formation in the initial step of autophagy (Kang et al., 2011). On the other hand, binding of Bcl-2 with Beclin-1 trigger autophagosome formation independently (Marquez and Xu, 2012). In the final step of autophagy, LC-3 and Dynein are involved in the interaction between autophagosomes and the lysosome (Monastyrska et al., 2009, Kimura et al., 2008). Several studies reported that the involvement of these proteins in activation of autophagy with nutrient depletion and oxidative stress in mammals and invertebrates (Kaushik and Cuervo, 2018, Moruno-Manchón et al., 2013). Nevertheless, there is little information available concerning the role of these proteins in calorie restriction and oxidative stress, by extension, the roles they may play in longevity in birds. Hence, the determination of these protein levels and their gene expression allow us to predict the stages of autophagy activation in relation to the given stressors in each experiments. However, in order to optimize the methods measuring basal protein levels and their gene expressions in birds, a preliminary study is required to.

In addition, transmission electron microscopy (TEM), a gold standard for detection of autophagy will be used to determine the ultrastructure of autophagy in the liver. The use of TEM is a valid and important method both for the qualitative and quantitative analyses of changes in various steps of autophagy activation (Ylä-Anttila et al., 2009), hence autophagy activation will be measured in each of our proposed experiments by TEM.

For Task-1, we will conduct experiments in groups of birds for the effect of different fasting times on activation of autophagy by determining above given protein and gene expression levels according to the procedures established in our preliminary experiments. Task-2 will be executed by manipulating oxidative stress using sulfoximine (inhibitor of GPx antioxidant) and measuring the concentrations of enzymatic and non-enzymatic antioxidants in blood and specific tissues (e.g, GPx, reactive oxygen species, superoxide dismutase, catalase etc.). To assess consequences of manipulation with increasing oxidative stress- dROM assay will be performed (Bryła et al., 2018). Finally, in Task-3, prolonged exposure to food deprivation and oxidative stress is required to study their effects on given protein and gene expressions involved in autophagy directly connecting longevity. Telomeres attrition, a physiological marker for longevity will be determined in blood samples collected before and after the stimulus (food deprivation and/or oxidative stress) and further in tissue samples at the end of the experiments according to the procedures given by Boonekamp et al., 2017.

For data analysis, we will use precise statistical test methods for each experiments perhaps ANCOVA. Utmost care will be taken while working with quantitative values of physiological traits, for example, controlling body mass, time of the day etc.

## 5.Literature

- Blagosklonny, M. V. (2010). Increasing healthy lifespan by suppressing aging in our lifetime: Preliminary proposal. *Cell Cycle*, 9(24), 4788–4794. <https://doi.org/10.4161/cc.9.24.14360>
- Boonekamp, J. J., Mulder, G. A., Salomons, H. M., Dijkstra, C., & Verhulst, S. (2014). Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. *Proceedings of the Royal Society B: Biological Sciences*, 281(1785), 20133287–20133287. <https://doi.org/10.1098/rspb.2013.3287>
- Bryła, A., Bury, S., Sadowska, E. T. & Bauchinger, U. (2018). Diurnal changes in oxidative damage and autophagy in Zebra finch. In: 4th International Conference of Cell Biology - Cracow, 11-12 May 2018.
- Filomeni, G., De Zio, D., & Cecconi, F. (2015). Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death and Differentiation*, 22(3), 377–388. <https://doi.org/10.1038/cdd.2014.150>
- Hickey, A. J. R., Jüllig, M., Aitken, J., Loomes, K., Hauber, M. E., & Phillips, A. R. J. (2012). Birds and longevity: Does flight driven aerobicity provide an oxidative sink? *Ageing Research Reviews*, 11(2), 242–253. <https://doi.org/10.1016/j.arr.2011.12.002>
- Kang, R., Zeh, H. J., Lotze, M. T., & Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death and Differentiation*, 18(4), 571–580. <https://doi.org/10.1038/cdd.2010.191>
- Kaushik, S., & Cuervo, A. M. (2018). The coming of age of chaperone-mediated autophagy. *Nature Reviews Molecular Cell Biology*. <https://doi.org/10.1038/s41580-018-0001-6>
- Kimura, S., Noda, T., & Yoshimori, T. (2008). Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes. *Cell structure and function*, 33(1), 109-122.
- Kon, M., & Cuervo, A. M. (2011). Chaperone-mediated Autophagy in Health and Disease. *FEBS Letters*, 584(7), 1399–1404. <https://doi.org/10.1016/j.febslet.2009.12.025>.Chaperone-mediated
- Marquez, R. T., & Xu, L. (2012). Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. *American Journal of Cancer Research*, 2(2), 214–221. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/22485198%5Chttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC3304572/pdf/ajcr0002-0214.pdf>
- Monastyrska, I., Rieter, E., Klionsky, D. J., & Reggiori, F. (2009). Multiple roles of the cytoskeleton in autophagy. *Biological Reviews of the Cambridge Philosophical Society*, 84(3), 431–448. <https://doi.org/10.1111/j.1469-185X.2009.00082.x>.Multiple
- Montgomery, M. K., Buttemer, W. A., & Hulbert, A. J. (2012). Does the oxidative stress theory of aging explain longevity differences in birds? II. Antioxidant systems and oxidative damage. *Experimental Gerontology*, 47(3), 211–222. <https://doi.org/10.1016/j.exger.2011.11.014>
- Moruno-Manchón, J. F., Pérez-Jiménez, E., & Knecht, E. (2013). Glucose induces autophagy under starvation conditions by a p38 MAPK-dependent pathway. *Biochemical Journal*, 449(2), 497–506. <https://doi.org/10.1042/BJ20121122>
- Ylä-Anttila, P., Vihinen, H., Jokitalo, E., & Eskelinen, E. L. (2009). Chapter 10 Monitoring Autophagy by Electron Microscopy in Mammalian Cells. *Methods in Enzymology* (1st ed., Vol. 451). Elsevier Inc. [https://doi.org/10.1016/S0076-6879\(08\)03610-0](https://doi.org/10.1016/S0076-6879(08)03610-0)

## 6. Table with budget of the project.

Item	Funds for each budget year (zł)			Total
	2019	2020	2021	
1. Direct costs, including:	11600	66000	66000	248000
1/ Salaries and benefits	36000	36000	36000	108000
2/ Equipment	20000	0	0	20000

3/ Other direct costs	60000	30000	30000	120000
2. Indirect costs	38400	26400	26400	91200
Total costs (1+2)	154400	92400	92400	339200

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

During first year, we need to order equipment for performing western blot analysis (power supply, chamber for electrophoresis, transfer, imaging etc.). High value of „other direct costs” are expected in first year of project because of requirement to buy antibodies for determination of protein concentration, chemicals for western blots, real time PCR, costs of TEM microscopy etc. Additionally, maintenance costs of zebra finch colonies have to be considered throughout the project (costs of seeds, cleaning products, repairing bird cages, personal protection etc.). Further, we expect to present our project findings in minimum of two international conferences probably in second or third year and minimum of three publications are anticipated.

## REVIEWS

---

### **Dr. hab. Piotr Skórka**

The idea of the project is fascinating. Scientific problem and research question are clearly stated and they justify undertaking the study. However, I think the principal investigators promises far too much than they can actually achieve. I am afraid that with the proposed study design the results can only explain the intra-specific variation in longevity in Zebra finch. Thus, it will not be the advance in explaining long life-span in birds comparing to mammals. To make this breakthrough real, experimental procedures should perhaps include a model mammal species (e.g. a mouse) as the reference point. Moreover, generality of the proposed research is limited. Why not to take, for example, three model bird species and three model mammal species and then apply the proposed experimental treatments? Wouldn't that be an approach providing excellent evidences rather than just clues?

I have also other critical comments that should be addressed in further version of the project. They are:

#### Point 1.

Description of the experimental procedures is very limited and fuzzy. Technical details are needed. For example following information is missing: (1) what is the sample size of birds and (2) their age and sex, (3) what will be experimental conditions, and (4) how bird welfare will be assured? Moreover, (5) all molecular procedures should be clearly described. How liver samples will be derived? Will this involve killing birds?

#### Point 2.

Authors state that their preliminary study showed overnight decrease in the hepatocytes size in zebra finch telling that it may indicate the involvement of overnight calorie restriction as a protective mechanism against oxidative damage. However, isn't that true for both birds and mammals? In my opinion, showing that the decrease was higher in birds than in mammals would be the confirmation for the existence of the autophagy mechanism.

#### Point 3.

Data analysis is described perfunctorily. Please, always try to describe this part in very details. List dependent and independent variables. Structure of the statistical model should be depicted. Potentially confounding variables should be identified and sample size must be given.

#### Point 4.

Budget is not explained at all. What are these costs, e.g. what PI plans to buy in order to successfully conduct the study?

### **Dr hab. Łukasz Michalczyk**

#### **Strengths:**

1. The subject is very interesting and understanding why birds, despite their significantly higher metabolic rate, live longer compared to mammals of similar size may have both scientific and applied implications beyond bird biology.
2. The Authors should be commended for including Gantt charts in the proposal.

### **Weaknesses:**

1. As far as I know, ageing by oxidative stress and by telomere shortening are different mechanisms, and the way the Authors link these two is unclear.
2. The Authors claim that “developing the link between oxidative, caloric restriction and induced autophagy to longevity will open up new gates to proposing specific solutions to improve longevity in general”. However, such links are already known. Thus, the putative novelty in impact must be more specifically described. Moreover, the Authors cannot ‘develop’ links or pathways, they may discover or describe them.
3. There should be a single leader who takes the entire responsibility for the project. If this is a consortium, then there should be a single coordinator who is responsible for all other members of the consortium.
4. It is considered good practice to propose specific journals in which the Authors would like to publish their papers.
5. The description of experiments is highly insufficient. In fact, I was unable to understand what exactly the Authors proposed to do. In addition to a proper description of experiments, even a simple graphic illustration of the experimental design would definitely help to understand the concept of the study.
6. The description of statistical methods is basically lacking. The statement “For data analysis, we will use precise statistical test methods for each experiment perhaps ANCOVA.” shows that the Authors have no clue how to analyse the data.
7. In general, the proposal is somewhat chaotic. The Authors would like to have a chance to obtain funding for their research, they must make much effort to make the proposal readable and understandable.
8. Last but not least, the English in the proposal definitely requires some serious polishing. There were quite a few moments when I was not sure what the Authors were saying.

### **Budget:**

1. There are no descriptions of tasks for individual team members, thus it is hard to assess whether the salaries are designed appropriately.
2. In contrast to title of the budget section, there is no cost breakdown. Moreover, there is no justification of the proposed costs. In other words, the budget is not justified.

## **Mgr Stanisław Bury**

The project is highly interesting. Arguments showing why the topic is worth to study are somehow provided, but in very chaotic way, making the whole project puzzling and sometimes not convincing. Moreover you do not have well balanced the content of the Objectives and Significance sections. The Objective contains quite a lot of information on the state of art and the gap of knowledge, but these are almost entirely missing in the Significance section. The Significance is starting from the information what are the expected outcomes, whereas it should start from the description of the state of knowledge, i. e. what is the bigger context, what is unknown and why your research is important for this field of science. Basically the Significance section is missing all that and is covered only by the informations on what you are going to do and achieve. Moreover you are not really convincing in showing the importance of the possible outcomes. Stating that you will have important contribution to ornithology suggests that your research has very narrow impact limited only to avian biology. This is quite striking when considering that your research are focused on aging and longevity, one of the biggest issues in the evolutionary biology and that you are referring in the Objectives to the recent Noble prize award.

The Objectives section starts with the information of a Noble prize for the discoveries in the role of autophagy. That would be a nice starting point, but afterwards you are jumping into completely different topic on bird longevity. There is no link between those first sentences, so I got confused in the very beginning of reading. Moreover you immediately said that birds are an exception, since they



achieve higher life span than similar sized mammals and only after that you refer to the general concept. I would suggest to start from invoking this concept, since this is one of the best known ideas proposed to explain the aging process and variation in longevity and then point on birds, as the exception. Later on you are coming back to autophagy but as a possible mechanism explaining longevity of birds, but again your justification why to expect so comes later. With this order one can immediately ask why not to study some more obvious mechanisms, like antioxidant capacity or ROS production that can differ birds from mammals.

Objective are finished with short description of hypothesis, but in my opinion it is also not well balanced. In this section you should say more about your goal, so spend more space on your specific question and emphasize them more.

## **Evgenii Baiakhmetov**

### **JUSTIFICATION FOR OVERALL ASSESSMENT**

The project is scientific due to pretend to cover the knowledge gap in controversial studies of longevity mechanisms in birds.

Principal investigators have clear specific hypotheses: the increased overnight fasting can activate autophagy in birds; the increased oxidative damage in birds can be removed by activated autophagy caused by increased caloric restriction; induced autophagy in calorie-restricted birds prevent oxidative damage resulting improved longevity in terms of decreased shortening of telomeres.

In addition, the project has the work plan, methods, references, and the budget description. That gives rise to a suggestion that reading of this an ambitious project was not a vain thing.

### **A. EVALUATION**

The application does not deserve top marks, but still has chances of success in the future if the project will be improved. Despite the impressive promised outcomes and well-written hypotheses, principal investigators did not clear up such parts of the grant proposal as methods (especially the sample size) and the budget of the project.

### **C. EVALUATION ESTIMATE**

Will the technician 1 and the technician 2 work due to the idea or will earn some money? Why direct costs for 2019 year (Page 5, Line 32) are “11600” instead of 116000? Based on the justification of the budget I cannot find a way how the direct costs were calculated.

### **D. EVALUATE VIABILITY**

For the current version of the project evaluate viability is very difficult due to the lack of detailed information, for instance, about the sample size, localities of the experiments. I also want to reference to Page 3 Line 22 “*our experiments under fully controlled conditions*”, how it will be conducted?

### **STRENGTHS OF THE PROPOSAL**

I like the ideas of the project. The proposal has the risk analysis of the outcomes of each task. The principal investigators have a preliminary data (see also WEAKNESSES OF THE PROPOSAL).

### **WEAKNESSES OF THE PROPOSAL**

The absolutely unclear budget (see EVALUATION ESTIMATE). I would add some of your preliminary data to the project due to the Googling approach for finding your conference data has not worked for me. Plus several minor remarks: it is really good to read about the Nobel Prize awarded research of Yoshinori Ohsumi, only in the text I did not find any references to him; a literal error (see Page 1, Line 30, “Yoshimori”); the word “*perhaps*” (see Page 4, Line 37) for me look very weird, I would remove it.

**Nevertheless, I wish good luck to the young scientists in a further scientific career.**

## **Michał Belcik**

The project has been written in due diligence, and it meets the criteria for a scientific proposal. It clearly meets the criteria for basic research. It is written in a good English, there are no grammatical errors or misuse of words.

Abstract is written in quite a compelling way, and it is a good and brief description of a whole research, and questions and assumptions underlying it. Research objective clearly describes what scientific questions authors are planning to answer with their research, and importance of research in this field is clearly underscored. Generally speaking, work plan is neat and organised. Authors are to be commended for formulating clear hypotheses and alternative hypotheses. Methods of research seem to be well described, although it is important to note that I am not an expert in this field. Budget seems to be concise, with every position generally well described.

That being said, I see some flaws in that proposal as well. To begin with, I am somehow not convinced about a knowledge gap authors are planning to address with their research questions. I am sure that there is one that authors are describing, and they cite other research to back up that claim, but I must admit I do not feel well informed about how big it is, especially when compared to the bigger picture of this field. It is also a little bit unclear to me what were the efforts of other researchers (if any) to address. Surely, if research in this field produces Noble Prize winners (as authors stated), then it must be a very active one, with lots of researches pursuing their scientific goals in it. Therefore it makes me hard to believe that such a promising topic (as authors describe it) remained completely unaddressed. Better clarification of a current state of knowledge on this issue would be in order. It is true, of course, that I am not an expert in that field, but a good scientists should be able to deliver their vision of own research topics, along with a compelling picture of a general state of knowledge in the field they are interested in.

My another concern is somehow connected to my previous paragraph. Given the popularity, or (in other words) “hotness” of the topic, why there was no one that addressed it? Is it due to the lack of general interest (which I found hard to believe in, as mentioned above), or is it due to some technical or other issues? That brings a question about a feasibility of the project, which can hardly be overstated given the fact that it comes with a significant budget proposed, that would eventually be funded from taxpayers money.

My next concerns are less significant one, and of more technical nature. I am not sure what are the tasks described in Table 1, and timing in Table 2 is somehow unclear. Another issue is that although hypotheses are formulated in a very good way, they should be more visible in the text, therefore making it easier to get back to them every time a reviewer finds a part of the proposal that are addressing them.

Generally speaking, I find this proposal interesting, and generally worth financing, but only after some concerns and uncertainties (mentioned above) would be answered by the authors.

## **Marta Grosiak**

Proposed project is well written, hypotheses and outline of experiment is described in clear and understandable way. The main research question in proposed project is well explained in the context of current knowledge (including mostly recently findings in the field of evolutionary physiology). The proposed explanation of found gap is really innovative and novel, but the impact of proposed research it isn't highlighted enough, especially in the more broader context such as bird's ecology. The structure and planning of this project is correct and well composed, what is presented in your well organized and considered work plan. Experiments are well planned, and each task is scientifically proven by given literature. Unfortunately, you skip the number of animals that you are planning to take and sacrifice in your project and general description about keeping and treating them. Because you probably have practical experience in the most of using methods, the estimated budget seems to be adequate to the planned project, but you should prove more details of direct costs item.

The strongest parts: materials and methods, work plan

The weakest parts: abstract, significance of project, justification of costs

### Project summary

10 - Organisms with higher MR or only endotherms?

In summary you present very well the background of current knowledge and reason of you research question and theoretical link between autophagy and longevity. The aim of project is written in clear way. But you don't present the experiment how you want to test presented hypothesis. You have suggestion about your model species but you don't say it in direct way. Probably it is possible to restrict the length of first part of explanation and describe a little about your general experimental set-up? How you want to solve your problem?

The second weak point is that you don't highlight the importance of the filling this gap of knowledge. Maybe it is good to put your big question in the ecological context e.g. light pollution in cities and its influence on caloric restriction and longevity of birds in urbanized areas?

### Research objective

32 – You should say that this size of organism is related to different metabolic rate, because you know it and in the rest of text you use this knowledge without explanation.

33 – You should emphasize that this adaptation is e.g. high sustained metabolic rate or increased aerobic rate because within list of other flight adaptation only these could be connected with birds longevity. On the other hand the group of active flying mammals due to their specific adaptation to energy conserving also comparatively longer (e.g. *Myotis brandtii* can achieve more than 38 years and weight 8-11g)

44 – you should provide references here, because you write about comparison of founding results on mammals, birds and particular species (zebra finch)

46 – Presenting contradictory explanation of the influence of phenomenon that you want to test show that you aware about other possible explanation and complexity of given problem.

2-6 – You have clear hypotheses but I am not sure that the last is like the prediction of hypothesis that induced autophagy in fasting birds improved longevity?

### Significance of the project

You don't have any references in this part, even you should present the state of current knowledge here. Here you have strong ideas that convince that this idea is really novel and needed, but you don't explain also why this particular project with your designed is such innovative? And I think, that this part should be much longer, presenting detailed innovation and expected outcome of this research in the context of other previous research.

### Work plan

The structure and planning of this project is correct and well composed. The research goals are understandable and present in clear way. The schedule presenting in a form of table is really good idea and present your well organized and considered work plan. I really like this part of your grant proposal.

### Materials and methods

Experiments are well planned, and each task is documented by given literature. You didn't mention the size of birds groups and the place where the experiment take place. You should have a separate paragraph about keeping and treating your animals. You focused only about the laboratory part of all experiment and skip description of practical work on birds. Probably you can explain your statistical model in more detailed way and divided to evaluate the results from each part of your research.

### Costs

Because you probably have practical experience in the most of using methods, the estimated budget seems to be adequate to the planned project. In this part you should present more detailed costs including each bigger category (e.g. equipment). I suggest to don't use "etc" when you list a little expensive laboratory staff costs more that few thousands.

## **Miriam Gonzalez Gonzalez**

### **1. Substantive evaluation of project**

The project addresses to a very interesting and hot topic, the autophagy which has received recently a Nobel Prize. What is more, they are concerned in studying it in a group of species that are not properly studied: the birds.

In this way, the authors presented four main hypotheses: a) increased overnight fasting can activate autophagy in birds, b) there is an effect on increased oxidative stress on autophagy in calorie restricted birds, c) increased oxidative damage in birds can be removed by activated autophagy caused by increased caloric restriction and d) induced autophagy in calorie-restricted birds prevent oxidative damage resulting improved longevity in terms of decreased shortening of telomeres. Unfortunately, I would suggest to the authors to express in a better way in the manuscript these hypothesis.

The gap of knowledge is in this manuscript a very good point, because very little known about this phenomenon in birds. But, I don't see any critical impact on the society of this study and it could be for example the importance that the poultry species have in the food industry. If we understand better their physiology we could adapt our infrastructures for producing healthier animals.

In relation with some assumption that is done in the background, I would say that the assumption that mammals do not have intensive fasting periods is risky. If so, why for example bears have so many fat deposits? We cannot make this assumption in such a general way. Authors predictions seem quite reasonable but more logical order of the scientific ideas should be provided in the future version.

In work plan, I have had problems for understanding the workflow of the experiments. Instead of the tables that give no relevant information, it would have been very good to add figures or tables for clarifying the order and division of the experiments.

## **2. Methodology**

In this part, the authors have explained exhaustively the risks analysis and possible explanations. On the other hand, few information is provided about the experiments itself (organization, control groups). This part is very messy. In addition, information about potential expression of genes and protein markers are provided, but all of them come from studies in mammals. It would have been interesting to stress this factor more. Also more information about the techniques would be very good. In addition, it has drowned my attention how the budget table has been done with very little information about the methods and techniques. The budget and justification needs to be properly justified (more information).

## **3. Evaluation and implementation**

Some careful consideration should be done for the improvements in the experimental design. However, the project itself seems to be realizable.

## **4. Costs of calculation**

I have absolutely no idea how the calculations for the budget are justified, so I cannot say if they are properly calculated.

## **5. Strengths of the Project**

- The novelty of the idea
- They have preliminary data which is a plus
- The results would be easily publishable in a high impact journal

## **6. Weaknesses of the Project**

- Not properly express the relevance of this investigation for the society or human being
- The methodology needs further details and better distribution and reasons
- The budget needs to be better justified
- It is difficult to follow the logical order, better development of some ideas should be done

## FINAL VERSION

---

Authors: Amadeusz Bryła, Jaya Sravanthi Mokkaapati

### **Investigating the role of autophagy under caloric restriction and oxidative stress for longevity in birds**

#### **Abstract**

In biology, ageing of birds is exceptional since albeit of their higher energy expenditure through flight than similar sized mammals, birds live longer. According to free radical theory of aging, organisms with higher metabolic rate should have higher oxidative stress, age faster and have lower life span, but surprisingly is not observed in birds. This lack of knowledge about mechanisms underlying slow ageing in birds irrespective of their high energy expenditure need to be addressed. Unlike mammals, birds (e.g., Zebra finch) fast comparatively long during each night suggesting the likely connection between calorie restriction and longevity. It is well explained in humans, rodents, and invertebrates that caloric restriction can induce autophagy, a conserved cellular process responsible for removing damage or non-functional cell components. Furthermore, induced process of autophagy can prevent oxidative damage substantially increasing the life span. Therefore, herewith we propose the development of a possible signalling pathway connecting the activation of autophagy under caloric restriction as a preventive mechanism of oxidative stress consequently resulting longevity in birds. Once the interlinks between activation of autophagy with caloric restriction and oxidative stress are established, their correlation with longevity in birds can be validated in terms of rate of telomeres shortening. The research findings of this project answer whether autophagy caused by caloric restriction is the underlying mechanism of longevity in birds, if yes, to what extent autophagy influence oxidative damage, and how these phenomena are interlinked within and ultimately to longevity in birds.

## 1. Research objective

In general, birds live much longer than mammals of the same size. It seems paradox, since many adaptations to flight should accelerate ageing giving short lifespan to birds. However, the cellular mechanisms responsible for this slow ageing processes in birds are not yet been investigated. It is known in humans, rodents, and other invertebrates that caloric restriction can induce autophagy, a conservative cellular process responsible for removing damage or non-functional cell components (Kon and Cuervo, 2011). Unlike rodents and other mammals which are active whole days, most of the birds (e.g., Zebra finch) eat only during day and comparatively fast long during each night. This repeatable innate calorie restriction in birds each night may subsequently activate autophagy and thereby causing long lifespan of birds. On the other hand, autophagy is potentially a counteracting mechanism to oxidative stress in glutathione peroxidases (GPx) depleted organisms (Filomeni et al., 2015). However, it is still not clear to what extent the physiological/molecular processes of removing oxidative damage increase the longevity in calorie restricted birds. This uncertainty about the pertinence of the oxidative stress theory in explaining longevity among calorie restricted birds motivated us to fill this knowledge gap by examining whether the activation of autophagy is the underlying cellular mechanism of longevity in birds.

In this research project, we want to address three hypotheses, first, the increased overnight fasting can activate autophagy in birds. In the second part of our work, we want to study the effect of increased oxidative stress on autophagy in calorie restricted birds, here we hypothesize that the increased oxidative damage in birds can be removed by activated autophagy caused by increased caloric restriction. Finally, the hypothesis we assume in the last part of our research is that induced autophagy in calorie-restricted birds prevent oxidative damage resulting improved longevity in terms of decreased shortening of telomeres.

## 2. Significance of the project

Addressing ageing is one of the major focusing field in the current scientific world. The Nobel prize awarded research on molecular discoveries in autophagy by Yoshimori Ohsumi paved the way to our current understanding of autophagy mechanisms involving caloric restrictions and ageing associated diseases (Kaushik and Cuervo, 2018). One of the theories explaining the differences in life span is free radical theory of ageing; ageing is a process caused by accumulation of damage by free radicals (Montgomery et al., 2012). The overall high metabolic rate of birds should lead to higher production of free radicals consequently should accelerate ageing but surprisingly is not observed in birds (Hickey et al., 2012). This can be explained by the fact that the oxidative damage can be reduced by the activation of autophagy. In birds, due to repeatable calorie restriction during each night can activate autophagy, a mechanism that provided cells with amino acids and substrates for energy production when nutrients are depleted. However, the underlying relationship among calorie restriction, oxidative stress and longevity in birds are still unclear.

The proposed study will let to understand the causal mechanisms of increased life span or longevity by explaining certain interactions between autophagy activation under caloric restriction and oxidative stress with the longevity in birds. In particular, we aim at demonstrating separate interlinks among food deprivation, removal effects of oxidative stress and induced autophagy ultimately resulting long life span in birds. From purely scientific point of view, it will be a major breakthrough in the biology of ageing particularly in bird physiology by developing possible interlinks between caloric restrictions, oxidative stress and autophagy with longevity as a cellular signalling pathway. Studies on cellular and molecular activities, and phenomenon like ageing always have applied aspects, as they are usually interrelated among living organisms. In this particular case such link is obvious, and potential impact of the study results on society cannot be overestimated: developing the link between oxidative, caloric restriction and induced autophagy to longevity will open up new gates to proposing specific solutions to improve longevity in general. Based on the project results, it is anticipated to develop a cellular signalling pathway linking stress induced autophagy to longevity.

### 3. Work plan

The ultimate goal of the project is to investigate the role of autophagy activation under caloric restriction and oxidative stress in increasing the longevity in birds. This general goal requires focusing on three specific tasks, determining the main specific goals and the work plan:

#### Task 1: Study of autophagy activation against different duration of caloric restriction

#### Task 2: Role of autophagy under increased oxidative stress in caloric restricted birds, and graph

#### Task 3: Investigation of autophagy activation under caloric restriction and oxidative stress in increasing longevity in birds.

It is clear from our pilot experiments that the circadian oscillation in oxidative damage (dROM) resulted overnight decrease in the hepatocytes size in zebra finch suggesting the involvement of overnight calorie restriction as a protective mechanism against oxidative damage (Bryła et al., 2018). However, before starting above said defined tasks, some preliminary experiments will be conducted to confirm the basal protein and gene expression levels of selected markers under food deprivation in birds. Below more detailed description of the specific tasks and the general work plan is given. Table 1 presents distribution of responsibilities of the project personnel and Table 2 – the timeline for major activities within the project. Certainly, before starting our experiments, we will apply to Local Institutional Animal Care and Use Committee for ethical permits.

GRAPH 1. PROPOSED EXPERIMENTAL DESIGNS FOR EACH INVOLVED TASKS

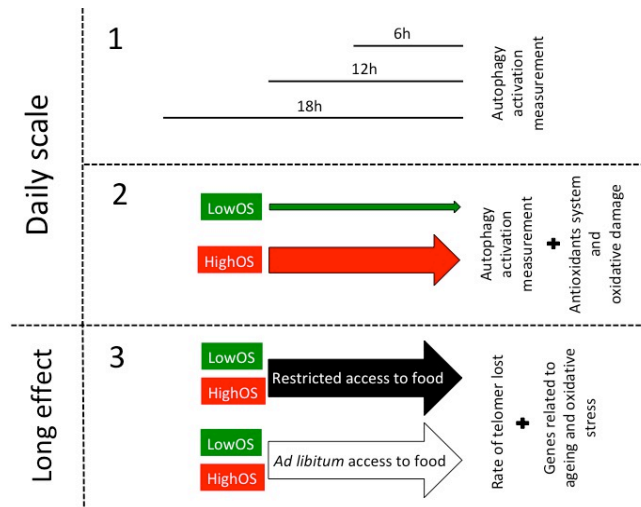


Table 1. Distribution of responsibilities of the project personnel: black – task leader; dark grey – major responsibilities; light grey – participation.

Team member	Project coordination	Task 1	Task 2	Task 3
Project leader 1				
Project leader 2				
Animal care				

Table 2. Time schedule for major activities within the project.

	2019	2020				2021			
Purchasing equipment and reagents									
Literature search									
Preliminary experiments									
1. Activation of autophagy									
2. Effect of autophagy on oxidative stress									
3. Effect on longevity									
Presentations/conferences									
Publications									



**The risk analysis** of the outcomes of each tasks are executed by proposing alternative hypothesis given herewith

**Task 1:**

If the autophagy is not activated with caloric restriction in birds, it may suggest that there is sovereign cellular process involved which can further be identified with specific markers for other mechanisms per se mTOR signaling pathway (Blagosklonny, 2010).

**Task 2:**

From the literature it is known that GPx, a major antioxidant is involved in activation of autophagy (Filomeni et al., 2015), but this mechanism has never been confirmed in birds. If the link between suppressed GPx levels with autophagy activation was established, it can suggest that autophagy is the underlying mechanism for removing damaged cell components under oxidative stress. On the other hand, if no activation of autophagy is observed under suppressed GPx levels, it makes even more interesting inferences that other mechanisms are involved in preventing oxidative damage other than autophagy.

**Task 3:**

There are several *in vitro* studies reported that increased metabolism under stress can increase telomere loss consequently shortening life span (Boonekamp et al., 2017). However, this prediction is not conclusive enough *in vivo*. With our experiments under fully controlled conditions in this task, we can answer questions concerning autophagy under the influence of oxidative stress on telomere shortening in calorie restricted birds (either positive or negative correlation can be established).

For publications, all three tasks together allow us to answer main research question, but on the other hand each task independently give significant contribution to field of experimental physiology specifically in birds. The advantage of this planned independent tasks is that they can give several articles connecting caloric restriction, autophagy, oxidative stress and ageing in birds, and methodology papers separately.

#### **4.Methods of research**

Proposed research will be conducted using zebra finch, *Taeniopygia guttata*, the common avian model organisms. Animals will be kept in cages inside climate chambers with fully controlled conditions: temperature  $31.4 \pm 0.1$  °C, and  $29 \pm 3\%$  humidity, light schedule 12:12 h besides time of experimental manipulation. All individuals will have *ad libitum* access to food in except time of experimental manipulation. Conditions were previously accepted several times by Local Institutional Animal Care and Use Committee. As described earlier, from our pilot experiments, shows decreased in hepatocytes size of zebra finch and decrease oxidative damage (dROM) during night suggest the involvement of **overnight caloric restriction as a protective mechanism against oxidative damage** (Bryła et al., 2018).

Transmission electron microscopy (TEM), a gold standard for detection of autophagy will be used to determine the ultrastructure of autophagy in the liver, pectoral muscle and pancreas. The use of TEM is a valid and important method both for the qualitative and quantitative analyses of changes in various steps of autophagy activation (Ylä-Anttila et al., 2009), hence autophagy activation will be measured in each of our proposed experiments by TEM.

In addition, molecular markers involved in autophagy are well established in mammals and invertebrates study. We will use western blot, and real-time PCR analysis of Beclin-1, autophagy related gene 5 (ATG-5), B-cell lymphoma 2 (Bcl-2), microtubule-associated protein light chain 3 (LC-3) and Dynein, which are the major marker involved in the signaling pathway of autophagy activation. Beclin-1 and ATG-5 are required for the autophagosome formation in the initial step of autophagy (Kang et al., 2011). On the other hand, binding of Bcl-2 with Beclin-1 trigger autophagosome

formation independently (Marquez and Xu, 2012). In the final step of autophagy, LC-3 and Dynein are involved in the interaction between autophagosomes and the lysosome (Monastyrska et al., 2009, Kimura et al., 2008). Several studies reported that the involvement of these proteins in activation of autophagy with nutrient depletion and oxidative stress in mammals and invertebrates (Kaushik and Cuervo, 2018, Moruno-Manchón et al., 2013). Nevertheless, there is little information available concerning the role of these proteins in calorie restriction and oxidative stress, by extension, the roles they may play in longevity in birds. Hence, the determination of these protein levels and their gene expression allow us to predict the stages of autophagy activation in relation to the given stressors in all experiments. However, in order to optimize the methods measuring basal protein levels and their gene expressions in birds, a preliminary study is required to.

**Task 1** we will conduct experiments in three groups (n=10) of birds for the effect of different fasting times (6h, 12h, 18h, control) on activation of autophagy by determining above given protein and gene expression levels (Graph 1, section 1).

**Task 2** will be executed by manipulating oxidative stress using sulfoximine (inhibitor of GPx antioxidant) (n=20 per group) and measuring the concentrations of enzymatic and non-enzymatic antioxidants in blood and specific tissues (e.g. GPx, reactive oxygen species, superoxide dismutase, catalase etc.). To assess consequences of manipulation with increasing oxidative stress- dROM assay will be performed (Bryła et al., 2018) (Graph 1, section 2).

**Task 3** prolonged exposure to food deprivation and modulated oxidative stress is required to study their effects on given protein and gene expressions involved in autophagy directly connecting longevity (n=10 per group). Telomeres attrition, a physiological marker for longevity will be determined in blood samples collected before and after the stimulus (food deprivation and/or oxidative stress) and further in tissue samples at the end of the experiments according to the procedures given by Boonekamp et al., 2017 (Graph 1 section 3).

For data analysis, separate statistical test methods will be used for each individual tasks since the type/number of variables are different for different tasks. In detail, ANCOVA will be used for Task 1 and task 2 in which measurement of activation of autophagy with quantitative values as dependent variable and caloric restriction/oxidative stress manipulation as categorical variable. Task 3 considers two factorial designs with manipulation of oxidative stress and caloric restriction (two categorical factors), telomere length and oxidative stress and genes expression will be used as covariates. Utmost care will be taken while working with quantitative values of physiological traits, for example, controlling body mass, time of the day etc.

## 5.Literature

- Blagosklonny, M. V. (2010). Increasing healthy lifespan by suppressing aging in our lifetime: Preliminary proposal. *Cell Cycle*, 9(24), 4788–4794. <https://doi.org/10.4161/cc.9.24.14360>
- Boonekamp, J. J., Mulder, G. A., Salomons, H. M., Dijkstra, C., & Verhulst, S. (2014). Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. *Proceedings of the Royal Society B: Biological Sciences*, 281(1785), 20133287–20133287. <https://doi.org/10.1098/rspb.2013.3287>
- Bryła, A., Bury, S., Sadowska, E. T. & Bauchinger, U. (2018). Diurnal changes in oxidative damage and autophagy in Zebra finch. In: *4th International Conference of Cell Biology - Cracow*, 11-12 May 2018.
- Filomeni, G., De Zio, D., & Cecconi, F. (2015). Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death and Differentiation*, 22(3), 377–388. <https://doi.org/10.1038/cdd.2014.150>
- Hickey, A. J. R., Jüllig, M., Aitken, J., Loomes, K., Hauber, M. E., & Phillips, A. R. J. (2012). Birds and longevity: Does flight driven aerobicity provide an oxidative sink? *Ageing Research Reviews*, 11(2), 242–253. <https://doi.org/10.1016/j.arr.2011.12.002>

- Kang, R., Zeh, H. J., Lotze, M. T., & Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death and Differentiation*, 18(4), 571–580. <https://doi.org/10.1038/cdd.2010.191>
- Kaushik, S., & Cuervo, A. M. (2018). The coming of age of chaperone-mediated autophagy. *Nature Reviews Molecular Cell Biology*. <https://doi.org/10.1038/s41580-018-0001-6>
- Kimura, S., Noda, T., & Yoshimori, T. (2008). Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes. *Cell structure and function*, 33(1), 109-122.
- Kon, M., & Cuervo, A. M. (2011). Chaperone-mediated Autophagy in Health and Disease. *FEBS Letters*, 584(7), 1399–1404. <https://doi.org/10.1016/j.febslet.2009.12.025>.Chaperone-mediated
- Marquez, R. T., & Xu, L. (2012). Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. *American Journal of Cancer Research*, 2(2), 214–221. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/22485198%5Chttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC3304572/pdf/ajcr0002-0214.pdf>
- Monastyrska, I., Rieter, E., Klionsky, D. J., & Reggiori, F. (2009). Multiple roles of the cytoskeleton in autophagy. *Biological Reviews of the Cambridge Philosophical Society*, 84(3), 431–448. <https://doi.org/10.1111/j.1469-185X.2009.00082.x>.Multiple
- Montgomery, M. K., Buttemer, W. A., & Hulbert, A. J. (2012). Does the oxidative stress theory of aging explain longevity differences in birds? II. Antioxidant systems and oxidative damage. *Experimental Gerontology*, 47(3), 211–222. <https://doi.org/10.1016/j.exger.2011.11.014>
- Moruno-Manchón, J. F., Pérez-Jiménez, E., & Knecht, E. (2013). Glucose induces autophagy under starvation conditions by a p38 MAPK-dependent pathway. *Biochemical Journal*, 449(2), 497–506. <https://doi.org/10.1042/BJ20121122>
- Ylä-Anttila, P., Vihinen, H., Jokitalo, E., & Eskelinen, E. L. (2009). *Chapter 10 Monitoring Autophagy by Electron Microscopy in Mammalian Cells. Methods in Enzymology* (1st ed., Vol. 451). Elsevier Inc. [https://doi.org/10.1016/S0076-6879\(08\)03610-0](https://doi.org/10.1016/S0076-6879(08)03610-0)

## 6. Table with budget of the project.

Item	Funds for each budget year (zł)			Total
	2019	2020	2021	
<b>1. Direct costs, including:</b>	116000	66000	66000	248000
<b>1/ Salaries and benefits</b>	36000	36000	36000	108000
<b>2/ Equipment</b>	20000	0	0	20000
<b>3/ Other direct costs</b>	60000	30000	30000	120000
<b>2. Indirect costs</b>	38400	26400	26400	91200
<b>Total costs (1+2)</b>	154400	92400	92400	339200

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

### Salaries and benefits:

- Salaries for each of two PI: 36 months x 1250 zł (PI will be responsible for: performing experiments western blot analysis, PCR, analysis of data sets, preparing manuscripts and attend to conferences), total 3 year costs: **90000 zł**
- Technical assistant:  
Animal care taker: person will be responsible for maintaining animal colony during whole project (36 months x 500zł): **18000 zł**

**Equipment:**

Western blot analysis (power supply, chamber for electrophoresis, transfer, imaging etc.): **20000zł**

**Other Direct costs:**

- 1) Summary costs of TEM analysis (preparation of samples and imaging)
  - a. Task 1 40 individual samples x 375zł = **15000 zł**
  - b. Task 2 40 individual samples x 375zł = **15000 zł**
  - c. Task 3 40 individual samples x 375zł = **15000 zł**
  - d. Diamond blade for electron and light microscopy = **14000 zł**
- 2) Analysis of protein concentration
  - a. Specific monoclonal antibodies for determination (Bcl-2, LC-3, ATG-5, Dynein, Beclin-1)  
5 x 2000zł = **10000 zł**
  - b. Secondary antibodies for westernblot analysis: **6000 zł**
  - c. Reagents and materials for western blot analysis (membranes, buffers etc.): **9000 zł** (3 year costs)
- 3) Real time PCR analysis
  - a. Design primers for analysis: **2000 zł**
  - b. Reagents, chemicals, materials (buffers, kits, SyberGreen, plates, tips etc.): **17000zł** (3 year costs)
- 4) Maintaining animals for 3 years (seeds, vitamins, safety protection, materials for repairing cages, cleaning materials etc.): **8000 zł**
- 5) 2 International conferences each for 2 persons and 3 publications: **9000 zł**

## “EUROPEAN HEDGEHOGS (ERINACEUS EUROPEAUS) RESPONSE TO CAR TRAFFIC IN RURAL AND URBAN AREAS”

---

Authors: Michał Bełcik, Marta Grosiak

### **Abstract**

Increasing human population is coupled with an increase in road traffic intensity, especially in developed urban areas. This has a significant effect on various wild mammal populations, one of those effects being road mortality. The hedgehog is one of the most frequently killed animal on roads. We hypothesize that the change of behavioral response to stressors associated with crossing road (light, noise of car engine) will appear more frequently among hedgehogs from more urbanized areas in comparison to those living in rural areas. We also hypothesize that the rate of road crossing in hedgehogs in urban areas will be lower. The main prediction is that behaviour of urban population will be “braver” and unique defense strategy of hedgehogs relying on rolling up will appear less frequently. To test these hypothesis we will select locations in several habitats in two areas with different urbanization status in Poland. The study will be performed on September and October during two years (2019-20). We will estimate general size of hedgehogs population and perform a simulation of incoming car to note their response for specific stressors. The GPS collaring will allow us to collect detailed data about hedgehog’s mobility and activity in different areas. Finding the significance of the behavioral changes in urban hedgehogs could be the signal of ongoing synurbization phenomenon, which was described in several population of wild species commonly living in the cities. The highly changed urban environment may be responsible for loss of their defensive and protective behavior what increased mortality. We expected that the results of this project will also highlight what lies at the base of many problems with “brave” behavior of larger animals in cities causes significant problems and large costs.

## SHORT DESCRIPTION OF THE RESEARCH PROJECT

### 1. Research objective

The growth of human population is associated with extremely rapid increase in urban areas, which are projected to triple by 2030. Ongoing urbanization creates a completely new types of habitats, which in most cases have negative effect on wild population, while for some other species in urban areas, it can create new microhabitats for some populations. This phenomena, described as synurbization, is associated with several morphological and/or behavioural changes in these populations. As a result, some species may be more abundant in cities due to higher rates of survival, better availability of food sources, and also lower predation risk. Nevertheless, human activity in cities provides higher rate of mortality and disturbance of wild animals. Increasing road density and road traffic are one of the factors, which significantly reduce number of animals in urbanized areas. It is important to note that while most of the species find it difficult to adapt to this new environment, some display a significant behavioural plasticity, allowing them to thrive in cities (Sol et al 2013). The European hedgehog is a nocturnal insectivore, present in higher densities in urbanized rather than in suburbanized areas. It is one of the very few mammals that have adapted to the urban environment, which may be caused by its ability to influence its behavior by the new information. In last decade many studies highlighted the increasing problem with elevated hedgehogs road mortality (Rondinini and Doncaster 2002), reducing density of its located populations by even more than 30% in some areas (Huijser and Bergers 2000). Because they are actively foraging and migrate for a quite long distances (Doncaster et al. 2001), the problem lies partially on abundance of invertebrate prey, but also they tend to avoid crossing roads while foraging (Rondinini and Doncaster 2002). In spite of that, increasing mortality suggests that some additional factor play an important role. We suggest that behavioral changes in hedgehogs more habituated to these areas with higher road traffic can explain their vulnerability.

**The main aim of our study is to test the hypothesis that urban environment with high density of road traffic is important factor driving behavioural changes in hedgehogs, what is directly affecting their survival rates.** We predict that urban hedgehogs will display a different behavioural response facing road traffic threats while crossing roads. We will have a two different approaches – one on performing a behavioural experiment trying to find a response for this stressor triggered by approaching car, and other on spatial patterns of movement and road avoidance. Specifically, we will test following hypotheses:

- I. Hedgehogs from areas of higher traffic intensity, will almost previously react by flee from incoming car, while those from rural areas will freeze or roll up.** Because many studies have investigated only avoidance of crossing roads (Dowding et al. 2010), but it can be associated mostly to avoiding asphalt, as an aversion to the synthetic surface (Mulder 1999). In this project we want to focus on describing the different modes of behavior response from approaching car. The experiment design will allow us to test a hedgehogs' response from two very specific stressors (light occurrence and noise of car engine). We predict that urban individuals will have faster response facing with all of these stressors. Rural hedgehogs should be more stressed by all these factors (more often roll up). We also want to check, that light stressor cause bigger response for rural population, due to lower light pollution in suburban areas.
- II. Hedgehogs in the urbanized area avoid crossing roads more often than individuals living in rural areas.** We will examine the size of their population and pattern of avoiding crossing roads by individuals from urbanized and rural populations.

## 2. Significance of the project

Human populations are increasing and becoming predominantly urban. By 2050, 66% of world population is projected to be urban (WUP2014). One of the biggest questions raised by this process is how wildlife will be affected by this new type of environment. Human-induced habitat loss due to urbanization is amongst one of the most frequently mentioned causes for biodiversity loss (Gaston et al. 2003). Increasing road density results in heavy fragmentation of wildlife habitats, resulting in the degradation of ecosystems and their functions (Ibisch et al. 2017). Due to the rapid nature of this changes, they may exceed a limit of tolerance for many species. However, many species that live in urban environment frequently differ in behaviour, compared to those from rural areas. Growing number of studies provide evidence that observed behavioural adjustments that could be reflected by phenotypic plasticity are needed to persist in urban habitats, however the underlying mechanism remain unclear (Sol et al. 2013). Only individuals of animals with specific traits, including proper behaviours, are able to colonize and have a stable populations in cities. Most of those species successfully adapted to deeply changed habitats are birds, mostly passerines like Great tit (Hu and Cardoso, 2010) or European pied flycatcher (Seppänen et al. 2011). There are few examples of mammals in this group, like Red foxes that modify their ranging activity (Baker et al. 2007) or Fox squirrels that invest less time in vigilance behaviour (McCleery 2009).

One of such mammals is European hedgehog (*Erinaceus europaeus*), which may serve as an interesting model species, with most of its range in Western Europe and present in a large variety of habitats. Its ground-dwelling behaviour reduces its ability to escape from threats and forces it to deal with the problem of growing road networks (Ibisch et al. 2017). In spite of that it is more abundant in cities than in rural environment (Hubert et al. 2011). So far there have been studies on hedgehog populations in cities (Hubert et al. 2011, Dickman and Doncaster 1987, Dowding 2010), how their dietary preferences or predator response has changed (Ward et al. 1997), or how hedgehogs movement patterns are altered by the presence and traffic intensity of roads (Huijser and Bergers 2000, Rondinini and Doncaster 2002). Nevertheless, there has been very few studies tackling this specific issue of how their defense response might vary when faced with a new type of threat, that is an incoming car (Mulder 1999). Any research on that topic could bring us closer to answering the issue of high hedgehog mortality on the roads (Huijser and Bergers 2000, Mulder 1999), therefore it could possibly be a foundation of the solution to that problem.

Behavioral responses to stressors associated with urban environment are poorly recognized, yet better recognition of them is crucial towards better understanding of the mechanisms defining some species as city dwellers and others, that are not. Hedgehogs have quite a unique defensive response (they roll-up in defence), which is sometimes suggested as an explanation of their high road mortality (Mulder 1999). Any changes in that response could help us in understanding how deeply the anthropogenic factors can influence animal behaviour. Specific character of this question, i.e. a response to a certain type of threat, compels us to simulate that threat in order to answer this issue. That in turn justifies the methods and experiments proposed, which are designed in order to minimize the hedgehogs stress.

Facing with the perspective of rapid development of cities and urban areas, the importance of understanding on how this influences a wildlife ecology and behavior could not be overstated. It is also a key element in devising effective conservation plans, that could help us to reduce human footprint on biodiversity loss. Our species could also serve as a model one, that could potentially show that significant behavioural changes can occur as an effect of urbanization, therefore pointing out for a further need of research in that field.

## 3. Work plan

In our study we work on European hedgehog, small mammal that is abundant in the cities. The initial stage of project will start on May 2019, and first month will be spent on creating team of people that have experience with working on small wild animals, and obtaining necessary permissions from

Regional Environment Protection Directory and Local Ethics Committee. Secondly, research areas and particular roads with neighboring sites will be chosen and all transects will be designated. In this state we will also define the status of traffic intensity. On half of the locations, the transect inspection with car simulation approach and hedgehogs collaring will start in September, and will last for a month. Every time the tracking will take place just after dusk. On the other half of the locations, inspections will start in October, and also last for a month. Simulating experiment and tracking of collared animals will finish in October, so for the rest of the year we will focus our attention to data analysis. In the next year, those locations that in the previous year were inspected in September, will in turn be inspected in October – and vice versa. The end of whole project is planned for December 2020.

Estimation of population density will allow us to test the assumption of higher abundance of hedgehogs in urban areas. Performing behavioral test for response to very specific factors (light, car engine noise) will allow us to tell whether each stressor is the main cause of fear, especially for hedgehogs that partly habituate in more urban areas in higher level of light pollution (I). Testing the rate of road crossing in each area deliver important information about general behavior pattern of avoiding roads (II).

In case if behavioural experiment would not show any significant differences, this would also provide an answer to the question of main cause of hedgehog road mortality, namely that other factors than behavioural may be involved. Lack of road avoidance would be in contrary to previous studies, so it could serve as an interesting starting point for discussion and further research. If there would be no difference in avoidance between urban and rural areas, it would show that in this case, urbanization effect is not in play. The results of behavioural experiment could be published in “Behavioural Ecology” or “Animal Behaviour”, whereas results of movement data analysis could be published in “Landscape and Urban Planning” or “Landscape Ecology”. Results from the whole project could be published in “Biological Conservation”.

#### 4.Methods of research

##### a.) *Research areas*

The study will be conducted in five cities (urban area) and in five villages (rural area), in Poland. Ten specific locations will be chosen randomly to represent each type of area, and then divided into few categories (tab.1). Each location will have minimum 25 ha and will be made from 50 m buffer zone built around roads. Those roads will differ in night traffic intensity (when hedgehogs are most active), so urban locations will be placed around roads of high intensity, while those in rural areas will be placed around roads of low intensity. Data representing roads will be obtained from the state spatial features geodatabase (BDOT). In addition to the information about the road nighttime traffic, we will verify it by counting vehicles per 1h during period between dusk and dawn.

Tab.1. Categories of locations for the study

<b>Urban</b>	<b>Rural</b>
Parks, Botanical Gardens	Gardens, Orchards, Fields
Gardens	Pastures
Green areas between settlements	Small woodlands

Locations will include habitats potentially preferred by hedgehogs, dominated by small scale grasslands with low density of woodland fragments (Hujiser and Bergers 2000). The size of experimental sites and distance from roads are based on hedgehogs home range size, which is about 10-50 ha (Hujiser and Bergers 2000). We will draw a line transects of approximately 5 km. on each location, which we will use to estimate the density of hedgehog populations with infrared binoculars and to perform our behavioral experiment.



*b.) Experiment design*

- (I) Field experiment will be carried out between September and October for two years (2019-20), for one month on every night. Such choice of period is necessary to avoid breeding season and winter hibernation (Rondinini and Doncaster 2002). On each investigated site person equipped with infrared binoculars, gloves, flashlight, portable loudspeaker and a GPS will walk along delimited transects. For each met hedgehog we will simulate approaching car, starting from the distance of about 10 m. Simulation will be based on either switching on the light (L), playing a playback with car engine noise (E) or combination of both (L+E), while simultaneously approaching the hedgehog. Simulation type will be switched between met hedgehogs, so for the first hedgehog met this night, L simulation will be used, E for the second, L+E for the third, then again L for the fourth etc. The exact elements of behavioral response and reaction time will be noted, and combined they will lead to one of the three types of response (tab.2.).

Tab.2. Different types of behavioral response

<b>Behavioral response</b>	<b>Description</b>
Flee	Hedgehog starts to flee continuously in any direction other than towards the person, in no more than 5 seconds
Freeze	Hedgehog will freeze (roll-up) at any point of his reaction
Other	Any other type of behavior

After this part, the hedgehog will be captured by hand and sexed, weight, check for parasites and injures. Animals will be marked individually by unique pattern of painting (special dye for animal's fur) (fig.1). On chosen locations, ten individuals weighting more than 550 g (Dowding et al. 2010) will be GPS collared. Collars will automatically fall off after a period of up to six days, so they can be reused for collaring of another individuals. All capturing and handling procedures will be performed under the license from Regional Environment Protection Directory and Local Ethics Committee.

- (II) Each collared animal will be tracked within period of up to six days (Rondinini and Doncaster 2002) and released immediately, the interval between GPS transmitter signals is 3 min. Analysis of obtained data deliver us the information of their mobility (migrated distances), habitat preferences (distances between center of activity and road), frequency of crossing road, rate of road mortality). Additionally, locations of all of the hedgehogs (both dead and alive) met close to the road edge or on those roads will be marked on GPS.

*c.) Statistical analysis*

We will use t-test to analyze the difference between density of urban and rural population. To randomize our urban and rural area selection we will apply randomized searching formula using a database of Poland cities. Because we have data only from 10 localities we will use pseudoreplication procedure. For the main analysis, we will use GLM models. First model (I) will include fixed effects of localization nested in areas type (urban vs. rural), type of using stressor (light, noise, light and noise) and interaction (area type x stressor type), and as a random effects we will take type of response (roll, freezy, other) and also mass of individuals, time of meeting, distance from the road and interactions between them. The second model (II) will be similar but as a random variable instead type of response we will take the rate of crossing action. Significance of the fixed effects was tested with F-tests. Significance of the random effects for which the variance estimate was positive was tested with the likelihood ratio test. All statistical analysis will be carried out in R software (R. version 3.4.4).

*d.) Analysis of GPS collar trajectories*

Tracking data will be analyzed in order to obtain information about the tendency to cross or not cross the roads. For each observed trajectory, 100 random walks from the same start point will be simulated (Rondinini and Doncaster 2002). Each walk will have the same number of points and the same frequency distribution of distances between consecutive points as the observed trajectory. The only random part will be the direction taken from one location to the next, and simulated locations will be constrained that it would not fall in impossible places (such as inside buildings, water bodies etc.). Differences between observed and simulated trajectories will be tested with Bonferroni-corrected paired-sample permutation tests (Rondinini and Doncaster 2002). The rest of the spatial data (i.e. marked positions of living and dead individuals) will be later used for the further analyses. For all of the spatial analyses, we will use ArcGIS ver. 10.3, R ver. 3.4.4 and Fragstats ver.4.2.

## 5.Literature

- Baker P. J., Dowding C. V., Molony S. E., White, P. C. L. & Harris, S. 2007. Activity patterns of urban red foxes (*Vulpes vulpes*) reduce the risk of traffic-induced mortality. *Behavioral Ecology* (18) 716-724.
- Doncaster C.P., Rondinini C., Johnson P.C.D. 2001. Field test for environmental correlates of dispersal in Hedgehogs *Erinaceus europaeus*. *Journal of Animal Ecology* (70), 33–46.
- Dowding C.V., Harris S., Poulton S., Baker et al. 2010. Nocturnal ranging behaviour of urban hedgehogs, *Erinaceus europaeus*, in relation to risk and reward. *Animal Behaviour* (80) 13-21.
- Gaston K.J., Blackburn T.M., Goldewijk K.K.2003. Habitat conversion and global avian biodiversity loss. *The Royal Society* (270) 1293–1300.
- Hubert P., Julliard R., Biagianti S., Pouille M.L. 2011. Ecological factors driving the higher hedgehog (*Erinaceus europaeus*) density in an urban area compared to the adjacent rural area. *Landscape and Urban Planning* 103: 34– 43.
- Huijser M.P, Bergers P.J.M. 2000. The effect of roads and traffic on hedgehog (*Erinaceus europaeus*) populations. *Biological Conservation* (95) 111-116.
- Ibisch P.L., Hoffmann M.T., Kreft S., Pe'er G. Kati V., Biber-Freudenberger L., Sala D.A.D. Vale M.M., Hobson P.R. Selva N. 2017. A global map of roadless areas and their conservation status. *Science* (354 ) 1423- 1427.
- Mccleery R.A. 2009. Changes in fox squirrel anti-predator behaviors across the urban–rural gradient. *Landscape Ecology* (24).
- Mulder J.L. 1999. The behavior of hedgehog on roads. *Lutra* (42) 35-37.
- Rondinini C., and Doncaster C.P. 2002. Roads as barriers to movement for hedgehogs. *Functional Ecology* (16), 504–509.
- Seppänen, J., Forsman, J., Mönkkönen M., Krams I., Salmi T. 2011. New behavioural trait adopted or rejected by observing heterospecific tutor fitness. *Proceedings of the Royal Society B* (278) 1736-1741.
- Sol D., Lapedra O., Gonzales-Lagos C. 2013. Behavioural adjustments for a life in the city. *Animal Behaviour* 1-12.
- United Nations, Department of Economic and Social Affairs, Population Division (2014). *World Urbanization Prospects: The 2014 Revision, Highlights* (ST/ESA/SER.A/352).



Fig.1. Individual marked European hedgehog with VHF transmitter.

## 6. Table with budget of the project.

Item	Funds for each budget year (PLN)			Total
	2019	2020	2021	
<b>1. Direct costs, including:</b>	$67\,600 + 33\,180 + 49\,000 = 149\,780$	$49\,000 + 79\,600 = 128\,600$		
<b>1/ Salaries and benefits</b>	67 600	79 600		
<b>2/ Equipment</b>	33 180	-		
<b>3/ Other direct costs</b>	49 000	49 000		
<b>2. Indirect costs</b>	$(49\,000 + 67\,600) * 0.4 = 46\,640$	$(79\,600 + 49\,000) * 0.4 = 51\,440$		
<b>Total costs (1+2)</b>	196 420	180 040		<b>376 460</b>

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

1. Direct costs:

1/ Salaries and benefits:

Salaries for main project performers

1500 PLN x 8 months x 2 persons = 24 000 PLN / per 1 th year

1500 PLN x 12 months x 2 persons = 36 000 PLN / per 2 th year

Salaries for all project performers (fee –for-task agreement)

2500 PLN + 9% \* 2500 PLN (insurance costs) x 2 months x 8 persons = 2725 PLN x 2 x 8 = 43 600 PLN / per year

## 2/ Equipment

Gloves – 3,50 PLN x 10 persons x 2 = 35 PLN  
Loudspeaker (Blow BT 460) – 80 PLN x 10 persons = 800 PLN  
Flashlight – (Olivat MAY) – 179 PLN x 10 persons = 1 790 PLN  
GPS – (Garmin Etrex) – 785 PLN x 10 persons = 7 850 PLN  
Faunabox (sunplast, 7.5 l) – 24,50 PLN x 10 persons = 245 PLN  
Weight (Medio Line, kg) – 262 PLN x 10 persons = 2 620 PLN  
Special dye for animal's fur (ManicPanic, 500 ml) = 50 PLN  
Bateries (GP) – 80 PLN x 10 persons = 800 PLN  
Infrared binocular (YUKON Night Vision No) x 10 persons = 1399 PLN x 10 persons = 13 990 PLN  
GPS collar (MicroLightTrack, 10g, ) = 5 collars x 1000 PLN = 5000 PLN  
(5 collars for 100 hedgehogs tracking (collar changes x10 per year, because each data collection takes 6 days (we have 60 days for each year))).

## 3/ Other costs

Daily allowance - 0,84 PLN x 5000 km x 10 persons = 42 000 PLN/ per year

Conferences – foreign conferences - 3500 PLN x 2 persons = 7 000/per year

---

## REVIEWS

---

### **Dr hab. Łukasz Michalczyk**

#### **Strengths:**

3. The subject is very interesting and timely.
4. Moreover, in addition to the clear scientific character, the project may also result in applications in conservation biology and urban planning.
5. The combination of experimental and field methods should be commended as such a comprehensive approach significantly increases the probability of obtaining sound conclusions.
6. The proposed experiments and observations are appropriate to test the proposed hypotheses.
7. The choice of target journals seems reasonable (but see below).

#### **Weaknesses:**

9. Whereas the choice of journals for behavioural and GPS results seems reasonable, it is not clear what will “results from the whole project” include.
10. Whereas organising some members of the research team and obtaining permissions from the Ethical Committee can be done after receiving funding, things such as the choice of study sites should be already presented in the proposal. At least one example, accompanied with relevant maps, should be provided in the proposal (this would show that the Authors planned the study carefully and it would also help to better understand the experimental design).
11. Table 1 suggests that individual urban and rural habitats are counterparts – is that true? If yes, this should be explained in the text.
12. There is no justification for two observation seasons (why is not one season sufficient or why not are not three rather than just two seasons proposed?).

13. The minimal distance between individual hedgehogs that assures the independence of observation is not specified. Given that both light and noise may scare other individuals in the area, not every encountered hedgehog should be tested (there should be a predefined minimal distance between hedgehogs that are going to be tested).
14. In addition to the type of site (urban vs rural), stress type (L vs E vs L+E), and various covariates (weight or distance from road), statistical testing in this study should include such important variables as site ID (1–10), habitat type (as listed in Table 1), observation day, observer ID, and study year. The type of hedgehog response to stimuli is not a random factor but *the response* (sic!) variable. The description of statistical analyses must be presented more clearly.
15. I do not understand the need for such a complex analysis of GPS data. Would not it be better to simply count the total number of road crossings and the proportion of time spent in the vicinity of roads within the entire observation period (i.e. 6 days per individual)? The choice of the current GPS analysis requires a sound justification.
16. It is better to say that statistical analyses will be carried out with the latest R version rather than to specify the version (a new version may be released when the data are analysed).
17. Last but not least, the English in the proposal definitely requires some serious polishing as there were quite a few moments when I was not sure what the Authors were saying.

#### **Budget:**

3. There are no descriptions of tasks for individual team members, thus it is hard to assess whether the salaries are appropriate.
4. There is no calculation for the 5 000 km. Where did this figure come from?
5. Other than that, the budget seems balanced and justified.

### **Prof. Wiesław Babik**

#### **Quality of Science**

This is potentially an interesting project aiming at solving a well defined and timely problem. Testable hypotheses and their predictions are given. State of the art is generally well described and project is well justified. The project has however two main weaknesses. First, work plan is not clearly written, it looks like part of the material should actually be in Methods. More importantly, work plan does not contain the outline of how the project goals, i.e., tests of the specific hypotheses will be achieved. Second, I could not understand how statistical analyses will be performed. It looks like no dependent variables were defined (shouldn't these be type of response in model I and rate of crossing in model II?). One has impression as if the authors have partially confounded fixed/random effect with explanatory/response variables. Additionally the models appear quite complex, with many explanatory variables and their interactions – couldn't the design be simplified without losing most important information?

#### **Significance: novelty and impact on the field**

The project is novel and is likely to have a substantial impact on the field. However, the authors could explain its broader significance in more detail. Currently there is only a few quite general sentences. For example, if behavioral differences are detected, this could be used to as a starting point to address

interesting questions about of response – adaptation through natural selection on genetic variation vs. phenotypic plasticity.

### **Budget**

Budget is feasible and calculated in considerable detail. Please note that usually equipment is a separate category from materials and disposables.

### **Feasibility**

It is currently difficult to assess how feasible is the project. For example, we don't know the samples size (the number of animals) the authors plan to investigate. The procedure of employing helpers is not described. There is also no risk analysis in the proposal.

### **Strengths**

- well defined aims and testable hypotheses
- well described state of the art
- novelty
- detailed plan for dissemination of the results
- precisely calculated budget

### **Weaknesses**

- Work plan not clearly outlined
- Major problems with statistical analysis
- Expected impact and significance not sufficiently described
- English could be improved

## **Mgr Stanisław Bury**

My major concern on the project is that I do not see clear indication of the gap of knowledge and justification why your research is important. In general you are starting from the statements that urban areas are growing, which increases the density of roads and road mortality of wild species, but even though some species can persist in cities with high abundance. Then you are jumping to hedgehogs stating that they are numerous in cities and highly subjected to road mortality, so they should have developed some protective behavior. Such statement does not yet justify what is the gap of knowledge, but instead you immediately go the specific topic of your study. Furthermore you inform that you will study behavior of hedgehogs, but without clear indication why it is worth to study any behavioral responses and why to expect them. In fact if hedgehogs do suffer very high road mortality, it indicates that there may be no changes in behavior towards avoiding cars or roads. High abundance of hedgehogs in cities can be in turn an outcome of higher availability of food, suggested already in your text and again this weakens the justification of your research. However the main concern comes with the sentence: „*We suggest that behavioral changes in hedgehogs more habituated to these areas with higher road traffic can explain their vulnerability*“. With this you are suggesting that you expect that in areas with high road traffic hedgehogs will exhibit behavior making them vulnerable. This is contradictory to you research hypothesis, since afterwards you are predicting something completely opposite, i. e. the behavioral reponse that should lead to lower vulnerability to road mortality, such as road avoidance and fleeing from cars. This is fundamental inconsistency that undermines the whole justification of the research. In my opinion the project needs to be strongly revisited.

## **Amadeusz Bryła**

Project proposal titled “European hedgehogs (*Erinaceus europaeus*) response to car traffic in rural and urban areas” meets all basic requirements for scientific proposal and criteria outlined in the call for proposals. However it seems necessary to provide some editorial changes in whole text that consists significant amount of misspellings, double space, and grammar mistakes. Proposal experiments meet criteria of basic research on animals’ behavior. Proposal researches focus on urban ecology that is fast developing branch of ecology what increase possibility of publication results of this research in several different journals as it was outlined in work plan. Nonetheless authors do not introduce in convincing way, why they focused on investigation a behavioral changes while they outlined that hedgehogs are actively avoiding crossing roads. Maybe this avoidance does not increase selection pressure to developing changes in behavior. High competition in the field of urban ecology makes it necessary to properly introduce scientific problem. Assessment of impact of this research on this field and society is not easily recognizable, is not outlined as strong point of this study. It seems necessary to perform pilot study or include data concerning variability in frequencies in two outlined behaviors (flee or freeze) in hedgehog’s population. Such data, were not included in proposal. Design of experiments looks well thought through, it provides all important information’s and shows authors experience with work in such setup. Authors include full description of statistical analysis which will be conducted, but it looks like that they forgot to include analysis of parasitism/ injuries (one of variables that they will determine in the field) and its effect on animal behavior. In design of experiment animals were supposed to wear GPS collars for 6 weeks, whereas in justification of budget you mentioned, “data collection takes 6 days”, it needs to be revised. You introduce needs of 8 assistants, but justification of budget did not include information why all experimental sites have to be monitored everyday. If it is connected with high migration of this animal I would expect some data proving this point in budget.

## **Evgenii Baiakhmetov**

### **JUSTIFICATION FOR OVERALL ASSESSMENT**

The project hypothesizes that the change of behavioral response to stressors associated with crossing road (light, noise of car engine) will appear more frequently among hedgehogs from more urbanized areas in comparison to those living in rural areas; the rate of road crossing in hedgehogs in urban areas will be lower.

Taking into account the enthusiasm of the authors to the problem of the hedgehogs mortality in urban/rural areas, I do not see a real scientific gap in knowledge. Do the results of the study pretend to have a significant effect for science? I have a lot of doubt. Furthermore, the authors did not provide the current information in the field (e.g. “*Road mortality of hedgehogs in Lower Silesia*” Orłowski and Nowak 2004; Rautio et al. 2012; Haigh et al. 2014; Rautio et al. 2016; Pettett et al. 2017).

### **A. EVALUATION**

I would suggest the authors to improve the background of the research topic. Could we assume that “brave” behavior of hedgehogs might be reflected in larger animals? I have not seen any information that supports this idea in the project.

### **C. EVALUATION ESTIMATE**

The budget should be revised due to the “Equipment” should not include such items like “gloves”, dye for animal’s fur, batteries. I would include these items to the other direct costs.

## **D. EVALUATE VIABILITY**

I could imagine possibility in realization of the project due to the detailed plane that was provided by the principal investigators, but without a strong background and visible outcomes it makes no sense.

## **STRENGTHS OF THE PROPOSAL**

I like the detailed plan and the statistical analysis of the project. The budget is also a quite detailed. The authors know exactly the journal names where they can publish their articles.

## **WEAKNESSES OF THE PROPOSAL**

The background of the problem is not fully analysed. The assumed significance also might not be mentioned as a strong side of the project. Plus several minor remarks: Page 1 Line 36 “*projected to triple by 2030*”, I would include a reference; Page 2 Line 41, not available in the Literature; Page 2 Line 51 “*Dickman and Doncaster 1987*”, not available in the Literature; Page 2 Line 52 “*Ward et al. 1997*”, not available in the Literature. **Nevertheless, I wish good luck to the young scientists in a further scientific career.**

## **Miriam Gonzalez Gonzalez**

### **1. Substantive evaluation of project**

The project addresses an interesting topic: how the human activity can affect the behavior of different species. The gap of knowledge seems to me, that it is we need to understand better how some animals are affected by different human activities as the traffic in roads, in order to design infrastructures that could respect the animal welfare. The authors hypothesized that a) “Hedgehogs from areas of higher traffic intensity, will almost previously react by flee from incoming car, while those from rural areas will freeze or roll up” and b) “Hedgehogs in the urbanized area avoid crossing roads more often than individuals living in rural areas”. Unfortunately, previously they suggested “suggest that behavioral changes in hedgehogs more habituated to these areas with higher road traffic can explain their vulnerability” which is contradictory. Anyway, the two hypotheses are developed during the work plan so I took them as the right ones. In addition, more stress should be done in the terminology before explaining the hypothesis. Developing the last point, you can have areas with high traffic intensity for example a highway in a rural area and urban areas with low traffic; I see this as a very weak point for the further development of the proposal.

### **2. Methodology**

The proposed methodology has been considered carefully. Despite this, I do not see any reason to choose the villages randomly and then classify them. It would be more productive just choose the ones with the proper characteristics. In addition, I think that will be poor correlation between the data obtained with this experimental approach and the real situation, because hedgehogs will probably memorize some routes and pathways and they will know for experience that in these roads some cars are approximating and will react different if they are surprised far away from roads. Finally, I do not see which valuable information will be of capturing the hedgehog and tracking their behavior after the experiment, this should be explained in more detail.

### **3. Evaluation of implementation**

I think that further explanation should be provided about the relevance for the society of this experiment. Especially because it is very common specie without any endanger situation, and the final costs of the investigation would be incredibly high.

### **4. Cost calculation**



The cost calculations are calculated properly for some points but for other not. For example, I do not understand why the principal investigators do not pay themselves during the full duration of the experiments. In addition, one conference per year (three years) I think it is excessive. Finally, maybe some specialized software should be taken in consideration and probably will necessary to pay for it. Finally, I do not understand why there are no costs on the third day, this assumption is very risky.

**5. Strengths of the project**

- Interesting methodological approach that can be carried out in other species
- All the investigations for preserving the biodiversity are important

**6. Weaknesses**

- The general structure of the manuscript could be improve
- Some sentences are contradictory
- The same terminology should be properly explained and used for making the work of the reviewers better
- Some inconsistencies in the work plan and methodology

## Jayasravanthi Mokkaapati

### EVALUATION OF PROPOSAL OF RESEARCH PROJECT –

#### European hedgehogs (*Erinaceus europaeus*) response to car traffic in rural and urban areas

- Has the proposal been written with all due diligence?  
-Yes
- Does the project meet the criteria of a scientific proposal?  
-Yes
- Does the project meet the criteria of basic research?  
-No
- Does the project meet other eligibility criteria outlined in the call for proposals?  
-Yes

#### A. EVALUATION OF THE PROJECT (WEIGHTING 75%)

##### A.1. EVALUATION OF PLANNED RESEARCH OR PROJECT TASKS (WEIGHTING 60%)

mainstream academic press/journals for a given field.

- ✓3 Good. Upon its completion the project results are likely to be published in specialist academic press/ journals.

**Justification:** Good. Although this project proposal clearly benefits the society to some extent, the methodological approach need to be revised against the following queries

- From Lines 50 to 57, page 2, it is not clearly justified how the defence response/behavioural change in hedgehogs can estimate their mortality on roads.
- Existing studies on hedgehog movement patterns altered by the presence and traffic intensity of roads questions the novelty of this research to some extent! (Lines 52-53, page 2)

##### A.2. ASSESSMENT OF THE PROJECT'S INNOVATIVE POTENTIAL AND ITS IMPACT FOR THE ADVANCEMENT OF THE SCIENTIFIC FIELD/DISCIPLINE (WEIGHTING 15%)

- Innovative nature of the proposed research:  
0. The project has no innovative elements.
- Impact of the research project on the advancement of the scientific field/discipline:  
0. The project will have no impact on the advancement of the scientific field/ discipline or the project has been submitted to a wrong panel.

**Justification:**

- In terms of advancement of scientific research, the proposed project doesn't involve any new technological or scientific developments or not modifying the any existing methods for further improvement.
- The proposed adaptive behavioural changes in hedgehogs need to be justified in evolutionary perspective.
- The findings of this study can't be used for generalisations.
- Specific protections goals for hedgehogs are not clearly mentioned.
- The lines 11 to 15 can't justify the effect of urbanization on biodiversity loss with this study since minimum of two different species from different populations are required to analyses biodiversity index.

## **B. EVALUATION OF THE QUALITY OF TEAM MEMBERS (WEIGHTING 20%)**

**Not given, hence not reviewed.**

## **C. ASSESSMENT OF PROJECT FEASIBILITY (WEIGHTING 5%)**

- **Assessment of the feasibility of the proposed project, including the principal investigator's qualifications, the structure of the research team, research facilities etc.:**

✓ 2 Good.

### **Justification:**

- Proposed project is clearly feasible to conduct the experiment. However, population size of hedgehogs per site have to framed clearly.
  - Regarding test methodology, from lines 30 to 42, it may not be possible to predict road mortality from ten individuals per site.
  - The effects of other phenomenon or variables like cannibalism, parasites on hedgehog death rate are needed to infer whether urbanization is solely causing major decline of hedgehog populations.
  - Simulation of urbanization with light and noise or their combination may not be enough. On the other hand, according to the experimental design (lines 16 to 26) each hedgehog response to all three stimuli can't be measured, hence it is may not be possible to properly analyse the results against in combination. Sample sizes may vary or may not be sufficient for each stimulus at the end of experiment. Justification is required.
- **Are the costs to be incurred well justified with regards to the subject and scope of the research?**  
-yes
  - **Does the proposal meet the criteria allowing for its re-submission in a subsequent edition of the PRELUDIUM and OPUS calls?**  
- yes

## **JUSTIFICATION FOR EVALUATION**

### **Strengths of the proposal:**

- The proposed research can clearly identify the damage to the hedgehog population by growing urbanization.
- Strong justification to feasibility and budget management of this research clearly shows the passion of the investigators in conducting this work.

- Findings of this project can be used to frame protection guidelines for hedgehogs.

**Weaknesses of the proposal:**

- Proposed research goal may not justify fully the significance of the project.
- More focussed work plan is required to estimate different variables on hedgehog population as a whole.
- Poor literature supporting the proposed test methods.

## FINAL VERSION

---

Authors: Michał Bełcik, Marta Grosiak

**Title: European hedgehogs (*Erinaceus europeaus*) response to car traffic in rural and urban areas**

### **Abstract**

Increasing human population is coupled with an increase in road traffic intensity, especially in developed urban areas. This has a significant effect on various wild mammal's populations, one of those effects being road mortality. For some other species in urban areas the synurbization phenomena is associated with several morphological and/or behavioural changes in these populations. The European hedgehog is one of the most frequently killed animal on roads, but also one of the most abundant species in cities. The highly changed urban environment may be responsible for loss of their natural defensive behaviour, but increasing stress resistance to factors associated with high traffic and acquiring new behavioural pattern could allow to reduce its road mortality. Finding the significance of the behavioral changes in urban hedgehogs could be the signal of ongoing synurbization phenomenon. The main aim of our study is to find out that whether their higher abundance in more urbanized areas can be explained by their higher avoidance of roads and by their changes of behavioural response, or by the combination of both. Firstly, we hypothesize that the ratio between avoiding and crossing roads will be higher in urban areas. We also expect that hedgehogs from areas of higher traffic intensity, will less often freeze or roll-up in response to stressors associated with crossing road (light, noise of car engine), than those from rural areas. To test this hypothesis, we will select ten locations in several habitats in ten areas with different urbanization status in Poland. The study will be performed in September and October during two years (2019-20). We will estimate general size of hedgehogs population and perform a simulation of incoming car to note their response for specific stressors. The GPS collaring will allow us to collect detailed data to analyze the ratio between road avoidance and road crossing. We expect that the results of this project help us to deeper understanding of the mechanisms defining some species as city dwellers. It would be also one of the most significant examples of changes in synurban animal behavior, that alleviate high mortality caused by urbanization.

## 1. Research objective

The growth of human population is associated with extremely rapid increase in urban areas, which are projected to triple by 2030. Ongoing urbanization creates a completely new types of habitats, which in most cases have negative effect on wild population, while for some other species in urban areas, it can create new microhabitats for some populations. This phenomena, described as synurbization, is associated with several morphological and/or behavioural changes in these populations. As a result, some species may be more abundant in cities due to higher rates of survival, better availability of food sources, and also lower predation risk. Nevertheless, human activity in cities provides higher rate of mortality and disturbance of wild animals. Increasing road density and road traffic are one of the factors, which significantly reduce number of animals in urbanized areas. It is important to note that while most of the species find it difficult to adapt to this new environment, some display a significant behavioural plasticity, allowing them to thrive in cities (Sol et al 2013).

The European hedgehog is a nocturnal insectivore, present in higher densities in urbanized rather than in suburbanized areas (Hubert et al. 2011). It is one of the very few mammals that have adapted to the urban environment, which may be caused by its behavior plasticity. Unlike birds that are able to avoid some threats by simply flying away, hedgehogs are restricted to ground-based movement, and by being small they are easy to be run over by cars. One of the examples of this altered behaviour maybe its avoidance of roads, which has been shown in many studies (Rondinini and Doncaster 2002, Dowding et al. 2010, Renthlei et al. 2017). It is important to note though, that accumulated evidence remains inconsistent about whether it applies to both avoidance of approaching and crossing roads, or only to avoidance of approaching the roads. In spite of those observed changes, many studies highlighted the increasing problem with elevated urban hedgehogs road mortality (Hof et al. 2009, Rautio et al. 2016, Orłowski and Nowak 2004), reducing density of its located populations by even more than 30% in some areas (Huijser and Bergers 2000). Such high mortality suggests that hedgehog populations in cities would decline, yet studies show that they remain abundant in that habitats. Higher road avoidance in urban populations might also be an insufficient explanation, since they do not appear to alleviate mortality rates in cities. We suggest that another factor, apart from the better food availability about lower predation, might be involved in keeping a hedgehog urban populations abundant.

**The main aim of our study is to find out whether an observed high hedgehog abundance in cities, in spite of significant road mortality, can be explained by their avoidance of roads, by their behavioural response, or by the combination of both.** We predict that urban hedgehogs will display a different behavioural response when facing road traffic threats and while crossing roads than rural ones. We will have a two different approaches – one on analyzing spatial patterns of movement and road avoidance, and other on performing a behavioural experiment trying to find a response for this stressor triggered by approaching car. Specifically, we will test following hypotheses:

- III. The ratio between avoiding and crossing roads will be higher in urban areas. We predict that in urban areas, a observed behaviour of road avoidance would be more frequent than in rural areas. In spite of that, due to the higher road density and higher population intensity in urban areas they would have to cross them more often, hence the higher crossing rate in urban than in rural areas.
- IV. **Hedgehogs from areas of higher traffic intensity, will less often freeze or roll-up in response, that those from rural areas.** Because many studies have investigated only avoidance of crossing roads (Dowding et al. 2010), but it can be associated mostly to avoiding asphalt, as an aversion to the synthetic surface (Mulder 1999). In this project we want to focus on describing the different modes of behavior response from approaching car. The experiment design will allow us to test a hedgehogs' response from two very specific stressors (light occurrence and noise of car engine). We predict that urban individuals should be less stressed

by all these factors (less often roll-up), since animals settled in urban environments tend to be more stress resistant (Surov et al. 2016, Herr et al. 2008). We also want to check if light stressor causes more frequent response for rural population, due to lower light pollution in suburban areas

## 2. Significance of the project

Human-induced habitat loss due to urbanization is amongst one of the most frequently mentioned causes for biodiversity loss (Gaston et al. 2003). Increasing road density results in heavy fragmentation of wildlife habitats, leading to the degradation of ecosystems and their functions (Ibisch et al. 2017). Due to the rapid nature of these changes, they may exceed a limit of tolerance for many species. However, many animals that live in urban environment frequently differ in behaviour, compared to those from rural ones. Growing number of studies provide evidence that observed behavioural adjustments, that could be reflected by phenotypic plasticity, are needed to persist in urban habitats, however the underlying mechanism remains unclear (Sol et al. 2013). Only individuals of animals with specific traits, including adjusted behaviours, are able to colonize and have stable populations in cities. Most of those species successfully adapted to deeply changed habitats are birds, mostly passerines like Great tits (Hu and Cardoso, 2010) or European pied flycatchers (Seppänen et al. 2011). There are few examples of mammals in this group, like red foxes that modify their ranging activity (Baker et al. 2007) or fox squirrels that invest less time in vigilance behaviour (McCleery 2009).

One of such mammals is a European hedgehog (*Erinaceus europaeus*), which may serve as an interesting model species. Most of its species range is located in Western Europe, and it is present in a large variety of habitats. Its ground-dwelling behaviour reduces its ability to escape from threats and forces it to deal with the problem of growing road networks (Ibisch et al. 2017). In spite of this potential vulnerability, it is more abundant in cities than in rural environment (Hubert et al. 2011). So far, there have been studies on hedgehog populations in cities (Hubert et al. 2011, Dickman and Doncaster 1987, Dowding 2010), how their dietary preferences or predator response has changed (Ward et al. 1997), or how hedgehogs' movement patterns are altered by the presence and traffic intensity of roads (Rondinini and Doncaster 2002, Dowding et al. 2010, Renthlei et al. 2017). However, there was no study that tried to address the question of hedgehog abundance in cities, despite its higher road mortality. Factors like alleviated pressure from predators and better food availability are often stated, but given this species' behavioural plasticity (typical for a synurban species), it is possible that other behavioural factors might be involved. Tendency to avoid roads in cities could be one of those factors, but different behavior when facing a threat might be another one. So far, there has been very few studies tackling this specific issue of how their defense response might vary when faced with a new type of threat, that is an incoming car (Mulder 1999). Any research on that topic could bring us closer to answering the issue of high hedgehog mortality on the roads (Huijser and Bergers 2000, Mulder 1999), and could bring a substantial impact to the field of urban ecology.

Behavioral responses to stressors associated with urban environment are poorly recognized, yet their better recognition is crucial towards deeper understanding of the mechanisms defining some species as city dwellers. Hedgehogs have quite a unique defensive response (they roll-up in defense), which is sometimes suggested as an explanation for their high road mortality (Mulder 1999). If behavioural differences are detected, this could be used as a starting point to address interesting questions about the nature of this change - whether, is it an adaptation through natural selection. It would be also one of the most significant examples of changes in synurban animal behavior, that alleviate high mortality caused by urbanisation. We expected that the results of this project will also highlight what lies at the base of many problems with “brave” behavior of larger animals (Sol et al. 2013) due to higher stress resistant in cities causes significant problems and large costs.

### 3. Work plan

Research areas and particular roads with neighboring sites have been already chosen, and at the beginning of May 2019 all transects will be designated (fig. 1). The initial stage of project will start from defining the status of traffic intensity. In this stage we will also create a team of people that have experience with working on small wild animals, and obtaining necessary permissions from Regional Environment Protection Directory and Local Ethics Committee because in our study we will work on European hedgehog, partially protected animal in Poland. Then, we are planning to perform preliminary study where we will investigate hedgehogs' population density on each chosen site.

On half of the locations, the transect inspection with car simulation approach and hedgehogs collaring will start in September 2019, and will last for a month. Every time the tracking will take place just after dusk. On the other half of the locations, inspections will start in October 2019, and will also last for a month. Simulating experiment and tracking of collared animals will finish in October, so for the rest of the year we will focus our attention on data analysis. In the next year, those locations that in the previous year were inspected in September 2020, will in turn be inspected in October 2020 – and vice versa. The end of the whole project is planned for December 2020.

- 1.) Performing the preliminary study of hedgehogs' population density on each site will give us the information about the possibility of conducting research and the availability of suitable habitats.
- 2.) Data about detailed estimation of population density (from performing our tracking) will allow us to test the assumption of higher abundance of hedgehogs in urban areas.
- 3.) Testing the ratio of road crossing and road avoiding in each area will deliver important information about general hedgehog's behavioral pattern of avoiding roads. It will allow us to test the I hypothesis.
- 4.) By performing behavioral test for response to very specific stressor which is approaching car, we will be able to test our II hypothesis about hedgehogs with higher stress resistant changing their behaviour.
- 5.) Performing behavioral test for response to very specific factors (light, car engine noise) will allow us to tell whether each particular stressor is the main cause of fear, especially for hedgehogs that partly habituate in more urban areas in higher level of light pollution (II hypothesis).
- 6.) Proving our hypotheses, we can think about future project where we will investigate possible adaptive and evolutionary basis of those changes.
- 7.) Proving our hypotheses, we can think about future project where we will investigate possible existence of such adaptive behavioural response in other urban species.

#### *Risk hypotheses:*

1. In case if founded, lower hedgehogs' density in urban areas would mean that problem with high mortality is more significant than stated in other publication.
2. Lack of road avoidance in urban areas would mean that it is a direct reason for high mortality and we can explain high hedgehogs' density in cities only by food availability or lack of predator's presence, without any behavioral adaptations to avoid direct danger associated with roads.
3. If there would be no difference in proportionally higher road avoidance in comparison to road crossing in more urbanized areas, it could mean that in rural areas hedgehogs avoid roads more frequently.
4. In case if found, higher frequency of roll-up behavior in hedgehogs living in cities would mean that urban environment provides more stress for these animals, or that these rural individuals fail to recognize dangers associated with cars.

The results of behavioural experiment could be published in “Behavioural Ecology” or “Animal Behaviour”, whereas results of movement data analysis could be published in “Landscape and Urban Planning” or “Landscape Ecology”. Results from the whole project could be published in “Biological Conservation”.

#### 4. Methods of research

##### a.) *Research team and permissions*

The initial stage of project will start on May 2019, and first month will be spent on creating team of people that have experience with field working on small wildlife. Because in our study we will work on European hedgehog, partially protected animal in Poland we will apply for necessary permissions from Regional Environment Protection Directory and Local Ethics Committee.

##### b.) *Research areas*

The study will be conducted in five cities (urban area) and in five villages (rural area), in Poland. Ten specific locations will be chosen randomly, and then in each of them we will chose ten specific sites reflecting the habitats potentially preferred by hedgehogs, dominated by small scale grasslands with low density of woodland fragments (Hujiser and Bergers 2000). These sites can be divided into few categories (tab. 1).

Tab. 1. Categories of chosen sites for the study

<b>Urban</b>	<b>Rural</b>
Parks, Botanical Gardens	Gardens, Orchards, Fields
Gardens	Pastures
Green areas between settlements	Small woodlands

Each site will have minimum 25 ha and will be make from 50 m buffer zone built around roads. Those roads will differ in night traffic intensity (when hedgehogs are most active), so sites in urban areas will be placed around roads of high intensity, while those in rural areas will be placed around roads of low intensity. In addition to the information about the road nighttime traffic, we will verify it by counting vehicles per 1h during period between dusk and dawn. The size of experimental sites and distance from roads are based on hedgehog's home range size, which is about 10-50 ha (Hujiser and Bergers 2000). We will draw a line transects of approximately 5 km. on each location. Then, we are planning to perform preliminary study where we will investigate hedgehogs' population density in each chosen sites to justify the possibility of conducting research and the availability of suitable habitats.

##### c.) *Animals*

In each site we want to perform behavioural experiment (II hypothesis) on minimum 18 and maximum 100 individuals in each year. The minimal distance between individual hedgehogs on which we will performed our experiment, will be  $\pm 30$  m on transects in urban area and  $\pm 60$  m. This approach will allow us to avoid startling all neighboring hedgehogs.

##### d.) *Experiment design*

###### First experiment (II hypothesis)

Field experiment will be carried out between September and October for two years (2019-20), for each night during one month. Such choice of period is necessary to avoid breeding season and winter hibernation (Rondinini and Doncaster 2002). On half of the locations, the transect inspection with car simulation approach and hedgehogs collaring will be performed in September 2019. On the other half of the locations, inspections will be performed in October 2019. In the next year, those locations that in the previous year were inspected in September 2020, will in turn be inspected in October 2020 – and vice versa, to avoid the effect of association between the site locations and different temperature condition in each month.

On each investigated site person equipped witch infrared binoculars, gloves, flashlight, portable loudspeaker and a GPS will walk along delimited transects. For each hedgehog encountered every 30 m (urban area) or 60 m (rural area), we will simulate approaching car, starting from the distance of about 10 m. Simulation will be based on either switching on the light (L), playing a playback with car engine noise (E) or combination of both (L+E), while simultaneously approaching the hedgehog. Simulation type will be switched between met hedgehogs, so for the first hedgehog met this night, L



simulation will be used, E for the second, L+E for the third, then again L for the fourth etc. The exact elements of behavioral response and reaction time will be noted, and combined they will lead to one of the three types of response (tab.2.).

Tab. 2. Different types of behavioral response

<b>Behavioral response</b>	<b>Description</b>
Flee	Hedgehog starts to flee continuously in any direction other than towards the person, in no more than 5 seconds
Freeze	Hedgehog will freeze (roll-up) at any point of his reaction
Other	Any other type of behavior

After this part, the hedgehog will be captured by hand and sexed, weight, check for parasites and injures. Animals will be marked individually by unique pattern of painting (special dye for animal’s fur) (fig.2). On half of chosen locations, 50 individuals (for each type of areas) weighting more than 550 g (Dowding et al. 2010) will be GPS collared. Collars will automatically fall of after a period of up to six days (Rondinini and Doncaster 2002), so they can be reused for collaring of another individual. Because we will have only five collars for tracking maximally 100 hedgehogs per year, we will change each collar ten times per year (each data collection takes six days and tracking will take 60 days per year). All capturing and handling procedures will be performed under the license from Regional Environment Protection Directory and Local Ethics Committee.

Second experiment (I hypothesis)

Each collared animal will be tracked within period of up to six days and released immediately, the interval between GPS transmitter signals is 3 min. Analysis of obtained data deliver us the information of frequency of their road avoidance and road crossing. We will also analyze their general mobility (migrated distances), habitat preferences (distances between center of activity and road) and rate of road mortality. Additionally, locations of all of the hedgehogs (both dead and alive) met close to the road edge or on those roads will be marked on GPS.

*e.) Statistical analysis*

We will use t-test to analyze the difference between density of urban and rural population. To randomize our urban and rural area selection we will apply randomized searching formula using a database of Poland cities.

Because we will have data only from 10 sites in every area’s type we will use pseudo-replication procedure (Hurlbert 1984). For the main analysis, we will use GLM models. First model (I hypothesis) will include the ratio of road avoidance/road crossing as response variable, and other variables: stress type (L vs E vs LE), type of site (urban vs rural), site ID (1-10), habitat type (listed in tab. 1), day, observer ID (1-18), year (2019 vs 2020) and covariates: the hedgehog mass and parasite presence. The second model (II hypothesis) will include the type of hedgehog as response variable, and other variables: type of site (urban vs rural), site ID (1-10), habitat type (listed in tab.1), day, observer ID (1-18), year (2019 vs 2020) and covariates: the road width. Significance of the fixed effects was tested with F-tests. Significance of the random effects for which the variance estimate was positive was tested with the likelihood ratio test. All statistical analysis will be carried out with the latest R version.

*f.) Analysis of GPS collar trajectories*

Tracking data will be analyzed in order to obtain information about the tendency to cross or not cross the roads. For each observed trajectory, 100 random walks from the same start point will be simulated in order to test whether observed frequencies in road avoidance and crossing are random, or present a certain pattern (Rondinini and Doncaster 2002). Each walk will have the same number of points and the only random part will be the direction taken from one location to the next, and simulated locations will be constrained that it would not fell in impossible places (such as inside buildings, water bodies etc.). Differences between observed and simulated trajectories will be tested with Bonferroni-corrected paired-sample permutation tests (Rondinini and Doncaster 2002). The rest of the spatial data will be

later used for the further analyses. For all of the spatial analyses, we will use latest versions of ArcGIS, R and Fragstats.

## 5.Literature

- Baker P. J., Dowding C. V., Molony S. E., White, P. C. L. & Harris, S. 2007. Activity patterns of urban red foxes (*Vulpes vulpes*) reduce the risk of traffic-induced mortality. *Behavioral Ecology* (18) 716-724.
- Doncaster C.P., Rondinini C., Johnson P.C.D. 2001. Field test for environmental correlates of dispersal in Hedgehogs *Erinaceus europaeus*. *Journal of Animal Ecology* (70), 33–46.
- Dowding C.V., Harris S., Poulton S., Baker et al. 2010. Nocturnal ranging behaviour of urban hedgehogs, *Erinaceus europaeus*, in relation to risk and reward. *Animal Behaviour* (80) 13-21.
- Gaston K.J., Blackburn T.M., Goldewijk K.K. 2003. Habitat conversion and global avian biodiversity loss. *The Royal Society* (270) 1293–1300.
- Hubert P., Julliard R., Biagianti S., Pouille M.L. 2011. Ecological factors driving the higher hedgehog (*Erinaceus europeus*) density in an urban area compared to the adjacent rural area. *Landscape and Urban Planning* (103) 34– 43.
- Hurlbert S.H. 1984. Pseudoreplication and the Design of Ecological Field Experiments. *Ecological Monographs* (54) 187-211.
- Huijser M.P, Bergers P.J.M. 2000. The effect of roads and traffic on hedgehog (*Erinaceus europaeus*) populations. *Biological Conservation* (95) 111-116.
- Ibisch P.L., Hoffmann M.T., Kreft S., Pe'er G. Kati V., Biber-Freudenberger L., Sala D.A.D. Vale M.M., Hobson P.R. Selva N. 2017. A global map of roadless areas and their conservation status. *Science* (354) 1423- 1427.
- Mccleery R.A. 2009. Changes in fox squirrel anti-predator behaviors across the urban–rural gradient. *Landscape Ecology* (24).
- Mulder J.L. 1999. The behavior of hedgehog on roads. *Lutra* (42) 35-37.
- Orłowski G., Nowak L. 2004. Road mortality of hedgehogs *Erinaceus spp.* in farmland in lower Silesia (South-Western Poland). *Polish Journal of Ecology* 52:377–382.
- Rautio A., Isomursu M., Valtonen A., Hirvelä-Koski V., Kunnasranta M. 2016. Mortality, diseases and diet of European hedgehogs (*Erinaceus europaeus*) in an urban environment in Finland. *Mammal Research* (61) 161–169.
- Renthlei Z., Borah BK., Trivedi AK. 2017. Effect of urbanization on daily behavior and seasonal functions in vertebrates. *Biological Rhythm Research* 48:789–804.
- Rondinini C., and Doncaster C.P. 2002. Roads as barriers to movement for hedgehogs. *Functional Ecology* (16) 504–509.
- Seppänen, J., Forsman, J., Mönkkönen M., Krams I., Salmi T. 2011. New behavioural trait adopted or rejected by observing heterospecific tutor fitness. *Proceedings of the Royal Society B* (278) 1736-1741.

Sol D., Lapiedra O., Gonzales-Lagos C. 2013. Behavioural adjustments for a life in the city. *Animal Behaviour* 1-12.



Fig. 1. Map with chosen locations.

Fig. 2. Individual marked European hedgehog with VHF transmitter.

## 6. Table with budget of the project.

Item			
	2019	2020	Total
<b>1. Direct costs, including:</b>	142 780	128 600	271 380
1/ Salaries and benefits	67 600	79 600	147 200
2/ Equipment	32 295	-	32 295
3/ Other direct costs	42 885	49 000	91 885
<b>2. Indirect costs</b>	44 194	51 440	95 634
<b>Total costs (1+2)</b>	186 974	180040	367 014

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

### 1. Direct costs:

#### 1/Salaries and benefits:

##### *Salaries for main project performers*

(TASKS: planning, preparing and conducting preliminary experiment, main experiments, statistical analyses, GPS data analyzes, conferences application and participation, writing articles)

1500 PLN (max PRELUDIUM salary) x 8 months x 2 persons = 24 000 PLN / per 1st year

1500 PLN (max PRELUDIUM salary) x 12 months x 2 persons = 36 000 PLN / per 2nd year

##### *Salaries for all project performers (fee –for–task agreement)*

(TASKS: experiment performing – in total 120 working nights, 8 h per 1 night)

2 500 PLN + 9% \* 2 500PLN (insurance costs) x 2 months x 2 persons = 2 725 PLN x 2 x 8 = 43 600 PLN / per year

## 2/ Equipment

Loudspeaker (Blow BT 460) – 80 PLN x 10 persons = 800 PLN

Flashlight – (Olivat MAY) – 179 PLN x 10 persons = 1 790 PLN

GPS – (Garmin Etrex) – 785 PLN x 10 persons = 7 850 PLN

Faunabox (sunplast, 7.5 l) – 24,50 PLN x 10 persons = 245 PLN

Weight (Medio Line, kg) – 262 PLN x 10 persons = 2 620 PLN

Infrared binocular (YUKON Night Vision No) x 10 persons = 1 399 PLN x 10 persons = 13 990 PLN

GPS collar (MicroLightTrack, 10g, ) = 5 collars x 1000 PLN = 5 000 PLN

## 3/ Other costs

Gloves – 3,50 PLN x 10 persons x 2 = 35 PLN

Special dye for animal's fur (ManicPanic, 500 ml) = 50 PLN

Batteries (GP) – 80 PLN x 10 persons = 800 PLN

Travel allowance – 140 PLN x 30 days x 10 persons = 42 000 PLN/ per year

Conferences – foreign conferences – 1 750 PLN x 2 persons x 2 conferences in 2<sup>th</sup> year = 7 000 PLN



WORK IN PROGRESS...



...AND LATER ON TRIP TO OBSERVATION TOWER ON MAGURKA MOUNTAIN