April 2022

NEJCZER.

Methodological workshop - Evolutionary biology - Grant writing

Deciphering anxiety in the brain

Nematode relationship with bark beetle and spruce

Stress level of wolves using green bridges

Ochotnica Górna 21.04.22-26.04.22

Cover made by Monika Hoffmann

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Research topics proposed and chosen by participants

1. How the diversity of nematodes vary in outbreaking and not outbreaking populations of spruce bark beetle? - JMG
2. Patterns of nematode diversity in relation to wolf migrations across Europe -SiD
3. Does ketogenic diet change the structure of perineuronal nets? – ZR
4. Does ketogenic diet reduce the risk of posttraumatic epilepsy? – ZR
5. How different diets affect susceptibility to anxiety disorders? – ZR & SyD
6. **Does anxiety network work similarly in different model species? - SyD**
7. Which proteins are synthesized within IPN neurons that innervate ventral hippocampus? - SyD
8. The role of adult sexual size dimorphism in shaping fitness components of the offspring - GB
9. **How does proximity to roads affect animal stress levels? – MH & SyD**
10. Do mitigation measures, such as green bridges, help endangered species to persist? - MH
11. Did pandemic enhance the spread of fake news and misinformation? - MH
12. How altitude and climatic conditions affect tardigrade diversity? - PKD
13. Factors by which domestic dogs provide thread to wildlife – PKD
14. Use of resources among different species of Yuhina genus – PKD
15. **Is the diversity of nematode, present in spruce bark beetle, dependent on a spruce trees condition? – JMG**
16. Nematodes distribution in a spruce forest according to the altitude – JMG
17. Search for beneficial mutations in order to reduce the risk of cancer – SiD
18. Application of game theory for human sexual selection – SiD
19. Can mimicry be reversed over the lifespan of a frog? – GB
20. Song learning in relation to paternal age in zebra finches – GB
21. The role of Nerve Growth Factor in the control of IPN-vHipp circuit - SyD

The topics in bold were chosen and described in project proposals.

Project 1: Nematode diversity present in Norway spruce forests as a possible biological control for spruce bark beetle pest

Draft project proposal

Title: Nematode diversity present in Norway spruce forests as a possible biological control for spruce bark beetle pest

Authors: Siri Devarakonda & Julia Morales-García

Summary: Control spruce bark beetle outbreaks has become a major issue in recent years as the effects of this pest on Norway spruce forests have been intensified by climate change. A true solution for this problem has not yet been achieved, but it is known that *Ips typographus* has numerous interactions with species of symbionts and parasites. Among them, one of the most common groups that can be found in association with this species are nematodes. Although several species of nematodes are known to be present in these ecosystems, it is not really known whether they have a positive or negative influence on spruce bark beetle outbreaks. In the present study, it is intended to know if there is a variation in the diversity of nematodes in Norway spruce trees in healthy and unhealthy conditions, and if this variation also occurs in the spruce bark beetle populations. In this way, it is intended to understand the relationships between the species, in order to design a more accurate forest management plan for the spruce bark beetle, based on the possible biological control carried out by the different species of nematodes, being able to approximate a solution for all the Norway spruce forests along Europe.

1) Scientific goal of the project

Norway spruce forests constitute the largest percentage of forests in Europe. In recent years, the species *Ips typographus*, better known as spruce bark beetle, has led to the loss of hundreds of hectares of these forests. This pest can easily outbreak when the beetle density is enough and in optimal environmental conditions, such as storms or droughts, which is why it has intensified in recent years due to climate change.

This pest is difficult to control because most of its life cycle is inside spruce trees. A possible assessment that has not yet been studied is the interaction of these beetles with nematodes that live in the bark of spruce trees. They are known to parasitize spruce bark beetles but is not really known if there is a negative or positive influence of this relationship on *Ips typographus* populations.

Considering that nematodes are biological indicators susceptible to changes in the ecosystem where they are found, our goal is to know if there is a difference between the species of nematodes present in healthy spruce trees, and in those that are weakened or close to the senescence and compare that diversity of nematodes with that found in the spruce bark beetle. With this we intend to better understand the relationships between nematodes and the spruce bark beetle, in different conditions of the spruce bark beetle hosts, to see if any nematode species could become an adequate biological control for this pest.

Hypothesis:

1. There is a difference in nematode diversity within the spruce tree in different ecological conditions.
2. There is any nematode species capable of assuming a biological control for spruce bark beetle.

2) Significance of the project

Pest forest management is an emerging need in terms of ecosystem conservation. These pests can outbreak, causing a high population growth in a relatively short period of time (Kausrud et al. 2012, Mezei et al. 2019). In the last years, the intensity of outbreaks in some forest pests has increased due to climatic variations, causing important economic and environmental losses (Ogris et al. 2010, de Groot et al. 2019).

Norway spruce forests in Europe (*Picea abies* L. Karst.) are a clear example of a species that suffer these pest outbreaks. Norway spruce is also affected by climatic change because forest composition and high temperatures increase the vulnerability of these trees against pests (Faccoli et al. 2014, de Groot et al. 2019).

Spruce bark beetle, *Ips typographus*, is a species from Curculionidae family, that is consider the most destructive forest pest along Europe (Mezei et al. 2019). It can outbreak easily in specific environmental conditions such a windthrow, droughts or storms (Faccoli et al. 2014, de Groot et al. 2019). Beetle density and health conditions of the host trees also influence the prevalence of spruce bark beetle to cause mass infestations for spruce trees, and their subsequent death (Ogris et al. 2010, Faccoli et al. 2014).

Fig 1. Example of bark-beetle infested trees in the High Tatra Mountains National Park in Slovakia. Bark beetle-infested trees are in the red rectangle. (Source: Mezei et al. 2019)

Spruce bark beetle is capable of building galleries inside trees, in which it breeds and lays its eggs. The larvae feed on the phloem of Norway spruces, making difficult the correct transport of nutrients throughout the tree. When the beetles become adults, they disperse to other trees where they reproduce and restart the cycle (Wermelinger 2004, Kausrud et al. 2012). When these populations are in an outbreak phase, the number of beetles is so high that they cause mass infestations in Norway spruce forests, causing great losses in these ecosystems (Wermelinger 2004, Kereselidze & Wegensteiner 2007, Ogris et al. 2010). In this way, the landscape is also altered, being able to distinguish patches throughout the forest where the Norway spruces have suffered the effect of this pest (Fig. 1).

These beetles are known to have multiple symbionts, many of them capable of conferring resistance against tree defences (Wermelinger 2004, Kereselidze & Wegensteiner 2007, Michalkova et al. 2012). Apart from these symbionts can also be found nematodes, whose diversity has been studied, but a clear interaction with beetles has not been clarified. These nematodes not only live inside of beetles being able to live in their gut or haemolymph, as is the case of *Controtylenchus diplogaster*, or under their elytra, like the genus *Bursatylenchus* (Kereselidze & Wegensteiner 2007, Burjanadze et al. 2015), but are also found in the wood of spruce trees, on which *Ips typographus* feeds (Renco et al. 2015).

Spruce bark beetle is capable of infesting and killing large hectares of healthy trees or take advantage of the weakened trees (Renco et al. 2015), however it is not known how the diversity of these nematodes changes as the state of the tree changes. Nematodes are considered a biological indicator of the quality of their hosts (Burjanadze et al. 2015, Renco et al. 2015). Knowing the relationship of these nematodes with the spruce trees will allow a better management of spruce bark beetle pest based on biological control, and a better understanding of the ecological dynamics of European forests.

3) Concept and work plan

1. Study area. A total of six National Parks in Poland.
   a. Babia Gora National Park
   b. Tatra National Park
   c. Gorce National Park
   d. Pieniny National Park
   e. Magurski National Park
   f. Biesczady National Park

2. Work plan.
   - Collect the samples of the spruce bark from each National Park in four patches of spruce forests depending on the condition: two in healthy spruce trees areas and two in unhealthy spruce trees areas along the forest. The area of the patch is divided into 2 km × 1 km and 20 bark samples from different trees are collected in 4×4×4 cm.
   - We plan to collect 50 spruce bark beetle individuals in the same sampling plots by hand or by cutting infested log sections from spruce trees.
   - Isolate the DNA from the samples and use amplicon sequencing method to with specific nematode primers. We plan to dual index the primers by running PCR twice with Illumina adaptor overhang nucleotide sequence for forward and reverse sequence. DNA will be run on a gel and amplicons are excised and purified using the QIAquick Gel Extraction kit (QIAGEN, Germany) according to the manufacturer's instruction.

4) Research methodology

1. Specific research goals:

- To identify the nematode diversity and presence/absence of different species in Norway spruce trees.
- To identify the presence of nematodes in Norway spruce trees inside the spruce bark beetles.
- To compare the nematodes in spruce bark beetle with Norway spruce trees nematodes.
- To study the positive/negative influence of the presence of these nematodes in the spruce bark beetle.

2. Results of preliminary:

- Preliminary results have shown the presence of the given nematodes in spruce bark beetle: *Contortylenchus typographi* (Tylenchida: Tylenchoidae), *Controtylenchus sp.* (Tylenchida: Tylenchoidae), *Bursaphelenchus sp. Parasitorhabditis sp.* (Rhabditida: Rhabditidae)
- Preliminary results have shown the presence of given nematodes in Norway spruce trees: *Acrobeloides nanus, Eudorylaimus silvaticus* and *Paratylenchus microdorus*.

3. Research, risk analysis:

- Possible bias of the nematodes in different samples.
- Contamination of the samples in DNA isolation and sequencing.
- Possibility of samples without nematodes.

4. **Project literature**

Burjanadze, M. Lortkipanidze, M. Supatashvili, A., Kajaia, G. 2015. Nematodes associated with bark beetle *Ips typographus* in Borjomi Gorge. Bulletin of the Georgian National Academy of Sciences 9(1): 163-166.

de Groot, M., Diaci, J., Ogris, N. 2019. Forest management is an important factor in bark beetle outbreaks: lessons for the future. Forest Ecology and Management 433: 467-474.

Faccoli, M., Bernardinelli, I. 2014. Composition and elevation of spruce forests affect susceptibility to bark beetle attacks: implications for forest management. Forests 5: 88-102.

Kausrud, K., Okland, B., Skarpaas, O., Gregoire, J.C., Erbilgin, N., Stenseth, N.C. 2012. Population dynamics in changing environments: the case of an eruptive forest pest species. Biological Reviews 87: 34-51.

Kereselidze, M., Wegensteiner, R. 2007. Occurrence of pathogens and parasites in *Ips typographus* L. from spruce stands (*Picea orientalis* L.) in Georgia. Insect Pathogens and Insect Parasitic Nematodes 30(1): 207-210.

Mezei, P.m Potterf, M., Skvarenina, J., Rasmussen, J.G., Jakus, R. 2019. Potential solar radiation as a driver for bark beetle infestation on a landscape scale. Forests 10(7): 604.

Michalkova, V., Krascsenitsova, E., Kozanek, M. 2012. On the pathogens of the spruce bark beetle *Ips typographus* (Coleoptera: Scolytinae) in the Western Carpathians. Biologia 67(1): 217-221.

Ogris, N., Jurc, M. 2010. Sanitary felling of Norway spruce due to spruce bark beetles in Slovenia: a model and projections for various climate change scenarios. Ecological Modelling 221: 290-302.

Renco, M., Cerevkova, A., Homolova, Z., Gomoryova, E. 2015. Long-term effects on soil nematode community structure in spruce forests of removing or not removing fallen trees after a windstorm. Forest Ecology and Management 356: 243-252.

Wermelinger, B. 2004. Ecology and management of the spruce bark beetle *Ips typographus* – a review of recent research. Forest Ecology and Management 202: 67-82.

Project schedule: anticipated tasks

No	Name and description of task	Expected completion dates	Expected cost (zl)
1.	The purchase of necessary equipment and materials	2023-2024	32.000
2.	Fieldwork in Poland	2023-2024	80.100
3.	Molecular analysis and other laboratory work	2024-2025	802.300
	Preparation of manuscripts and conference presentation, participation in conferences.	2023-2025	30.000
		Total	**944.400**

Proposed budget:

No.	Items	Funds for each budget year (zl)			
		2023	2024	2025	**Total**
1	Direct cost, including:		680.000	46.700	
	1. Salaries and benefits	160.000	60.000	60.000	
	2. Equipment	10.000			
	3. Other direct costs	50.000	9.000	9.000	
2.	Indirect costs	6000	4.000		
3.	Total costs (1+2)	226.000	753.000	115.700	**1.095.000**

Direct details of the cost items

1) Salaries and benefits
   Principal Investigator (1 person) – 36 months: 180.000 zl
   Field assistants (10 persons) – 3 months: 60.000 zl
   Technical assistant (2 persons) – 10 months: 40.000zl

2) Equipment
   Laptops Intel Core i7-9750H six-core processor (6x4.5Ghz): 30.000 zl
   For data analysis.

3) Other direct costs
   a. Expenses related to the field work in Poland. March-May 2023.
   A total of 3 months (90 days) and 10 people.

- Diets: 30 zl (person/day): 30x90x10= 27.000 zl
- Travel costs (private cars): 5 travels to one National Park to the next one and go and come to Krakow.
  Total: 100 zl rent x 90 days + 100 zl/gas x 5 trips + 200 zl/gas come and go to Krakow = 9.700 zl x 3 cars = 29.100 zl
- Accommodation: 20 zl/person/day: 20x90x10= 18.000 zl
- Other expenses for setbacks in field: 6.000 zl

b. Materials for chemicals and molecular methods for analysing the DNA (1680 Samples). Cost:

- DNA isolation kits: 680.000 zl
- PCR Primers: 4.200 zl
- PCR kits: 42.500
- DNA Sequencing in Illumina: 75.600 zl
- Equipment for isolation of DNA and PCR (Eppendorf tubes, pipets tips): 300 zl
- Office materials (paper, printer refills, etc.): 2.000 zl
- Expenses related to travelling within Poland: 4.000 zl

c. Fee and travel costs related to participation in international conferences:

- July 22nd 2023 International Conference on Forest Entomology and Insect Physiology (ICFEIP) in Tokyo, Japan: 15.000 zl
- Aug 9th 2024 International Conference on Biodiversity and Forest Entomology (ICBFE) in New York, United States: 15.000 zl

Reviews

Aneta Arct – abstract review

The guidelines of most research projects suggest that abstract should include the most important information on the project allowing the experts requested to review the project to assess their competencies to perform the review. Unfortunately, this abstract does not meet the requirements (lack of clear project objectives and a brief description of the methods). Moreover, I suggest writing a more appealing abstract that describes how the project can contribute to understanding the relationships between the nematodes and spruce bark beetle, stressing its novelty. By the way, it is not understandable to me what the aim of the project is, whether the project will be a study the influence of nematodes on spruce bark beetle outbreaks or if there is a variation in the diversity of nematodes in Norway spruce trees in healthy and unhealthy conditions. The 'opening line' should always relate to the topic being presented and the context within which it is being shared. However, the first sentence suggests that it that the project will refer to spruce bark beetle outbreaks. The Latin name should appear right after the species name is first used in the text.

Mariusz Cichoń

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

The project aims at checking out whether nematodes may constitute an important biological agent to control population outbreaks of bark beetle pest which may severely affect forest health. The question is important as the losses in wood production and biodiversity might be substantial due to action of bark beetles. The chemical control via pesticides is costly and limited in action, so possibilities of biological control of the population of bark beetles may constitute an important solution in forest management. I am not an expert in this area, but the research proposal sounds like an important and original one. The potential impact of the proposed study may have important consequences for forest management providing that the research will bring results indicating good candidate nematode species for control of bark beetle population.

Unfortunately, the proposal is weakly structured in terms of explaining what will be done and why, and what result will lead to achieve the goal of the project. The goals, themselves, are weakly defined and the hypotheses are loosely related to these goals and, in my opinion, too general. I might wrongly read the intentions of the authors, so please forgive me if I wrongly got your intentions.

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

Although this proposal sounds like highly applicable, I consider it as belonging to basic studies. The aim of the project sounds like not to establish a new ways of pest control, but rather to study diversity of nematodes in tree trunks and within the bettles, even without considering whether these nematodes do any harm to the pest. So, the only output of the project would be publications and conference communications. Again, I am not an expert in this field, so I can not asses on potential quality of the results and suited journals. Definitely, the results should be good enough to publish in indexed international journals in the area of entomology and foresty. The authors also plan to attend two important international conferences with presentations of the project output.

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

The project is fully feasible. It will generate some data on nematode diversity and this by itself will be publishable. I do not understand why the study is planned in national parks, and why the authors consider 6 national parks in Poland, and why those, and why only in Poland. I can not find any arguments in the proposal explaining whether three are any expectations concenrning this choice, or these are only repetitions, and if so, why these repetions are required. There are also no arguments for the sample size, why do you need so many samples: 4 plots in each locality, 20 bark samples and 50 beetle samples from each plot. This sounds a lot. Are you sure you will find healthy plots in the localities already heavely infected? Will you control for severity of infection somehow?

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

The costs of the project is very high and the project is very big. 13 persons involved! Too big for the potential outcome of the project. The budged is not carefully planned, the costs in the project schedule does not math to the project budget and the explanations of the costs also do not match the budget.

5. **Strengths of the proposal**

Important biological question that may potentially lead to establishing a new biological control of pest population. Preliminary data.

6. **Weaknesses of the proposal**

Weakly structured text of the project, the goals and hypotheses are too general. Definitely too high project budget and not very well thought.

Monika Hoffmann

1. Assessment of scientific quality of the research project

Norway spruce is one of the predominant tree species in Europe. It is an important tree species in terms of economy and ecology. One of the greatest threats apart from climate change to this tree species is the spruce bark beetle. Destabilized forests provide ideal conditions for the outbreak of this pest and a true solution how to control the outbreaks has not been found yet. A solution could be to understand what role nematodes play in relationship to spruce bark beetle outbreaks. This project aims to identify if there is a difference in nematode diversity and spruce tree in different ecological conditions and their interaction. Research on nematodes is available but their role and interaction with spruce and spruce bark beetle is not known. This research will be conducted in six national parks in Poland. In each region four sites will be selected with two different tree conditions. Two sites with healthy trees and two sites with unhealthy trees. From each site the bark sample of a tree, and 50 bark beetle individuals will be collected, and their DNA will be analysed. The method appears to be straight forward. This study is well structured. The references show that this topic is of international importance, as this kind of research has not been done yet. Although nematodes in spruce bark beetle were studied, their specific relation is unknown. The study will be conducted in Poland, but the results could be of international relevance.
In my opinion, the DNA analysis is described too briefly, as I do not understand how the positive and negative influences will be tested. The first part of the document is well elaborated, although some references could be included in the scientific goals, in the second half of the proposal it lacks some more detailed descriptions. This project is of scientific relevance especially for Poland due to the study site selection.

2. Assessment of potential impact of the research project

This project could be of international importance if successfully implemented. It is rare that a forest beetle is known outside of the scientific community. This shows that if a pest control would be extracted from this research as one of the outputs, it would have an impact not only in the scientific community but also for forest practitioners and for forest management in Europe. If this method could be applied to other bark beetle species it would gain in potential impact as it could be applied in North America as well. It is a very specific research field that has not yet been studied in this form and would be of great importance both in basic science and in its application, since the spruce is one of the most important trees both from an ecological and economic point of view in Europe. Climate change will continue to affect the trees and the possibility of stopping an infestation would be a major breakthrough and would find a place both at international conferences and in high quality publications. Due to the narrow scope of the study area this could negatively affect the international importance. It is a very specific study field but due to the threat bark beetles pose to forests this study shows great potential.

3. Assessment of feasibility of the research project

The feasibility of this research project appears to be well designed. Within three years this project can be successfully executed. I am guessing that trees infested with the spruce bark beetle will probably be easy to detect in order to determine the exact study locations. The extraction of the study material is not complicated, and the methodology give the impression to be straight forward. I am not familiar with the timeline of DNA research, but I am concluding that the author is an expert in this field and knows the time to examines the data and that one year is sufficient time to analyse the data.

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

The total expenditures for the project, as well as for the specific tasks appear to be plausible and justified. The summing of the total cost is not adding up. 700 zl are missing in the summary of the total cost.

5. Strengths of the proposal

The project is well justified in terms of importance in basic science and in applied science, and the research questions are clearly outlined. The goal is precisely stated, and the language is well formulated, and it is written fluently. For a three-year project the design is reasonable. The goals and significance of the project are very well structured.

6. Weaknesses of the proposal

In Europe, there are 11 biogeographical regions that could affect the results of the study. Although Poland is mostly located in the continental ecozone and only in the south in the alpine region, all national parks were selected in the alpine ecozone. Therefore, the coverage is narrow and may not be as representative in terms of international importance, given the geographic distribution of spruce trees. This could also affect the relevance in terms of publication. The research methodology could be outlined in more detail instead of bullet points. The hypothesis could be more specific. The unhealthy tree condition should be considered as possible influence of the data. I think that pine has the highest percentage of forest in Europe after spruce, but this should be checked. It would be advisable to give some references in the scientific goals part of the project proposal. Some minor errors in the layout and wording should be corrected.

Pritam Kumar Dey

Title of the project

In my opinion the title of the project is nice and justifying the goals what they wants to achieve by this study, but it is necessary to put scientific names of the organisms on which you are working. Addition of the scientific names (with higher taxonomical position) is needed.

1. Assessment of scientific quality of the research project

Authors were able to justify the goals and what they want to achieve by this project but there is lack of scientific evidence which is very necessary to give proposal more scientific support.

As an example, in line Number 29, authors mentioned a very interesting fact "They are known to parasitize spruce bark beetles but is not really known if there is a negative or positive influence of this relationship on Ips typographus populations.", which is justifying the goal of the project, but they did not mention any references which is making their goals weak in scientific point of view.

In line Number 22; "Norway spruce forests" Scientific name is necessary.

In line Number 22-26; Facts mentioned here is very much important to be supported by references to strengthen the goals of this project.

In line Number 32-36; I would suggest reframing the question without changing the meaning to make it clearer and more understandable.

In line Number 41-44; two hypotheses have been written in present tense which making it already proven. Reframing the hypothesis in future tense with more clear predictions is needed.

2. Assessment of potential impact of the research project

The research project has identified the significance and potentiality, but they failed to carry the impact and in larger scale. Under the "2) Significance of the project" authors have mentioned many sentences which is not suitable for this section which should be under Concept and Work Plan.

In line Number 52-83; this part is much more about the description about the whole organisms and description of description caused by them. Author should think about more about the significance and possible impact. I would also suggest broadening the aspect of the study by making connection with other fields which will make it more significant.

In terms of minor corrections, I would like to raise few points and would suggest the authors to think about them and change it:

In line Number 47; "Pest Forest management" or Forest Pest Management? Which is the appropriate?

In line Number 47; "In the last years" these kinds of words are indefinite and should not be used if you do not have support it, both references given here not indication the last year anyway.

3. Assessment of feasibility of the research project

The proposed study is feasible to achieve goals but for that it is very necessary to check symbiotic relationship. Under the given work plan especially under the research methodology it is not clear how does authors will establish the interactive relationship between nematodes diversity and the spruce bark beetle.

In line Number 93-139; Work plan and Research Methodology is very general; I would highly suggest improvements under these sections.

Under the Concept and Work Plans authors should describe very little about their model organisms. This sections also lack of explanations on selection of study sites which is a very important part of the research methodology. In line Number 102-106; authors should clarify the sample sizes which not very clear.

"Research Methodology" should be highly improved in terms of technicality and scientific point of view. There is complete lack how they will do the research. Under the section of "Results of Preliminary" authors did not use any references from the previous studies which is making this section insignificant in scientific point of view.

In line Number 137; This point of the risk assessment is not very clear, and authors should either elaborate or reframe it accordingly to give more clarity.

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

The budget of the study is well justified but I would like to give some comments about the "salaries and benefits sections" authors mentioned only about one for PI section where there are two authors for this grant, authors should justify or rethink about this point. I did notice any list of field equipment but this study have field work, authors should think about it.

5. Strengths of the proposal

Spruce bark beetle outbreaks has become a major problem for mass destructions of Norway spruce forest, which is becoming a major problem for sustainability for European forest ecosystem and its conservation. In this grant proposal authors are willing to find out the solution for Spruce bark beetle outbreaks in corelations with the nematode diversity which is very interesting idea and will be very significant for conservation and ecology.

6. Weaknesses of the proposal

Major problem of the proposal is its implications, broadening the impacts in global scale, research methodology and work plan.

Gokul Bhaskaran

Summary

Line 6: Please change to Controlling (instead of control).

1. Assessment of scientific quality of the research project

The quality of the proposed project is at good standards and the broader domain of studying three different species will certainly increase better biological and empirical knowledge.

Line 24: the authors have indicated a very important issue, and had made a very strong argument – but the reference/previous study(s) reporting such results should be cited and that is missing here. Line 25: storms and droughts, and other periodic weather was always present with or without climate change, so bringing a very dynamic term "climate change" (in order to receive more attention) cannot be accepted, rather the authors should have emphasized how the periodicity in storms/droughts or its variation due to climate change had impacted the beetle and in turn changed their traits/characteristics that led to the loss of forest patch.

As we know in nature, there is always an arms-race. The race continues until there is a co-evolution leading to the most evolutionary stable strategy. It is fair to think that nematodes might a have a role in shaping these relations. However, in Line 30: the proposed plan makes counterintuitive thoughts, without even justifying how the relation between beetles and spruce trees might have shaped a sudden jump to a third species feels the project is extrapolated to make it looks big. However, we completely agree that we are in search of the positive or negative interaction especially as nematodes are present both in the tree and beetle. So, a suggestion here is to reflect on the how well the interaction between trees and nematodes alone cannot provide greater inferences.

2. Assessment of potential impact of the research project

Undoubtedly, a massive project like this will gain global attention and will inspire young researchers to think out of the box. Although, the study is focused within Europe, the study pattern can be adopted to a range of plant-animal interaction in various regions.

Both pest and the victim are a part of our ecosystem, so a total eradication shall not be the aim of the project (and it is not possible). However, in a nutshell in order to highlight the necessity of pest control, its eradication is resonated at few places (and agreed that the project not claiming it). The authors must have a caution to have a look in to this matter. A simple way to overcome this little mis-leading thought is to indicate how the results from the project will help to implement possible solutions. The authors pointed out the importance of population and ecosystem conservation but missed out to reflect how these can be possible in real sense.

Line 88: Agreed, but how will this project directly help in management (the applied part: after knowing the diversity – how it can help).

3. Assessment of feasibility of the research project

Studying from six geographically varying arenas is a very strong way to collect huge data and will lead to a better interpretation, but I am partially worried about the practicality in it – however, I appreciate the assertiveness of the authors. Since, the molecular tools will be widely used I assume that the authors will collect back-up samples in case to overcome any technical caveats.

The description of collecting tree barks is nicely justified, but evidently how the collected sample will be transported and stored before performing molecular part remains as a mystery. The various study arena can differ in their real-time temperature/moist conditions and these parameters are also inconsistent over the

seasons, also the entire collection from a study site can last over several days. So, it is very vital to realize the processing of the collected materials and if the samples collected is not taken care, the whole purpose may get diluted. Additionally, it is essential to emphasize on the transport system (cold-chain/any) because it shall be addressed in the budget.

In the methodology it is not a harm to highlight the research goals, but where is actual methods that you will be using for the project (like brief steps of molecular techniques). The data analysis might look simple for the people who are involved in the project, but how and where (tools both statistical and software) it can be achieved is not mentioned or emphasized.

Preliminary results are essentially motivating this study to carry on. There can be less sampling density from the area of patch which shall also be considered as potential risk factor (so that the authors and the team can be mentally prepared to have multiple areas; if required). Line 127: Please consider changing to "Results of preliminary studies" instead of Results of preliminary.

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

The equipment price (laptop) is hugely represented. The current price of the mentioned specific equipment can be purchased with 50% of the mentioned money. On top of that, the authors are not purchasing any software/storage devices/graphic cards which is suspicious. For a project like this, it is very important to make sure that the work results are accessible to everyone, very sadly in the budget the authors have not devoted any amount for the publication (open access). In the table provide here "Preparation of manuscripts and conference presentation, participation in conferences" 30000 PLN is budgeted. But in the description provided, this 30000 PLN is divided for the conference expenses. It is very evident that the authors have under represented the budget at crucial places, and overestimated to things as mentioned intially. Overall, the two tables provided in the budget section and the further details are providing a lot of detailed information – but not clear and easy to understand – for instance in the first table the total is 944400 PLN and in the second table the total is 1095000 PLN, so what is the final amount of money that is required still remains unclear and in a way mislead the granting body. These confusions are leading to a factor that the budget divisions need a extra care and manipulations shall be avoided. If the cost is uncertain please include the money under sections which is relevant, becasue we want this project to be fullfilled.

5. Strengths of the proposal

Forest conservation are gaining less popular at many research centers, the current challenge is to bring that temper of having more and more scientific thoughts on these field of research. This project is a strong step in doing so, and can have an overall impact to this research area. Empirical evidence from the study systems will help to better understand their biology and their ecological settings.

6. Weaknesses of the proposal

Conservation is the need of the hour, but the purpose should be met with the studies that are framed under this theme. Unfortunately, the proposed project does not looks really justifying the conservation aspects. Specifically, the data collections require travelling inside the forest may harm the natural habitat and behaviour of other small and large animals. Additionally, the presence of attacking animals needs to be taken precaution as the animals can directly harm field researchers. For this, the authors should come with some solid measures (including aerial monitoring prior to forest visit or geographical identification etc.), and these points are missing in the risk factors.

The project description overall lacks detailed information, including references, and the three species interaction details. It is well appreciated in using the contemporary next-generation techniques is answering

questions – but the authors missed in addressing the some fundamental behavioural/ecological observations (like the healthy and unhealthy tree patch size, vegetational dynamics). It is well understood that a study is not proposed to cover all the aspects, but please also keep in mind that sometimes basic and curiosity driven observation can make sense and the whole purpose shall not be limited to just the usage of expensive techniques.

One of the strong weakness is the budget proposed, with many reasons it lack a proper arrangement (mentioned in detail above). The lack of information arrangement is also reflected in the detailing of the project under various sub-headings (in research methodology instead of elaborating the method – the goals are highlighted). Similarly, under the significance of the project, the authors prioritize giving additional information than showing the natural implications that potentially arise as a result of the study.

Overall, the proposed project highlighted several key terms (appreciated) but missed out to justify that the results can make an impact (it is not obligatory to have immediate applications) but the way the authors outlined the contents and how the results will be interpreted is missing and the feasibility of the project is at least partially diluted due to the mismanagement of budget.

Final project proposal

Title: Nematode diversity present in Norway spruce (*Picea abies* L. Karst.) forests as a possible biological control for spruce bark beetle, *Ips typographus* (Linnaeus, 1758) (Coleoptera: Curculionidae) pest

Authors: Siri Devarakonda & Julia Morales-García

Summary: Norway spruce (*Picea abies* L. Karst.) forests are under threat from **s**pruce bark beetle (Linnaeus, 1758) outbreaks. The chemical control via pesticides is costly and limited in action, so possibilities of biological control of the population of bark beetles may constitute an important solution in forest management. It is known that *Ips typographus* has numerous interactions with species of symbionts and parasites. Among them, one of the most common groups that can be found in association with this species are nematodes, but it is not really known whether they have a positive or negative influence on spruce bark beetle outbreaks. The present project aims at checking out whether nematodes may constitute an important biological agent to control population outbreaks of bark beetle pest which severely affect forest health. Amplicon and metagenomics sequencing analyses will be carried out on populations of spruce bark beetle in different forests throughout Europe. This can contribute to a more accurate forest management plan for the spruce bark beetle, based on the possible biological control carried out by the different species of nematodes, being able to approximate a solution for Norway spruce forest ecosystems.

1) Scientific goal of the project

Norway spruce (*Picea abies* L. Karst.) forests constitute the largest percentage of forests in Europe. In recent years, the species *Ips typographus* (Linnaeus, 1758) (Coleoptera: Curculionidae) better known as spruce bark beetle, has led to the loss of hundreds of hectares of these forests (Caudullo et al. 2016). This pest can easily outbreak when the beetle density is enough and in optimal environmental conditions, such as storms or droughts, which is why it has intensified in recent years due to climate change, due to the change in temperatures and conditions in the ecosystems (Kausrud et al. 2012, Mezei et al. 2019).

This pest is difficult to control because most of its life cycle is inside spruce trees (Wremelinger 2004). A possible assessment that has not yet been studied is the interaction of these beetles with nematodes that live in the bark of spruce trees. They are known to parasitize spruce bark beetles but is not really known if there is a negative or positive influence of this relationship on *Ips typographus* populations (Burjanadze et al. 2015, Renco et al. 2015).

Considering that nematodes are biological indicators susceptible to changes in the ecosystem where they are found (Kereselidze & Wegensteiner 2007, Burnajadze et al. 2015), our goal is to know if there is a difference between the species of nematodes present in healthy spruce trees, and in those that are weakened or close to the senescence and compare that diversity of nematodes with that found in the spruce bark beetle. With this we intend to better understand the relationships between nematodes and the spruce bark beetle, in different conditions of the spruce bark beetle and spruce bark beetle hosts, to see if any nematode species could become an adequate biological control for this pest.

Hypothesis:

1. There will be a difference in nematode diversity within the spruce tree in different ecological conditions depending on the health of the spruce tree.

2. There will be any nematode species that present a negative influence on spruce bark beetle outbreaks, capable of assuming a biological control for this pest.

2) Significance of the project

Forest pest management is an emerging need in terms of ecosystem conservation. These pests can outbreak, causing a high population growth in a relatively short period of time (Kausrud et al. 2012, Mezei et al. 2019). In the last years, the intensity of outbreaks in some forest pests has increased due to climatic variations, causing important economic and environmental losses (Ogris & Jurc 2010, de Groot et al. 2019).

Norway spruce forests in Europe are a clear example of a species that suffer these pest outbreaks. Norway spruce is also affected by climatic change because forest composition and high temperatures increase the vulnerability of these trees against pests (Faccoli & Bernardelli 2014, de Groot et al. 2019).

Spruce bark beetle, *Ips typographus*, is a species from Curculionidae family, that is consider the most destructive forest pest along Europe (Mezei et al. 2019). It can outbreak easily in specific environmental conditions such a windthrow, droughts or storms (Faccoli & Bernardelli 2014, de Groot et al. 2019). Beetle density and health conditions of the host trees also influence the prevalence of spruce bark beetle to cause mass infestations for spruce trees, and their subsequent death (Ogris & Jurc 2010, Faccoli & Bernardelli 2014).

Fig 1. Example of bark-beetle infested trees in the High Tatra Mountains National Park in Slovakia. Bark beetle-infested trees are in the red rectangle. (Source: Mezei et al. 2019)

Spruce bark beetle is capable of building galleries inside trees, in which it breeds and lays its eggs. The larvae feed on the phloem of Norway spruces, making difficult the correct transport of nutrients throughout the tree. When the beetles become adults, they disperse to other trees where they reproduce and restart the cycle (Wermelinger 2004, Kausrud et al. 2012). When these populations are in an outbreak phase, the number of beetles is so high that they cause mass infestations in Norway spruce forests, causing great losses in these ecosystems (Wermelinger 2004, Kereselidze & Wegensteiner 2007, Ogris & Jurc 2010). In this way, the landscape is also altered, being able to distinguish patches throughout the forest where the Norway spruces have suffered the effect of this pest (Fig. 1).

These beetles are known to have multiple symbionts, many of them capable of conferring resistance against tree defences, such as *Ceratocystis polonica* or *Wolbachia* (Viiri & Lieutier 2004, Michalkova et al. 2012). Apart from these symbionts can also be found nematodes (Wermelinger 2004, Kereselidze & Wegensteiner 2007)., whose diversity has been studied, but a clear interaction with beetles has not been clarified. These nematodes not only live inside of beetles being able to live in their gut or haemolymph, as is the case of *Controtylenchus diplogaster*, or under their elytra, like the genus *Bursatylenchus* (Kereselidze & Wegensteiner 2007, Burjanadze et al. 2015), but are also found in the wood of spruce trees, on which *Ips typographus* feeds (Renco et al. 2015).

Spruce bark beetle is capable of infesting and killing large hectares of healthy trees or take advantage of the weakened trees (Renco et al. 2015), however it is not known how the diversity of these nematodes changes as the state of the tree changes. Nematodes are considered a biological indicator of the quality of their hosts (Burjanadze et al. 2015, Renco et al. 2015), but also symbionts are good biological indicators (Viiri & Lieutier 2004, Michalkova et al. 2012). To check the influence of the nematodes in spruce bark beetle, we are going to also check the conditions of these beetles when the nematode is present. One way to check the proper function of the beetle system is checking the presence of symbionts. In that context, we are going to fish for sequences of symbionts that we already know. If the symbionts are there when the nematodes are, there will be no influence of the nematodes in the spruce bark beetle. But, if in contrary there is a lack of symbionts in the spruce bark beetle populations when the nematodes are there, it means that the nematodes are affecting the system of the beetle, so then they will have an influence and we could use it as a biological control.

Know these interactions will allow a better management of spruce bark beetle pest based on biological control, and a better understanding of the ecological dynamics of European forests.

3) Concept and work plan

1. Study area. A total of six National Parks along Europe: (a) Djurö National Park (Sweden), (b) Langsua National Park (Norway), (c) Gorce National Park (Poland), (d) Thayatal National Park (Czech Republic), (e) Dobratsch National Park (Austria), (f) Dolomites National Park (Italy).

2. Work plan.
   - Collect the samples of the spruce bark from each National Park in four patches of spruce forests depending on the condition: two in healthy spruce trees areas and two in unhealthy spruce trees areas along the forest. The area of the patch is divided into 2 km × 1 km and 10 bark samples from different trees are collected in 4×4×4 cm.
   - We plan to collect 20 spruce bark beetle individuals in the same sampling plots by hand or by cutting infested log sections from spruce trees.
   - The samples will be kept in airtight vacuum bags to maintain the conditions of the samples until they reach the laboratory.
   - Isolate the DNA from the samples.
   - Use amplicon sequencing method to with specific nematode primers. We plan to dual index the primers by running PCR twice with Illumina adaptor overhang nucleotide sequence for forward and reverse sequence. DNA will be run on a gel and amplicons are excised and purified using the QIAquick Gel Extraction kit (QIAGEN, Germany) according to the manufacturer's instruction.
   - Use metagenomics sequencing to identify all symbiont species in each sample of spruce bark beetle and test the conditions of them in presence/absence of the nematodes.

4) Research methodology

1. Specific research goals:
- To identify the nematode diversity and presence/absence of different species in Norway spruce trees.
- To identify the presence of nematodes in Norway spruce trees inside the spruce bark beetles and compare them with Norway spruce trees nematodes.
- To study the positive/negative influence of the presence of these nematodes in the spruce bark beetle.

2. Results of preliminary studies:
- Preliminary results have shown the presence of the given nematodes in spruce bark beetle: *Contortylenchus typographi* (Tylenchida: Tylenchoidae), *Controtylenchus sp.* (Tylenchida: Tylenchoidae), *Bursaphelenchus sp. Parasitorhabditis sp.* (Rhabditida: Rhabditidae)
- Preliminary results have shown the presence of given nematodes in Norway spruce trees: *Acrobeloides nanus, Eudorylaimus silvaticus* and *Paratylenchus microdorus*.
- Selected preliminary results have shown the presence of the given symbionts that have a positive influence in spruce bark beetle: *Ceratocystis polonica* and *Wolbachia*.

3. Research, risk analysis:

Contamination of the samples in DNA isolation and sequencing can be fixed if we count with a laboratory proper control. In the same way, we can have the possibility of sample without nematodes, and that is why we have several samples from each area and from each patch. Replications allow us to give more accurate results.

5) Project literature

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Renco, M., Cerevkova, A., Homolova, Z., Gomoryova, E. 2015. Long-term effects on soil nematode community structure in spruce forests of removing or not removing fallen trees after a windstorm. Forest Ecology and Management 356: 243-252.

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Wermelinger, B. 2004. Ecology and management of the spruce bark beetle *Ips typographus* – a review of recent research. Forest Ecology and Management 202: 67-82.

Project Schedule – anticipated tasks

No	Name and description of task	Expected completion dates	Expected cost (PLN)
1.	The purchase of necessary equipment and materials	2023-2024	47200
2.	Fieldwork in Poland	2023-2024	70600
3.	Molecular analysis and other laboratory work	2024-2025	55400
4	Preparation of manuscripts and conference presentation, participation in conferences.	2023-2025	40000
5	Salaries and benefits	2023-2025	282.000
6	Indirect costs	2023-2025	99040
		Total	**594240**

Proposed budget

No.	Items	Funds for each budget year (PLN)			
		2023	2024	2025	Total
1	Direct cost, Including:				
	1) Salaries and benefits	94000	94000	94000	282000
	2) Equipment	23200	12000	12000	47200
	3) Other direct costs	79600	64400	22000	166000
2.	Indirect costs	39360	34080	25600	99040
3.	Total costs	236160	204480	153600	**594240**

Direct details of the cost items:

1) Salaries and benefits
   Principal investigator person months, 36 months 182.000 PLN.
   Field assistants 10 persons – 3 months 60.000 PLN.
   Technical assistant 2 persons – 10 months 40.000 PLN.
2) Equipment
   Laptops Intel Core i7-9750H six-core processor (6x4.5Ghz) 2 x 10.000 PLN = 20000 PLN for data analysis.
   External hard disks 5TB 2 x 600 PLN = 1200 PLN.
   Maintenance of equipment = 20000 PLN.

- Office materials (paper, printer refills, etc.) (2023-2024): 6.000 PLN (2000 PLN/year).

3) Other direct costs

Expenses related to the field work March-May 2023. 3 months (90 days) and 10 people:

- Diets: 30 PLN/person/day = 30x90x10 = 27.000 PLN.
- Travel costs (car): 60 PLN gas car x 90 days = 5400 PLN. 60 PLN rent car x 90 days = 5400 PLN. Total x2 cars = 21600 PLN.
- Accommodation: 20 PLN/person/day = 20x90x10 = 18.000 PLN.
- Other expenses for setbacks in field: 4.000 PLN.
  Total expenses for field work (2023): 70600 PLN.
- Materials for chemicals and molecular methods for analysing the DNA (720 samples):
  DNA isolation kits: 39.900 PLN.
  PCR Primers: 4.200 PLN.
  PCR kits: 11.000 PLN.
- Equipment for isolation of DNA and PCR (Eppendorf tubes, pipets tips): 300 PLN.
  Total expenses for lab work (2024): 55400 PLN.

- Fee and travel costs related to participation in international conferences: 18.000 PLN.
  Jul 22-2023 International Conference on Forest Entomology and Insect Physiology (ICFEIP). Tokyo, Japan.
  Aug 09-2024 International Conference on Biodiversity and Forest Entomology (ICBFE) - New York, United States.
- Publication in Ecology letters: 22.000 PLN.

Project 2: Deciphering anxiety pathway among model species

Draft project proposal

Title: Deciphering anxiety pathway among model species

Authors: Sylwia Drabik, Gokul Bhaskaran

Summary:

Research objectives

Anxiety disorders constitute the largest group of mental disorders, yet a very low percentage of patients receive clinical treatment. The first and foremost reason for this challenge is the lack of empirical studies. Therefore, studies aiming to discover neurochemical, neuroanatomical, and cellular mechanisms underlying these conditions are of high importance.

Proposed project targets interpeduncular nucleus (IPN) – ventral hippocampus (vHipp) axis, which consists of structures that are essential in controlling anxiety. IPN, a midbrain structure lying just ventral to the ventral tegmental area, is involved in discriminating between the novel and familiar stimuli, signals unpleasant symptoms of drug cessation, and plays a key role in anxiety behaviour. IPN densely innervates vHipp, limbic system structure implicated in contextual fear memory formation, avoidance, and anxiety response.

Moreover, the project will concentrate on the role of nerve growth factor (NGF) signalling in the overmentioned circuit. NGF was discovered as a neurotrophin responsible for neurites growth and survival, however, more recent discoveries showed its role in anxiety-related behaviours. Notably, NGF levels in both the brain and bloodstream were shown to significantly differ in stressful conditions in animals and humans. At the same time, IPN is a structure with one the highest level of expression of mRNA for high-affinity nerve growth factor receptors TrkA, and vHipp is enriched with TrkA immunoreactive fibres. However, the role and characteristics of the NGF signalling in IPN-vHipp axis remains unclear.

The implementation of the proposed project will allow for a detailed electrophysiological and neurochemical characterisation of the IPN-vHipp circuit as well as description of the functional connectivity between IPN and vHipp. Moreover, during the planned project. Additionally, whole-brain projections of IPN neurons innervating vHipp will be traced, which will allow for the assignment of this IPN neuronal population to the specific functional brain circuits.

We aim to pursue this research with three important model systems (Rat, Zebra finch and Zebrafish) to unveil the effect of anxiety. Furthermore, human IPN samples will be used in one part of planned project to gain information about possible clinical implication of found results.

Research methodology

In the current project various research methods will be used. Anxiety in all animal species will be evoked using classical paradigm of social isolation. Furthermore c-Fos expression and immunofluorescence staining will be performed in order to characterize IPN cells innervating vHipp. With the usage of novel viral tools neural tract-tracing studies will be performed. High-throughput technique - RNAScope will be used to characterize biochemically IPN neurons. Data analysis will be executed in Matlab environment and CED scripts (electrophysiological research), ImageJ (dendrite and 3D Sholl analysis), L-Measure (morphological parameters). Subsequent statistical analysis will be held in Prism7 software (GraphPad).

Expected impact of the research project on the development of science

Answering the main questions of this study will significantly expand current knowledge about the IPN and vHipp axis, which is of potential significance for understanding neurobiological basis of anxiety control and anxiety disorders. Moreover, a better understanding of the NGF role and action in the proposed circuit, as well

as detailed characterization of NGF-sensitive IPN neurons, may bring us closer to untangle the complicated mechanism underlying anxiety signalling. We consider our study as a next step for biomedical research and also in shaping fundamental and advanced evolutionary concepts.

I. Scientific goal of the project

Anxiety disorders constitute the largest group of mental disorders and currently approximately one third of the global population according to large population-based surveys (Bandelow et al., 2015) or 21,7% according to the WHO estimates, suffers from them (Charlson et al., 2019). Moreover, anxiety disorders show high comorbidity with other anxiety and mental conditions. They are diagnosed both in adults and children, however, there is a decrease in prevalence following older age. Unfortunately, only one third of affected people receive drug treatment, and according to the current estimations, only half of cases are being recognized (Bandelow et al., 2015). Anxiety disorders form a big family of disorders including panic disorder with or without agoraphobia, Generalized Anxiety Disorder (GAD), Social Anxiety Disorder (SAD), separation anxiety disorder and specific phobias (Bandelow et al., 2015). Patients suffering from anxiety related disorders are usually treated as outpatients and they receive less care and attention from their therapist in comparison to other mental disorders (Maestripieri et al., 1990). The lack of proper therapies and treatments partly arises from the limited knowledge about the neural basis of anxiety disorders. **Therefore, studies aimed at uncovering neurochemical and cellular mechanisms underlying anxiety related behaviours are of high importance.**

Recently, nerve growth factor (NGF), the first discovered member of the neurotrophin family, primarily known from its involvement in neurites growth, cells survival and neurotransmitter production, has gained attention in the context of anxiety disorders research. NGF level in bloodstream is affected by stressors and anxiety like behaviours in humans (Lambiase et al., 1994) and animals (Maestripieri et al. 1990). A massive release of NGF from salivary glands was observed in fighting males and lactating females during nest-defence (Berry et al., 2012; Maestripieri et al., 1990), and changed NGF level in the brain of animals after aggressive behaviours and psychosocial stressful events was described (Berry et al., 2012). **Despite the increasing number of data showing NGF role in anxiety related behaviours, its specific site of action as wells as possible functions are not well established yet.**

In the adult brain, several areas were shown to synthetise NGF: hippocampus, olfactory bulb, neocortex, septum, diagonal band of Broca, nucleus basalis of Meynert, striatum, hindbrain, medulla oblongata, hypothalamus and cerebellum (Korsching et al., 1985). From overmentioned brain areas, ventral hippocampus (vHipp) seems to be a principal component of anxiety and emotional behaviour control system and its lesions have an anxiolytic effect (Trivedi et al., 2004). Moreover, "anxiety cells" were described in ventral, but not dorsal part of hippocampus (Jimenez et al., 2018). **Importantly, vHipp is densely innervated by fibres immunoreactive for TrkA – receptor for NGF** (Sobreviela et al., 1994), **however, the source of this innervation remains undiscovered.**

Brain structure densely innervating vHipp (Lima et al., 2017) and involved in anxiety signalling is interpeduncular nucleus (IPN) (DeGroot et al., 2020). Importantly, IPN is one of a handful brain structures enriched with TrkA receptors in mice (Allen Brain Atlas, 2020) and preliminary data presented in the current project show a direct, postsynaptic effect of NGF administration on IPN neurons activity**. Therefore, this project's main hypothesis is that vHipp is under the influence of NGF sensitive IPN innervation and that IPN-vHipp axis plays an important role in anxiety signalling.**

II. Significance of the project

Despite emerging data there is still a need for research concerning the role of IPN-vHipp axis in control of anxiety related behaviours, as well as an involvement of NGF in modulating this neuronal axis activity. Therefore, **the goal of this study is to identify the source of NGF in the area of IPN, investigate anatomical and functional connectivity of IPN-vHipp pathway and characterise IPN NGF sensitive neurons innervating vHipp at both morphological and electrophysiological levels. Moreover, the influence of NGF administration on the neuronal activity of IPN neurons innervating vHipp will be verified.** In this project we three different animal systems will be used: all the experiments will be held on Sprague-Daweley rats, zebra finches and zebra fish. Moreover, thanks to the cooperation with laboratory in Sydney (Australia)

we will be able to perform RNAscope assay on human tissue. Various neuroscientific techniques will be used to answer the questions of the current project. Planned research will combine electrophysiological recordings with optogenetics, immunohistochemical staining and viral based tract tracing methods to bring us closer to uncovering neuronal mechanisms involved in IPN control of vHipp, and to describe NGF influence on IPN-vHipp axis among species. Some anatomical studies will be performed using human brain tissue, to ascertain the translational relevance of the planned preclinical studies.

III. Concept and work plan

1. Measuring activity of the cells within IPN in all species; behavioural experiments followed by c-Fos analysis

Social separation is described as a stressful stimulus for fish, rats and zebra finches (Shams et al., 2017, Beery and Kaufer, (2015), Emmerson and Spencer, (2017)).Therefore, to evoke anxiety in all animals they will be kept in separate cages (aquarium in case of fish) for 90 minutes. IEGs and their protein products are expressed following neural activity, among them cfos and Fos expression are the most widely used indicators post-mortem (McReynolds et al., 2018). This techique will be used to verify the presence of supposed activity of IPN after exposure to stresful stimuli.

2. Biochemical characterization of IPN neurons innervating vHipp and active during anxiety; bahavioural studies, stereotaxic surgeries, RNAscope.

In order to **identify and characterise the IPN neurons innervating the ventral part of hippocampus**, the retrograde viral based tracing combined with the electrophysiological recordings will be used. Surgery on adult male rats will be conducted and AVV retrograde viral vector driving mCherry expression (AAVrg-hSyn-mCherry) will be injected bilaterally into the vHipp. Three weeks after the surgery, the same behavioural stressors as in experiment 1 will be induced to animals. 60 minutes after RNA scope procedure will be held with the usage of kit dedicated for each species. RNAScope is an in situ hybridization assay for detecting RNA in formalin-fixed paraffin-embedded tissue that is commercially accessible. The RNAScope assay consists of target probes and a signal amplification system composed of a preamplifier, amplifier, and label probe. The probe is typically labeled with a fluorochrome or an enzyme, so it can be detected either by using a fluorescent microscope or with a bright field microscope. Using this procedure we will be able to answer the questions weather IPN cell active in anxiety are innervating vHipp, do they have receptors for NGF, CFR (corticotropin releasing factor, a canonical stress modulator) and weather they are GABA or Glutamatergic (thanks to checking the presence of absence of mRNA for vGlut2 and vGAT1).

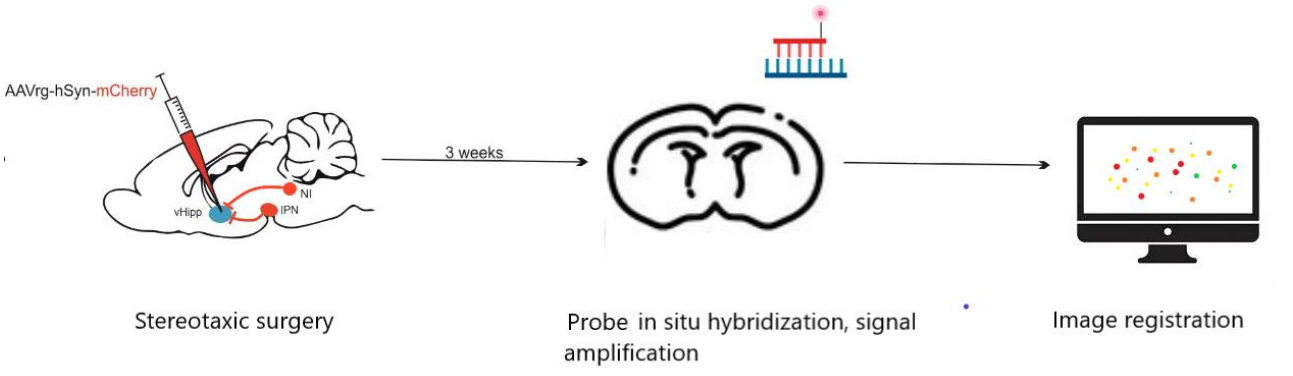


Fig. 3 Experimental scheme showing the injection site and major steps in RNAscope procedure.

3. Biochemical characterization of human IPN neurons; RNAscope

RNAscope on human frozen IPN samples obtained in The Florey Institute of Neuroscience and Mental Health (Melbourne, Victoria, Australia) will be performed. Sonds for TrkA, CRFR1, VGlut2, vGlut1 (just like in experiment 2) and two additional ones for CCK and RLN3 will be used. This part of the project will help us

to ascertain the translational relevance of the planned preclinical studies in animals and assuring evolutional conservativness of the circuit.

4. Electrophysiological and neuroanatomical characterization of IPN neurons innervating vHipp and verification of sensitivity of neurons constituting IPN-vHipp axis to NGF in all species; patch clamp electrophysiological experiments.

In order to identify and characterise the IPN neurons innervating the ventral part of hippocampus, the retrograde viral based tracing combined with the electrophysiological recordings will be used. Surgery on adult male rats will be conducted and AVV retrograde viral vector driving mCherry expression (AAVrg-hSyn-mCherry) will be injected bilaterally into the vHipp (just like in the previous experiment). Three weeks after the surgery, electrophysiological patch clamp experiments will be performed.

During whole-cell patch clamp recordings of mCherry expressing IPN neurons, their electrophysiological and anatomical features will be characterised. Responsiveness of IPN neurons to acute and prolonged exposure to the NGF will be verified. Firstly, during patch clamp experiments, responsiveness of IPN neurons directly innervating vHipp (mCherry expressing) to acute (lasting approximately 3 minutes) administrations of NGF will be verified, secondly recordings of IPN neuronal activity will be performed from slices pre-incubated for an hour in ACSF containing equivalent to short administrations concentration of NGF. The procedures with some technical changes will be held for all the animal species. Importantly, in our preliminary studies (Fig. 1) we observed that NGF administration induces whole cell inward current in IPN neurons, what proves that NGF can act in a neurotransmitter-like mode in the IPN of rat. Results of these experiments will answer the question whether NGF acts like a neurotransmitter in the IPN and will allow to characterise the nature of the response of IPN neurons innervating vHipp to the NGF.

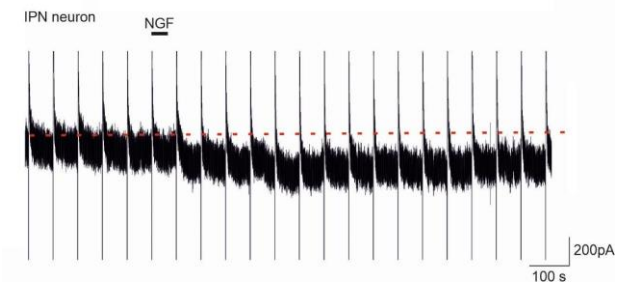


Fig. 2 NGF administration induced whole-cell inward current in IPN neurons. Voltage clamp recording of IPN neuron activity (command potential -50mV) before and after (indicated by bar) the administration of NGF (5ml, 250ng/ml).

To answer the question **whether IPN neurons innervating vHipp are directly or indirectly sensitive to NGF**, animals with specific expression of mCherry in IPN neurons innervating vHipp will be used. Responsiveness of IPN neurons innervating vHipp to the NGF administration will be recorded and subsequently repeated in the presence of voltage sensitive sodium channel blocker and GABA and glutamate ionotropic receptors antagonists. These experiments will allow to identify if the action of NGF on IPN has a pre- or postsynaptic nature. In specific series of experiments selective agonists and modulators of TrkA receptors will be tested and their specificity will be further verified by administrating receptor antagonists. Due to biocytin presence in the recording micropipette, **anti-biocytin staining of recorded neurons will allow to assign them to specific IPN location** within the IPN as well as to preform Sholl analysis of the biocytin filled neurons and to characterize their anatomical features such as number of branches, bifurcations and area occupied by their dendrites.

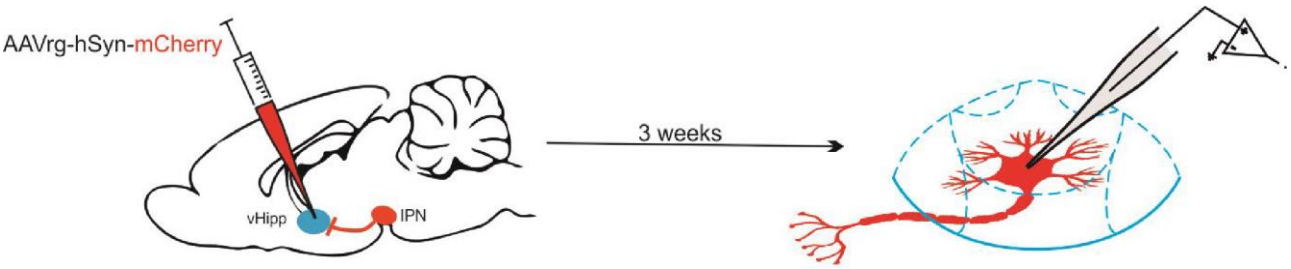
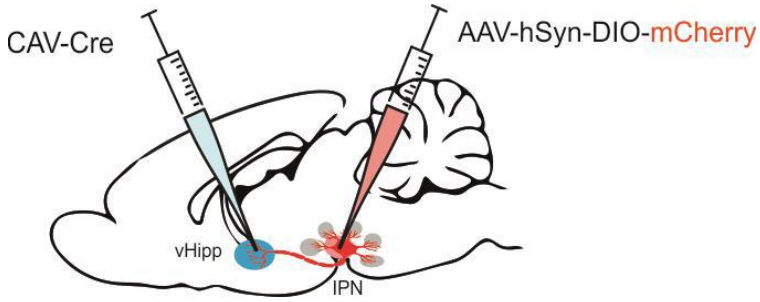


Fig. 3 Experimental scheme showing the injection site, used virus and structure to be recorded on the example of rat's brain.

5. Characterization of the whole brain axonal endings distribution of IPN neurons that innervate vHipp; neural tract-tracing and immunohistochemical studies.

Characterization of projections of IPN neurons innervating ventral hippocampus. In order to describe whole brain axonal endings distribution of IPN neurons innervating vHipp, CAV-Cre retrograde virus (CAV-2 vector harbouring the CMV promoter that leads to Cre recombinase expression) will be injected bilaterally to ventral hippocampus and anterograde Cre-dependent AAV-hSyn-DIO-mCherry will be injected to IPN of the same animal. Immunofluorescence anti-mCherry staining will reveal the whole brain fibre distribution of IPN neurons innervating the vHipp. The procedure with all the necessary technical corrections will be repeated for zebra fish and zebra finches. Mapping the structures controlled by this discrete IPN neuronal population, will allow to further hypothesise about the functional importance of IPN-vHipp axis as well as checking weather the function predicted is conservative among species.

Fig. 4 Experimental scheme showing the injection sites and used viral vectors.



EXP. NO.	METHODS	EXPECTED RESULTS AND OBSERVATIONS
1	Viral injections, Social isolation, Immunohistochemistry	Identification of IPN cells active in anxiety, checking if the localization is evolutionary conservative among species examined
2	Social isolation, RNAscope	Biochemical characterization of IPN neurons innervating vHipp and active in anxiety, checking similarities among species
3	RNAscope	Biochemical characterization of human IPN neurons
4	Viral injections, Patch clamp recordings, Immunohistochemistry	Characterization of electrophysiological and neuroanatomical properties of IPN neurons innervating vHipp and verification of their sensitiveness to NGF; checking if mechanism is evolutionary conservative
5	Viral injections, Immunohistochemistry, Neural tract-tracing	Characterization of whole brain axonal endings distribution of IPN neurons innervating vHipp, checking if the distribution is evolutionary conservative

Fig. 5 Chart showing the summary of methods used in each part of the project and expected results and observation driven from each experiment.

IV. Risk Assessment

PI experience in described research techniques and results of presented preliminary studies support successful completion of the study. Intra brain injections are the aspect of the highest risk of failure so there will likely be a need to refine and repeat the surgeries. Therefore, the number of animals planned for these parts of the project, where intra-brain injections are planned, is overstated. There of course is a chance of negative results appearing in the first experiment planned. However, cfos transcription assumed as always being associated with neural activation no always is the case. For example, substantia nigra activity has been measured following kindled seizure induction but corresponding changes in Fos were not observed (Labiner et al., 1993). Therefore, even if no cFos positive cells will be visible within IPN in the first experiment, the rest will be continued and other markers of neural activity will be taken into account.

V. Research methodology

All the described experiments will be held at the Department of Neurophysiology and Chronobiology at the Institute of Zoology and Biomedical Research at the Jagiellonian University. Experiments will be conducted on 70 Sprague-Dawley rats (6-8 weeks at the start of each experiment) and 100 zebra fish (5-7 days at the start of experiment) both species of animals will be bred and housed at the Institute of Zoology and Biomedical Research, Jagiellonian University. Moreover, 80 zebra finches (12-14 weeks at the start of each experiment) which will be bred and housed at the Institute of Environmental Sciences, Jagiellonian University will be used. Moreover, thanks to the collaboration with Prof Andrew Gundlach from The Florey Institute of Neuroscience and Mental Health (Melbourne, Victoria, Australia) we will be provieded with frozen human IPN samples for RNAscope procedures.

Social anxiety induction: As the animals used in our experiments are social in nature, isolating them will induce stress. For this, rats will be kept in individual cages in isloated arena to prevent any auditory, olfactory or visual cues from other rats. Zebrafishes before experiment will be singly transferred to pool that is freshly filled with water, and each tank will be masked from one another which further strength in level of isolation. Zebrafinches will also be isolated singly in individual cages. All the isolations will be for an hour before the start of the experimental procedures.

Neural tract-tracing and immunofluorescence staining: In order to identify interpeduncular nucleus neurons innervating ventral hippocampus, after bilateral intra-vHipp injections of viral vector (Tab. 1) rats will be given three weeks recovery period. After that, rats will be deeply anesthetised with pentobarbital, killed by transcranial perfusion with 300ml of saline followed by 400 ml of 4% formaldehyde and their brains will be dissected and postfixed for 24h. Zebra finches and zebrafish will undergo the same procedure with relatively smaller amounts of liquids (100ml/130ml for zebra finches and 10ml/15ml for zebra fish) and. After post-fixation period coronal sections, 30μm thick, will be cut on a freezing microtome (Bright Instruments). In experiments aiming at identification **of the whole brain axonal endings distribution of IPN neurons that innervate vHipp,** after viral vector injections slices will be incubated with mouse anti-mCherry. For **experiments aimed to identify cFos positive cells in IPN** mice anti-cFos will be used.. Afterwards slices will be incubated with secondary anti-mouse Cy3, anti-rabbit A488 for 18h in 4 ºC. After patch-clamp recordings sections will be stained against biocytin. All the described sections will be mounted on glass slides, coverslipped with Vectashield containing DAPI (Vector Laboratories) and imaged with the usage of fluorescent microscope (Axio Imager M2, Carl Zeiss).

Multiplex Fluorescent in situ Hybridization (RNAscope): in situ hybridization using an RNAscope HiPlex Assay [Advanced Cell Diagnostics (ACD), Hayward, CA, United States] with RNAscope HiPlex Alternate Display Module (ACD; for AF488, Atto550 and Atto647 detection) will be conducted on brain sections from 3 male Sprague-Dawley rats, 3 male Zebra Finches, 3 male danio rerio fish and 3 samples from male human brains 3 weeks after the injection of the retrograde viral vector encoding mCherry protein, into the vHipp. All procedures will be performed following the manufacturer's instructions, with preparation and pretreatment for fresh frozen samples. Briefly, animals will be anesthetized with isoflurane and decapitated. Their brimmediately collected, frozen on dry ice and stored at –80°C. For each brain, three 16 μm sections containing IPN were cut at –20°C, using a cryostat (Cryocut CM 1800, Leica Microsystems, Wetzlar, Germany), and mounted onto Superfrost-Plus slides (Thermo Fisher Scientific, Braunschweig, Germany). The slides were stored at –80°C until a 1 h fixation in a freshly-prepared solution of 4% formaldehyde in PBS (pH 7.4, initially 4°C) at room temperature (RT), followed by washing in PBS and dehydration in ethanol solutions of increasing concentration (50, 70, and 100%). Dehydrated sections will be stored at –20°C overnight and the next day will be air-dried, outlined with Immedge Hydrophobic Barrier Pen (Vector Laboratories, Burlingame, California, United States) and incubated with Protease IV pretreatment solution (ACD) for 30 min at RT. After washing in PBS, the sections will hybridized for 2 h at 40°C with a solution of HiPlex probes.

Surgical procedures and viral vectors injections: Before surgery adult Sprague-Dawley rats will be anaesthetised with isoflurane and placed into a motorized stereotaxic instrument for rats. Viral vectors will be injected bilaterally into vHipp: -5.2; 4.5; -8 (AP; ML; DV, mm from bregma) and (in experiment 4) into IPN: : -6.2; 0.0; -9.0. Zebrafish will be anestetised with buffered tricaine methanesulfonate (MS222) and zebrafinches with ketamine and xylazine solution intraperitoneal injection. Brain coordinates of both structures in for birds and fish need to be calculated. After the surgery, animals will be allowed to recover for three weeks (time necessary for full expression of the transduced genes).

Whole-cell patch clamp electrophysiological studies: Experiment will be performed 3 weeks after viral injections. Brains will be prepared and placed in recording chamber of Axioskop FS2 microscope (Zeiss). Whole cell current clamp and voltage clamp recording with specific tests for electrophysiological properties of neurons will be performed. All the drugs will be administrated via bath perfusion. All the necessary equipment for patch-clamp recordings in available at the host Department.

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VII. Table with budget of the project

	Amount in PLN
Direct costs, including	1202500
- personnel costs and scholarships	
- research equipment/device/software cost	
- other direct costs	
Indirect costs, including:	15000
- indirect costs of OA	
- other indirect costs	
Total costs	1217500

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

Details of direct cost items:

a. Salaries and benefits

Principal Investigator salary = 3000 PLN/month*36 = 108000 PLN

Technical Assistant (1) salary = 1500 PLN/month*36 = 54000 PLN

Collective investigator (1) salary = 2000 PLN/month*36 = 864000 PLN

b. Equipment

Stereotaxic apparatus for birds + fishes = 5000 PLN

Computer system + high graphics support + storage + software = 5000 PLN

c. Materials

RNAScope Kits (for all study systems, the kit also will have buffers and basic chemicals) = 100000 PLN

Chemical reagents necessary to accomplish research objectives (for example, biocytin and extrAvidin - visualization of registered neurons, Flouoroshield - to coverslip the slices, viral vectors needed in surgeries (AAVrg-hSyn-mCherry), primary and secondary antibodies needed for immunostainings) = 1000 PLN

Small laboratory supplies (Expendable goods needed in research e.g. borosilicate glass pipets, eppendorf tubes, pipette tips, well-plates) = 1000 PLN

Stationery supplies (Printing cartridges, toners, paper) = 500 PLN

d. Travel

International Conference to Australia and USA (Plane ticket, daily allowance, visa) = 30000 PLN

Training Workshops (ticket, daily allowance) = 8000 PLN

Other direct costs:

Purchase/maintenance of study animals (includes food, cages) + Bio-hazard waste management = 21000 PLN

Sample transportation/shipment = 5000 PLN

Details of Indirect cost items:

Publication of the study results (open access journals) = 15000 PLN

Reviews

Aneta Arct – abstract review

The abstract acts as a surrogate or synopsis of your project, doing almost as much work as the thousands of words that follow it in the body of the main text. I believe that a clear division into three sections of your abstract is a very good choice. However, in my opinion the first paragraph of your abstract is not concise, readable, and quantitative, during reading I miss the point of the project. Too much detail is given in a chaotic manner in this section. Nevertheless, after reading the abstract I found that research seems very interesting.

Julia Morales-Garcia

Title of the project

Short and catchy title with a known topic ("anxiety"). A little bit pretentious (you do not decipher nothing yet).

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

Of course, anxiety disorders are a social and scientific topic of great interest. The project itself is quite important due to the subject to be investigated. However, it has some slightly pretentious aspects that I proceed to specify.

In the Research objective's part, paragraph three raises the complexity of the topic to a point where non-experts will not really understand what is being explained there. I suggest authors try to find other terms for "vHipp" or "TrkA", synonyms, or even write the full name, because it would make it easier to read and understand.

In the fourth paragraph, line 25 there is a sentence that does not make any sense: "Moreover, during the planned project.". Authors are encouraged to proofread the text once written.

In the last paragraph the authors write "We aim to pursue this research with three important model systems (Rat, Zebra finch and Zebrafish) to unveil the effect of anxiety". Doing experiments with three species you cannot say that you are going to unveil the effect of anxiety. Make this part more specific because it is not accurate with your experiment.

The part of scientific goal of the project is over-explanatory. You really do not need that many details. For example, in the third paragraph, it is not necessary to mention all the brain structures that can be found in the adult brain. On the other hand, it would be much more intuitive to know the hypotheses of the project if a subsection was dedicated to it, because they are not well appreciated. They are highlighted in bold like other important ideas, making it difficult to understand.

The authors know the most appropriate methods to carry out their study. They do not use only one method, but rather combine several to contrast their results in the most meaningful way. In an international context, the project is truly important, since it has the participation and cooperation of a laboratory in Australia, combining other possible methods that may be specific to researchers in laboratories in other countries, giving the project greater significance.

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

Nowadays, researching common problems such as anxiety disorder is a social and economic necessity at an international level, so the authors have chosen to study a subject of great importance that will allow them to

achieve great results and obtain quality research publications. It is a project that can have a great impact, and that is well chosen and planned.

3. Assessment of feasibility of the research project

The methods of the project are very well explained. It is appreciated that the authors understand the process and know how each of the steps should be carried out to achieve the objective. Despite the great amount of detail, it is generally understood what is going to be done.

The division of the methodology into different sections makes it easier to read and understand. The risk assessment is justified and explained correctly.

Despite not being an easy methodology to carry out, the authors have similar preliminary studies, and the international help of other laboratories, which gives the project security to put it into practice.

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

Although the Budget of the project is properly justified, a table is missing that specifies how much budget is going to be used each year; that is, how that budget is going to be divided over the three years that the project lasts. From a general point of view, the chemical reagents and primary and secondary antibodies cost only 1.000 PLN, which seems like a low budget, since these types of materials usually cost a lot of money.

5. Strengths of the proposal

The topic itself is already of great importance. If to this is added the fact that the importance is clearly highlighted throughout the proposal, and that the methods are also organized in an understandable and justified way, it is assumed that the proposal has great advantages to be able to be accepted.

The authors have added graphs and tables to facilitate the interpretation of the text, even for people who are not experts in the field. For such an ambitious project, the budget is appropriately adjusted, giving the project even more advantages to be carried out.

6. Weaknesses of the proposal

Regarding presentation, the proposal does not comply with the format, because it exceeds five pages and does not have the appropriate line spacing. In addition, the part of bibliographical references can hardly be read unless you zoom in, so authors are suggested to summarize the text. Some aspects are too complex, or unnecessary as has been commented throughout the review. If the authors suppress the unimportant parts, they could adjust the proposal to the required format, and make scientists who are not specialists in this topic understand the importance of their project.

An important issue: where is the summary? Authors should write a summary of the entire proposal.

Authors are requested to PLEASE check the grammar and writing, there are dozens of minor errors:

Line 34: double space between Words.

Line 112: double space between words.

Line 116: techique = technique.

Line 117: stresful = stressful.

Line 120: bahavioural = behavioural.

Line 130: labeled = labelled.

Line 142: sonds = sondes.

Line 145: conservativness = conservativeness.

Linea 210 – Risk Assessment paragraph: “cfos” and “cFos” are the same? Should it be written in italic (as in the “Measuring activity of the cells within IPN…” part)?

Line 226: double space between words.

Line 230: provieded = provided.

Line 233: isloated = isolated.

Line 236: no space between words.

Line 247: “will be used..” ¿¿??

Line 259: “brimmediately” ¿¿??

Line 271: anestetised = anesthetized.

Piotr Łukasik

1. Assessment of scientific quality of the research project

Poor. The authors ask questions about the mechanisms of anxiety disorders – an important societal issue. They claim that the knowledge about the neural basis of anxiety disorders is limited (which I have no grounds to assess, but can agree with), and propose to address them using a series of sophisticated experiments. Unfortunately, I had a hard time understanding what the authors actually want to do. The proposal is very methodological and written using a very technical language, full of acronyms and specialized vocabulary, and I have been unable to follow it fully. I have found the critical section "concept" particularly hard to access, but have struggled with other portions of the document, beyond the third paragraph of the Introduction.

I also feel that the authors have failed to provide critical pieces of information. After reading the proposal, I am not sure about the project goals. I also do not know the rationale for different experiments, how they fit into the current state of knowledge, or what are the expected results. While I lack technical expertise to understand the methodological details, the authors failed to provide an overview of what they want to do – general description, sample sizes, or expected results. Without these critical pieces of information, I am unable to score the proposal favorably.

Some key recommendations:

- Identify precisely the gaps in the knowledge that your project will address
- Make sure to state clearly the project goals (2-3 preferred?) and explain how they fit into the state of knowledge in your field
- Make it easy to readers to understand the relationship between goals and research tasks
- Use language accessible to the general biology audience.
- Use English grammar/style editing software

2. Assessment of potential impact of the research project

This is not clear – the authors provided little space for a non-neurologist to understand the impacts. While I can agree with the overall claims about "limited knowledge about the neural basis of anxiety disorders", I would really like to read how this project builds upon and expands on research to date, addressing major but well-defined unknowns in the field. "Significantly expand current knowledge" is a claim that is easy to make, but the authors need to be more specific on the expected outcomes of their work, and why it should interest the panel. The Significance section includes a statement *"the goal of this study is to identify the source of NGF in the area of IPN, investigate anatomical and functional connectivity of IPN-vHipp pathway and characterise IPN NGF sensitive neurons innervating vHipp at both morphological and electrophysiological levels"* – but I would say that few biologists would be able to say how this relates to impacts and broader significance.

3. Assessment of feasibility of the research project

Based on information provided, I am unable to assess that. In my view, the "Concepts / Research Overview" section is the place where I expected that this point will be made clear – but it was not. I would say that Figure 5 is virtually the only part of this section that belongs there, with the overall level of detail in this section hugely excessive.

In the "Concepts" section, the authors should provide a very clear description, written in a language accessible to the general biology audience, of:

- What are the main tasks, and what are their expected outcomes. Here, concepts are much more important than methodological details!
- How these tasks are related to project goals
- Who, when, and where will execute the tasks

- How your experience, skills, methods, preliminary data, and collaborations will give you high likelihood of success.

Helpful visual aids in this section are a flow chart and a Gantt chart.

You may want to elaborate on technical aspects and use somewhat more specialized terms in the Research Methodology section – but remember that they should be described in ways that are accessible to biologists from different fields.

The proposal also lacks any information about ethical issues and procedures, important given that experiments with live vertebrates are at the core of this project.

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

No. The budget is incomplete and poorly explained, and the numbers do not add up.
The budget table is incomplete. By far the major expense is the "collective investigator salary" for 36 months, but it has the Total over an order of magnitude greater than the sum of monthly amounts, and no information on who is that "collective investigator" or what is their role role. There is no information on the roles or employment basis of the other personnel (Part-time employment? Salary supplement?).
The description of the position "RNAScope Kits" seems very generic: we do not know how many kits you will buy, from whom, if these are standard or custom products, what is the unit costs, for how many samples they will last… The costs of Equipment and Chemical Reagents seems low. The authors request a large budget for attending "Conference to Australia and USA" – what conferences, why you need to travel specifically to such distant/expensive locations rather than attending probably more cost-effective European events, or who is going. There are no indirect costs of the host institution.

5. Strengths of the proposal

- Topic of broad general interest
- Plans to use different model organisms

6. Weaknesses of the proposal

- Very difficult and technical language makes the text inaccessible to non-specialists
- Organization and structure, lack of clear relationship among goals and tasks/experiments
- Poor integration of the planned work into "state of the art" in the field
- Lack of general work plan

Zuzanna Rauk

1. Assessment of scientific quality of the research project

The aim of the project is to investigate cellular and molecular underpinnings of anxiety-related behaviours. On the basis of the specialist literature the authors indicate that anxiety disorders constitute a serious problem among global population and that difficulties with developing efficient therapies arise from limited knowledge about physiological basis of anxiety. This statement fully justifies the idea of conducting the project. The authors propose to focus on two structures - IPN and vHipp and NGF as a signaling molecule between the regions, which is understandable as the structures and molecule were proven to be engaged in anxiety processing. The novelty of the project comes from the fact that NGF signaling has never been evaluated in the context of this neuronal circuit and because it is planned to elucidate the exact mechanism of the communication between structures using molecular and electrophysiological techniques. The research hypothesis is clearly formulated and revelatory. Its validity is supported by the results of preliminary electrophysiological research on IPN neurons sensitivity to NGF, which revealed that they respond to NGF administration with changes in activity. Advanced methodologies that authors plan to use are appropriate to achieve particular research goals described in the proposal. The idea of analyzing the IPN-vHipp connectivity in different model species an human samples is interesting, however the authors did not argued the choice of particular species in the study. It should be also explained more clearly why they suppose that anxiety processing can be similar in these species.

2. Assessment of potential impact of the research project

The broad approach to the research problem allows to assume that its realization will bring a lot of interesting data regarding the role of IPN-vHipp axis in anxiety behaviours, as well as the importance of NGF in the signaling process between the regions. The project has real impact on understanding anxiety-related mechanism at the very basic level which is of high importance in the context of developing new therapeutic approaches. As the authors will utilize highly advanced and innovative technologies to verify the hypotheses the project will probably result in high-quality publications. Its realization will contribute to the development of different fields including neurobiology of anxiety, comparative biology and translational biology, as different species including human will be scrutinized in terms of biochemical characterization of IPN neurons.

3. Assessment of feasibility of the research project

The chosen methodology is appropriate to achieve proposed research goals, and will provide detailed information about the structures of interest. A combination of RNA scope technique, patch clamp experiments and Sholl analysis will result in a clear image of IPN neurons activity, morphology and biochemical properties. A behavioural experiment with stress induction will show if IPN is active in anxiety-related situation in 3 model species and in combination with patch-clamp and RNA scope will provide information about conservativeness of IPN-vHipp circuit. It is possible to obtain negative result in the experiment related to IPN activity in different species (no changes in cFos level) and the author is aware of this contingency. Nevertheless, she claims that the problem can be solved by taking into account other markers on neuronal activity, which indicates the author has good knowledge of the field. The study has a very complex structure and many experiments are planned, therefore it is good that technical assistant and collective investigator are going to be involved in conducting the studies, because it increases the chances of completion the project until scheduled time.

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

The budget of the project is clear, cost of equipment and materials are well assessed. A large sum was planned to be spent on RNAscope experiments, which is however justified, as the price of single RNAscope kit is high and the analyses will be conducted on the tissue of four model species and human samples.

5. Strengths of the proposal

The first strong point of the project is an interesting and innovative hypothesis that may help to elucidate unclear role of NGF in anxiety disorders. The strength of the project is also that the role of NGF and IPN-vHipp connections will be assessed at different levels, as the authors have planned a biochemical analysis of molecules of interest, anatomical and electrophysiological assessment of neuronal cells as well as behavioural tests. The approach to the research problem is very broad, which constitutes an occasion for authors to learn new, advanced methodologies and improve previously gained skills. Moreover, if the results of human tissue will prove promising, it is probable that the cooperation with laboratory in Sydney (Australia) will develop and result in establishing bigger international project, focused on anxiety signaling in humans. The proposal is well structured, graphs are clear and help to understand the idea of described methods.

6. Weaknesses of the proposal

The author does not explain clearly the idea of testing NGF role and IPN-vHipp connections in different species. I would recommend to formulate a separate hypothesis for that point. In the research plan there are some minor inconsistences. In Experiment 1 it is not clear if the author is going to perform viral injections in this point, as they are mentioned in methods chart but not in the experiment description. One abbreviation has no extension (IEGs). Moreover, in the description of Experiment 1 I would add the information about immunofluorescent staining for cFos and how the microscopic image will be analyzed to compare cells activity between animals. Viral injections are mentioned in the description of Experiment 2, but not in the methods chart.

Final project proposal

Title: Deciphering anxiety pathway among model species

Authors: Sylwia Drabik, Gokul Bhaskaran

Summary:

Research objectives

Anxiety disorders constitute the largest group of mental disorders, yet a very low percentage of patients receive clinical treatment. The first and foremost reason for this challenge is the lack of drug therapies that would base on neurobiological mechanism, as most of treatments concentrates on observable symptoms of the disease. Therefore, studies aiming to discover neurochemical, neuroanatomical, and cellular mechanisms underlying these conditions are of high importance.

Proposed project targets interpeduncular nucleus (IPN) – ventral hippocampus (vHipp) axis, which consists of structures that are essential in controlling anxiety. IPN, a midbrain structure lying just ventral to the ventral tegmental area, is involved in discriminating between the novel and familiar stimuli, signals unpleasant symptoms of drug cessation, and plays a key role in anxiety behaviour. IPN densely innervates vHipp, limbic system structure implicated in contextual fear memory formation, avoidance, and anxiety response.

Moreover, the project will concentrate on the role of nerve growth factor (NGF) signalling in the overmentioned circuit. NGF was discovered as a neurotrophin responsible for neurites growth and survival, however, more recent discoveries showed its role in anxiety-related behaviours. Notably, NGF levels in both the brain and bloodstream were shown to significantly differ in stressful conditions in animals and humans. At the same time, IPN is a structure with one the highest level of expression of mRNA for high-affinity nerve growth factor receptors TrkA, and vHipp is enriched with TrkA immunoreactive fibres. However, the role and characteristics of the NGF signalling in IPN-vHipp axis remains unclear.

The implementation of the proposed project will allow for a detailed electrophysiological and neurochemical characterisation of the IPN-vHipp circuit as well as description of the functional connectivity between IPN and vHipp. Additionally, whole-brain projections of IPN neurons innervating vHipp will be traced, which will allow for the assignment of this IPN neuronal population to the specific functional brain circuits.

We aim to pursue this research with three important model systems (Sprague-Daweley rat (Rattus norvegicus), Zebra finch (Taeniopygia guttata) and Zebrafish (Danio rerio)) to check if the anxiety mechanism functions similarly among them. Furthermore, human IPN samples will be used in one part of planned project to gain information about possible clinical implication of found results obtained in animal models.

Research methodology

In the current project various research methods will be used. Anxiety in all animal species will be evoked using classical paradigm of social isolation. Furthermore c-Fos expression and immunofluorescence staining will be performed in order to characterize IPN cells innervating vHipp. With the usage of novel viral tools neural tract-tracing studies will be performed. High-throughput technique – RNAScope will be used to characterize biochemically IPN neurons. Data analysis will be executed in Matlab environment and CED scripts (electrophysiological research), ImageJ (dendrite and 3D Sholl analysis), L-Measure (morphological parameters). Subsequent statistical analysis will be held in Prism7 software (GraphPad).

Expected impact of the research project on the development of science

Answering the main questions of this study will significantly expand current knowledge about the IPN and vHipp axis, which is of potential significance for understanding neurobiological basis of anxiety control and

anxiety disorders. Moreover, a better understanding of the NGF role and action in the proposed circuit, as well as detailed characterization of NGF-sensitive IPN neurons will bring us closer to untangle the complicated mechanism underlying anxiety signalling. We consider our study as a next step for biomedical research and also in shaping fundamental and advanced evolutionary concepts.

I. Scientific goal of the project

Anxiety disorders constitute the largest group of mental disorders and currently approximately one third of the global population according to large population-based surveys (Bandelow et al., 2015) or 21,7% according to the WHO estimates, suffers from them (Charlson et al., 2019). Moreover, anxiety disorders show high comorbidity with other anxiety and mental conditions. They are diagnosed both in adults and children, however, there is a decrease in prevalence following older age. Unfortunately, only one third of affected people receive drug treatment, and according to the current estimations, only half of cases are being recognized (Bandelow et al., 2015). Anxiety disorders form a big family of disorders including panic disorder with or without agoraphobia, Generalized Anxiety Disorder (GAD), Social Anxiety Disorder (SAD), separation anxiety disorder and specific phobias (Bandelow et al., 2015). Patients suffering from anxiety related disorders are usually treated as outpatients and they receive less care and attention from their therapist in comparison to other mental disorders (Maestripieri et al., 1990). The lack of proper therapies and treatments partly arises from the limited knowledge about the neural basis of anxiety disorders. **Therefore, studies aimed at uncovering neurochemical and cellular mechanisms underlying anxiety related behaviours are of high importance.**

Recently, nerve growth factor (NGF), the first discovered member of the neurotrophin family, primarily known from its involvement in neurites growth, cells survival and neurotransmitter production, has gained attention in the context of anxiety disorders research. NGF level in bloodstream is affected by stressors and anxiety like behaviours in humans (Lambiase et al., 1994) and animals (Maestripieri et al. 1990). A massive release of NGF from salivary glands was observed in fighting males and lactating females during nest-defence (Berry et al., 2012; Maestripieri et al., 1990), and changed NGF level in the brain of animals after aggressive behaviours and psychosocial stressful events was described (Berry et al., 2012). **Despite the increasing number of data showing NGF role in anxiety related behaviours, its specific site of action as wells as possible functions are not well established yet.**

In the adult brain, several areas were shown to synthetise NGF: hippocampus, olfactory bulb, neocortex, septum, diagonal band of Broca, nucleus basalis of Meynert, striatum, hindbrain, medulla oblongata, hypothalamus and cerebellum (Korsching et al., 1985). From overmentioned brain areas, ventral hippocampus (vHipp) seems to be a principal component of anxiety and emotional behaviour control system and its lesions have an anxiolytic effect (Trivedi et al., 2004). Moreover, "anxiety cells" were described in ventral, but not dorsal part of hippocampus (Jimenez et al., 2018). **Importantly, vHipp is densely innervated by fibres immunoreactive for TrkA – receptor for NGF** (Sobreviela et al., 1994), **however, the source of this innervation remains undiscovered.**

Brain structure densely innervating vHipp (Lima et al., 2017) and involved in anxiety signalling is interpeduncular nucleus (IPN) (DeGroot et al., 2020). Importantly, IPN is one of a handful brain structures enriched with TrkA receptors in mice (Allen Brain Atlas, 2020) and preliminary data presented in the current project show a direct, postsynaptic effect of NGF administration on IPN neurons activity. **Therefore, this project's main hypothesis is that vHipp is under the influence of NGF sensitive IPN innervation and that IPN-vHipp axis plays an important role in anxiety signalling.**

It is supposed that anxiety signalling is evolutionary conserved process, moreover, we know that all the described structures are present not only in mammalian but also birds' and fish brains. **Another aim of the project will be to examine differences and similarities of this pathway among species which could potentially lead us to the conclusion that it would be enough to conduct several experiments on zebra fish, as their similarity to rat is sufficient**. Obtaining such a result would save a lot of time and money as zebrafish are easier to be propagated and cheaper to be bread than rodent models.

II. Significance of the project

Despite emerging data there is still a need for research concerning the role of IPN-vHipp axis in control of anxiety related behaviours, as well as an involvement of NGF in modulating this neuronal axis activity. Therefore, **the goal of this study is to identify the source of NGF in the area of IPN, investigate anatomical and functional connectivity of IPN-vHipp pathway and characterise IPN NGF sensitive neurons**

innervating vHipp at both morphological and electrophysiological levels. **Moreover, the influence of NGF administration on the neuronal activity of IPN neurons innervating vHipp will be verified.** As the aim of the project is to examine conservativness of vHipp-IPN axis in evolution one exmaplary species will be taken into consideration from 3 divisions (mammals, birds, fish). Therefore all the experiments will be held on Sprague-Daweley rats, zebra finches and zebra fish. Moreover, thanks to the cooperation with laboratory in Sydney (Australia) we will be able to perform RNAscope assay on human tissue. Planned research will combine electrophysiological recordings with optogenetics, immunohistochemical staining and viral based tract tracing methods to bring us closer to uncovering neuronal mechanisms involved in IPN control of vHipp, and to describe NGF influence on IPN-vHipp axis among species. Some anatomical studies will be performed using human brain tissue, to ascertain the **translational relevance of the planned preclinical studies.**

Scientific hypothesis:

- IPN cell are active in anxiety in all examined species
- IPN neurons innervating vHipp have similar expression of mRNA for NGF and CRF receptors in all examined species and humans
- vHipp is under the influence of NGF sensitive IPN innervation
- IPN cells innervating vHipp project to strucures involved in stress signalling in all examined models

III. Concept and work plan

1. Measuring activity of the cells within IPN in all species; behavioural experiments followed by c-Fos analysis

Social separation is described as a stressful stimulus for fish, rats and zebra finches (Shams et al., 2017, Beery and Kaufer, (2015), Emmerson and Spencer, (2017)). Therefore, to evoke anxiety in all animals they will be kept in separate cages (aquarium in case of fish) for 90 minutes. IEGs and their protein products are expressed following neural activity, among them *cfos* and Fos expression are the most widely used indicators post-mortem (McReynolds et al., 2018). This techique will be used to verify the presence of supposed activity of IPN after exposure to stresful stimuli.

2. Biochemical characterization of IPN neurons innervating vHipp and active during anxiety; bahavioural studies, stereotaxic surgeries, RNAscope.

In order to **identify and characterise the IPN neurons innervating the ventral part of hippocampus,** the retrograde viral based tracing combined with the electrophysiological recordings will be used. Surgery on adult male rats will be conducted and AVV retrograde viral vector driving mCherry expression (AAVrg-hSyn-mCherry) will be injected bilaterally into the vHipp. Three weeks after the surgery, the same behavioural stressors as in experiment 1 will be induced to animals. 60 minutes after RNA scope procedure will be held with the usage of kit dedicated for each species. RNAScope is an *in situ* hybridization assay for detecting RNA in formalin-fixed paraffin-embedded tissue that is commercially accessible. **This procedure will answer the questions weather IPN cell active in anxiety are innervating vHipp, do they have receptors for NGF, CFR (corticotropin releasing factor, a canonical stress modulator) and weather they are GABA or Glutamatergic** (thanks to checking the presence of absence of mRNA for vGlut2 and vGAT1).

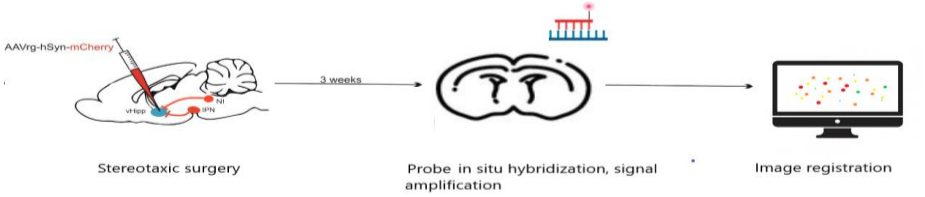


Fig. 3 Experimental scheme showing the injection site and major steps in RNAscope procedure.

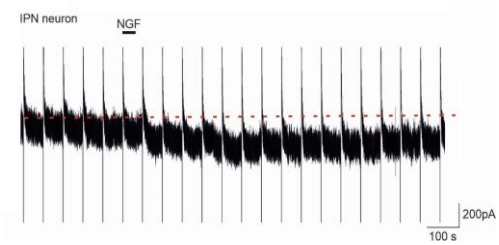
3. Biochemical characterization of human IPN neurons; RNAscope

RNAscope on human frozen IPN samples obtained in The Florey Institute of Neuroscience and Mental Health (Melbourne, Victoria, Australia) will be performed. T**his part of the project will help us to ascertain the**

translational relevance of the planned preclinical studies in animals and assuring evolutional conservativness of the circuit.

4. Electrophysiological and neuroanatomical characterization of IPN neurons innervating vHipp and verification of sensitivity of neurons constituting IPN-vHipp axis to NGF in all species; patch clamp electrophysiological experiments.

In order to **identify and characterise the IPN neurons innervating the ventral part of hippocampus,** the retrograde viral based tracing combined with the electrophysiological recordings will be used. Surgery on adult male rats will be conducted and AVV retrograde viral vector driving mCherry expression (AAVrg-hSyn-mCherry) will be injected bilaterally into the vHipp (just like in the previous experiment). Three weeks after the surgery, electrophysiological patch clamp experiments will be performed.



During whole-cell patch clamp recordings of mCherry expressing IPN neurons, their electrophysiological and anatomical features will be characterised. Responsiveness of IPN neurons exposure to the NGF will be verified. During patch clamp experiments, responsiveness of IPN neurons directly innervating vHipp (mCherry expressing) to administrations of NGF will be verified. The procedures with some technical changes will be held for all the animal species. Importantly, in our preliminary studies (Fig. 1) we observed that NGF administration induces whole cell inward current in IPN neurons, what proves that NGF can act in a neurotransmitter-like mode in the IPN of rat. **Results of these experiments will answer the question whether NGF acts like a neurotransmitter in the IPN and will allow to characterise the nature of the response of IPN neurons innervating vHipp to the NGF.**

Fig. 2 NGF administration induced whole-cell inward current in IPN neurons. Voltage clamp recording of IPN neuron activity (command potential -50mV) before and after (indicated by bar) the administration of NGF (5ml, 250ng/ml).

To answer the question **whether IPN neurons innervating vHipp are directly or indirectly sensitive to NGF**, responsiveness of IPN neurons innervating vHipp to the NGF administration will be recorded and subsequently repeated in the presence of voltage sensitive sodium channel blocker and GABA and glutamate ionotropic receptors antagonists. These experiments will allow to identify if the action of NGF on IPN has a pre- or postsynaptic nature.

Due to biocytin presence in the recording micropipette, **anti-biocytin staining of recorded neurons will allow to assign them to specific IPN location** within the IPN as well as to preform Sholl analysis of the biocytin filled neurons and to characterize their anatomical features such as number of branches, bifurcations and area occupied by their dendrites.

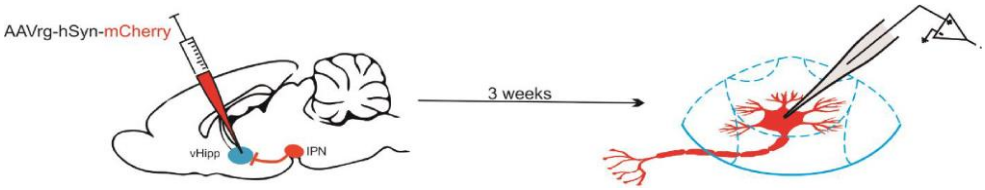


Fig. 3 Experimental scheme showing the injection site, used virus and structure to be recorded on the example of rat's brain. .

5. Characterization of the whole brain axonal endings distribution of IPN neurons that innervate vHipp; neural tract-tracing and immunohistochemical studies.

Characterization of projections of IPN neurons innervating ventral hippocampus. In order to describe whole brain axonal endings distribution of IPN neurons innervating vHipp, CAV-Cre retrograde virus (CAV-2 vector harbouring the CMV promoter that leads to Cre recombinase expression) will be injected bilaterally to ventral hippocampus and anterograde Cre-dependent AAV-hSyn-DIO-mCherry will be injected to IPN of the same animal. Immunofluorescence anti-mCherry staining will reveal the whole brain fibre distribution of IPN neurons innervating the vHipp. The procedure with all the necessary technical corrections will be repeated for zebra fish and zebra finches. Mapping the structures controlled by this discrete IPN neuronal population, will allow to further **hypothesise about the functional importance of IPN-vHipp axis** as well as **checking whether the function predicted is conservative among species.**

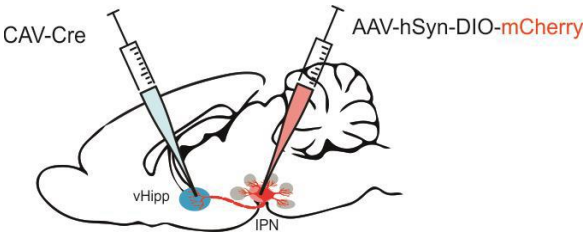


Fig. 4 Experimental scheme showing the injection sites and used viral vectors.

EXP. NO.	METHODS	EXPECTED RESULTS AND OBSERVATIONS
1	Viral injections, Social isolation, Immunohistochemistry	Identification of IPN cells active in anxiety, checking if the localization is evolutionary conservative among species examined
2	Social isolation, RNAscope	Biochemical characterization of IPN neurons innervating vHipp and active in anxiety, checking similarities among species
3	RNAscope	Biochemical characterization of human IPN neurons
4	Viral injections, Patch clamp recordings, Immunohistochemistry	Characterization of electrophysiological and neuroanatomical properties of IPN neurons innervating vHipp and verification of their sensitiveness to NGF; checking if mechanism is evolutionary conservative
5	Viral injections, Immunohistochemistry, Neural tract-tracing	Characterization of whole brain axonal endings distribution of IPN neurons innervating vHipp, checking if the distribution is evolutionary conservative

Fig. 5 Chart showing the summary of methods used in each part of the project and expected results and observation driven from each experiment.

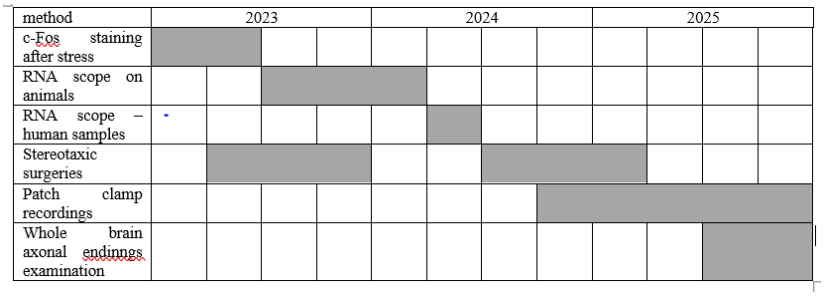


Fig. 6. Experiments timeline

IV. Risk Assessment

PI's experience in described research techniques and results of presented preliminary studies support successful completion of the study. Intra brain injections are the aspect of the highest risk of failure so there will likely be a need to refine and repeat the surgeries. Therefore, the number of animals planned for these parts of the project, where intra-brain injections are planned, is overstated. There of course is a chance of negative results appearing in the first experiment planned. However, cfos transcription assumed as always being associated with neural activation no always is the case. For example, substantia nigra activity has been measured following kindled seizure induction but corresponding changes in Fos were not observed (Labiner et al., 1993). Therefore, even if no cFos positive cells will be visible within IPN in the first experiment, the rest will be continued and other markers of neural activity will be taken into account.

V. Research methodology

All the described experiments will be held at the Department of Neurophysiology and Chronobiology at the Institute of Zoology and Biomedical Research at the Jagiellonian University in accordance with the Ethical Committee Agreement. PI and technical assistant have experience with work on overmentioned species and documents allowing them to perform all the described procedures on animals. Moreover, two additional workshops increasing their competences are planned at the very beginning of the project. Experiments will be conducted on 70 Sprague-Dawley rats (6-8 weeks at the start of each experiment) and 100 zebra fish (5-7 days at the start of experiment) both species of animals will be bred and housed at the Institute of Zoology and Biomedical Research, Jagiellonian University. Moreover, 80 zebra finches (12-14 weeks at the start of each experiment) which will be bred and housed at the Institute of Environmental Sciences, Jagiellonian University will be used. Moreover, thanks to the collaboration with Prof Andrew Gundlach from The Florey Institute of Neuroscience and Mental Health (Melbourne, Victoria, Australia) we will be provided with frozen human IPN samples for RNAscope procedures.

Social anxiety induction: As the animals used in our experiments are social in nature, isolating them will induce anxiety. Rats will be kept in individual cages in isolated arena to prevent any auditory, olfactory or visual cues from other rats. Zebrafishes before experiment will be singly transferred to pool that is freshly filled with water, and each tank will be masked from one another which further strength in level of isolation. Zebra finches will also be isolated singly in individual cages. All the isolations take place 90 minutes before the start of the experimental procedures.

Neural tract-tracing and **immunofluorescence staining:** In order to **identify interpeduncular nucleus neurons innervating ventral hippocampus,** after bilateral intra-vHipp injections of viral vector rats will be given three weeks recovery period. After that, all animals will be deeply anesthetised with pentobarbital, killed by transcranial perfusion and their brains will be dissected and postfixed for 24h. After post-fixation period coronal sections, will be cut on a freezing microtome (Bright Instruments). In experiments aiming at identification of **the whole brain axonal endings distribution of IPN neurons that innervate vHipp**, after viral vector injections slices will be incubated with mouse anti-mCherry. For **experiments aimed to identify cFos positive cells in IPN** mice anti-cFos will be used. After patch-clamp recordings sections will be stained against biocytin. All the described sections will be mounted on glass slides, coverslipped with Vectashield containing DAPI (Vector Laboratories) and imaged with the usage of fluorescent microscope (Axio Imager M2, Carl Zeiss).

Multiplex Fluorescent in situ Hybridization (RNAscope): in situ hybridization using an RNAscope HiPlex Assay [Advanced Cell Diagnostics (ACD), Hayward, CA, United States] with RNAscope HiPlex Alternate Display Module (ACD; for AF488, Atto550 and Atto647 detection) will be conducted on brain sections from 3 male Sprague-Dawley rats, 3 male Zebra Finches, 3 male danio rerio fish and 3 samples from male human brains 3 weeks after the injection of the retrograde viral vector encoding mCherry protein, into the vHipp. All procedures will be performed following the manufacturer's instructions, with preparation and pretreatment for fresh frozen samples.

Surgical procedures and viral vectors injections: Before surgery adult Sprague-Dawley rats will be anaesthetised with isoflurane and placed into a motorized stereotaxic instrument for rats. Viral vectors will be injected bilaterally into vHipp: -5.2; 4.5; -8 (AP; ML; DV, mm from bregma) and (in experiment 4) into IPN: -6.2; 0.0; -9.0. Zebrafish will be anesthetised with buffered tricaine methane sulfonate (MS222) and zebra finches with ketamine and xylazine solution intraperitoneal injection. Brain coordinates of both structures in for birds and fish need to be calculated using stereotaxic atlas for particular species and checked with preliminary surgeries with the usage of fluoro-stainings. After the surgery, animals will be allowed to recover for three weeks (time necessary for full expression of the transduced genes).

Whole-cell patch clamp electrophysiological studies: Experiment will be performed 3 weeks after viral injections. Brains will be prepared and placed in recording chamber of Axioskop FS2 microscope (Zeiss). Whole cell current clamp and voltage clamp recording with specific tests for electrophysiological properties of neurons will be performed. All the drugs will be administrated via bath perfusion. All the necessary equipment for patch-clamp recordings in available at the host Department.

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VII. Table with budget of the project

	Amount in PLN
Direct costs, including (I+II+III+IV)	**410000**
- personnel costs and scholarships (I)	234000
- research equipment/device/software cost (II)	112000
- Travel costs (III)	38000
- other direct costs (IV)	26000
Indirect costs, including (V+VI)	**82000**
- indirect costs of OA (V)	15000
- other indirect costs (VI)	67000
Total costs (I+II+III+IV+V+VI)	**492000**

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

Details of direct cost items:

Category	Description	Amount
Salaries and benefits	Principal Investigator (1)	5000 PLN/month*36 = 180000 PLN
	Technical Assistant (1)	1500 PLN/month*36 = 54000 PLN
Equipment	Stereotaxic apparatus for birds + fishes	5000 PLN (including VAT)
	Computer system + high graphics support + storage + software	5000 PLN (including VAT)
	RNAScope (colocation of cells innervating vHipp and c-fos) + TrKA (receptors for NGF) + CRFR1 + VGLUT2	100000 PLN (including VAT)
Materials	Chemical reagents (biocytin and extrAvidin - visualization of registered neurons, Flouoroshield - to coverslip the slices, viral vectors needed in surgeries (AAVrg-hSyn-mCherry), primary and secondary antibodies	10000 PLN (including VAT)

	needed for immunostainings)	
	Small laboratory supplies (Expendable goods needed in research e.g. borosilicate glass pipets, eppendorf tubes, pipette tips, well-plates)	10000 PLN (including VAT)
	Stationery supplies (Printing cartridges, toners, paper)	500 PLN (including VAT)
Travel	International Conference (FENS, IBRO) registration fee (2500 PLN) + tickets (3000 PLN) + daily allowance (250 PLN) + accommodation (1750 PLN) = 7500 PLN * 2 individual *2 conference	30000 PLN (including VAT)
	Training workshops (WONWEP, EMBO Neurobiology Workshop) registration fee (500 PLN) + tickets (750 PLN) + daily allowance (250 PLN) + accommodation (500 PLN) = 2000 PLN * 2 individual * 2 workshops	8000 PLN (including VAT)

Other direct costs:

Purchase/maintenance of study animals (includes food, cages) + Bio-hazard waste management = 21000 PLN
Sample transportation/shipment = 5000 PLN

Details of Indirect cost items:

Publication of the study results (open access journals) = 15000 PLN
Host institute = 67000 PLN

Project 3: Behavioural and physiological changes induced by the road proximity in gray wolf

Draft project proposal

Title: Behavioural and physiological changes induced by the road proximity in gray wolf.

Authors: Zuzanna Rauk, Pritam Kumar Dey, Monika Hoffmann

Summary:

The negative impact of roads on different species is well documented. Although the behavioural changes are known, they were not tested with physiological measures. In this project we want to analyse if wolf packs have a different stress response according to whether they live close or far away from roads. We will analyse if the location affects their behaviour and reproduction success. We will collar 60 individual wolves from three geographic regions (Asia, Europe, North America) and take hair samples to analyse accumulation of cortisol and steroids. The following hypotheses will be tested: (1) Road proximity increases the chronic stress level in gray wolves; (2) Wolf packs living to the road proximity will have smaller movement radius or unusual temporal activity and lesser reproductive success in comparison with the packs living in roadless areas; (3) Wolf populations using or living close to the green bridges will have lower chronic stress level in comparison with the packs living in roady areas but similar or higher chronic stress level living in comparison with the packs living in roadless areas whereas heart rate will be lower while using the bridges in comparison to using the roads. The wolves will be tracked for the period of the whole project and 6 times within the first 18 month samples will be collected. Spatial analysis will be conducted with ArcGIS software and the hair samples will be analysed with ELISA.

1) Scientific goal of the project

Approximately one million species are at risk of extinction given the accelerated rate of environmental change (IPBES 2019). Across the globe, habitat fragmentation is one of the greatest threats to biodiversity (Butchart et al. 2010). Fragmentation has altered more than 50% of the Earth's landscapes (Keeley 2019) and the road network is shaping the environment on a global scale (Ibisch et al 2016). Ecosystems are influenced by road infrastructure in a diverse, very complex, and time delayed way. The effects extend far beyond the road itself. The ecological impact of roads on ecosystems, species, and populations is well documented (Laurance & Balmford 2013). Only in the last 20 years have the effects of roads on wildlife gained significant attention and are referred to as "road ecology". Direct impacts of road construction include the fragmentation and isolation of landscapes, the physical alteration of sites, dust and salt, heavy metal pollution, changes to the microclimate, noise, light, behavioural changes, and animal mortality caused by vehicle collision (Kaphegyi et al. 2012). Roadless areas play a significant role in maintaining ecosystem functionality and supporting biodiversity and ecological processes. They may also support species movements, like long-distance dispersal.

In this project, we will try to understand the correlation between the animal stress level due to proximity to roads and their behavioural changes. One of our objectives will help to understand different levels of stress caused by areas with high road density, areas with the green bridges, and roadless areas (here areas at least 50km away for a road). We will analyze the hair cortisol accumulation and steroid hormones as a measure of reproductivity and the migration pattern of Canis lupus (gray wolf).

Their wide range distribution and living in pack nature will make them suitable to address our objectives more efficiently. Thanks to our robust sampling plan (Six sampling regions across two continents) where we will take three individuals (one male, one female and cub irrespective of their sex) from at least three wolf packs living in three different regions with roads, with green bridges and without roads. With comparative analysis of data coming from wolf pack (size and demography), GPS collaring, GIS, Cardiac logger, cortisol from hair samples, we will able to answer our questions:

1. ***Q1. Do wolf packs living in areas affected by roads have a high chronic level of stress in comparison to wolves living in roadless areas? H1.*** *Road proximity increases the chronic stress level in gray wolves.*
2. ***Q2. Does chronic stress related to road proximity affect the behaviour (temporal and spatial movement patterns) and reproductive success in gray wolves? H2.*** *Wolf packs living to the road proximity will have smaller movement radius or unusual temporal activity and lesser reproductive success in comparison with the packs living in roadless areas.*
3. ***Q3. Do green bridges reduce acute as well as chronic stress levels in gray wolves? H3.*** *Wolf populations using or living close to the green bridges will have lower chronic stress level in comparison with the packs living in roady areas but similar or higher chronic stress level living in comparison with the packs living in roadless areas whereas heart rate will be lower while using the bridges in comparison to using the roads.*

2) Significance of the project

Many studies have shown that road proximity affects wild animals physiology. Experimentally arranged exposure to human-generated noise induces a physiological stress response in female wood frogs and impaired female travel towards a male breeding chorus in the field, which may negatively affect the population persistence (Tenessen et al., 2014). Noise was also shown to small mammals and large carnivores (Malo et al. 2012). Gray wolves are known to avoid roads and settlements and breed less successfully in the areas of high road density (Merill, 2000). It is however unknown if everyday crossing the roads puts the animals under chronic stress, which lowers animals' reproductiveness and increases the risk of population decline. Our study will let us identify a potential relationship between living in roady areas and chronic stress in wolves and make conclusions about the effect of road proximity on wolves' reproductivity. Green bridges (GB) are the

communication facilities that can mitigate the impact of roads on wildlife. Ford et al. proved that construction of green bridges restored connectivity in the landscape and helped to recolonize eastern Germany with gray wolf, roe deer, red deer and wild boar (Plaschke et al., 2020). There is however no data about the effect of green bridges on wolves' physiological parameters. In the present study we will compare the immediate stress response in wolves crossing the roads and using green bridges, as we assume that using such facilities is less stressful for animals. Moreover, chronic stress indicators will also be analyzed in wolves in areas with high number of GBs, to verify the hypothesis that restored connectivity in the landscape reduces the stress level in wolves. Analysis of the relationship between road crossing and animals' physiology and behaviour is a completely novel approach in wolf biology. This experiment will contribute to the development of conservation biology, as it may potentially identify green bridges as a new tool to protect gray wolves and re-introduce them into traffic-affected areas.

3) Concept and work plan

(general work plan, specific research goals, results of preliminary research, risk analysis);

In this project we focus on the *Canis lupus* gray wolf species. The distribution of wolves stretches from Eurasia to North America. We selected three broader geographic regions. Wolf populations will be analyzed in North America, Asia and (Geographic) Europe. Within these regions we have three subcategories. We divided the locations in roadless areas (population home range at least 50 km away from any roads), areas with high road density (highways part of the wolf's home range) and presence of green bridges in the home range of the wolf population. For each subregion we select 5 wolf populations (see map figure 1). Per population we will collar 4 individuals (2 male and 2 female). Two "breeders" (formerly known as alphas) and 2 yearlings or subadults. Per region we will analyse 20 wolves and in total 60 wolves. This study utilized GPS tracking collars, deployed on gray wolves through aerial net gunning, to analyze their movement patterns. Telemetry will be analysed statistically using GIS software.

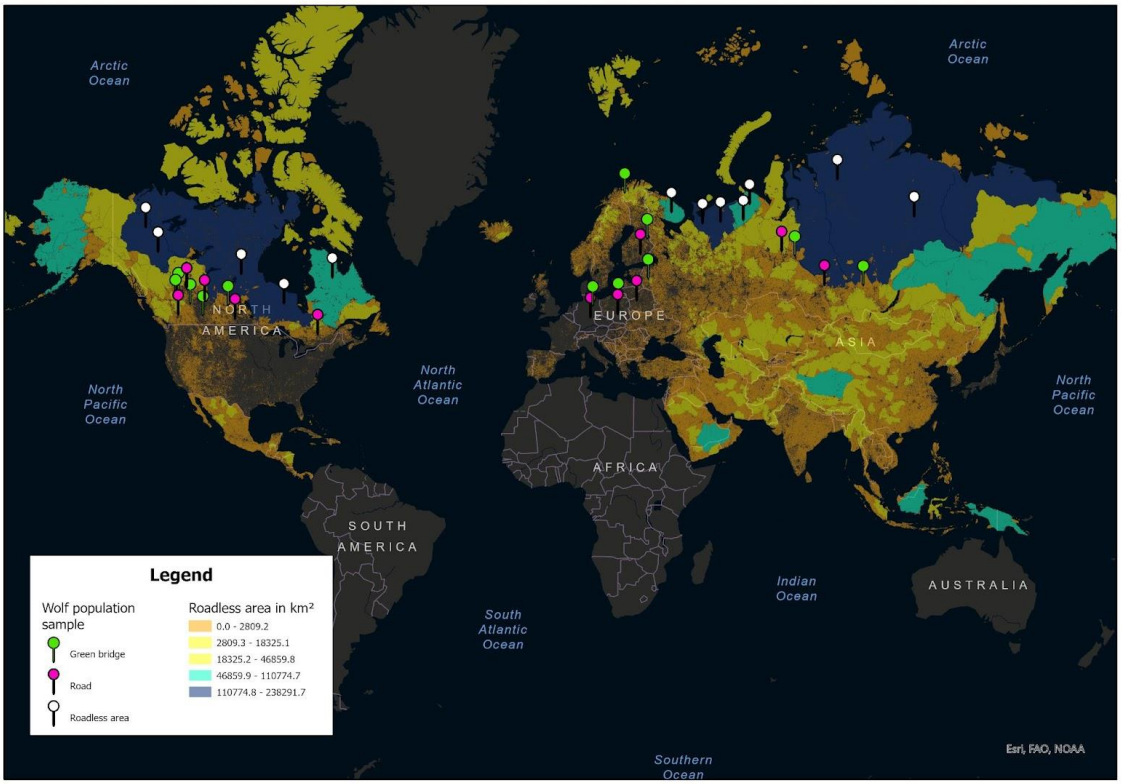

Figure 1: Map of wolf populations sites in roadless areas.Green pins are wolf packs that use green bridges, pink pins are packs that live close to roads, and white pins are wolf packs that live in roadless areas.

All movement data will be continuously transmitted to the three labs per geographic region, cleaned and anlaysed according to the density of movement, travel distances and day and night activity.

4) Research methodology

Field work

The animals will be captured, anesthetized and outfitted with radicollars as well as cardiac biologgers implemented subcutaneously in a peristernal location. We will use the model that was successfully used in HR measurements in American black bears and contain software that allows linking the discrete events captured by the GPS collar to changes in HR (Medtronic Inc.,Reveal® XT Model 9529, Minneapolis, MN; specifications: 9 cc; 8 × 19 × 62 mm; 15 g). For the next 18 months the measurements of heart rate will be conducted. Every 3 months animals will be captured and anesthetized for body mass measurements (Hook balance (WAGMA Poland) and hair samples collection, for the assessment of cortisol accumulation as an indicator of chronic stress. This approach was proved to reliably reflect prolonged stress exposure both in moose and in wolves, in contrast to blood cortisol level which fluctuations depend on multiple factors. Moreover, steroid hormones accumulation can be analyzed in hair, as a measure of reproductiveness (Bryan et al., 2014). 3 hair samples will be collected, one for cortisol analysis and two for testosterone and progesterone. Hair will be collected from the neck by cutting the hair as close to the skin as possible using a clean knife or electric clipper over an area of approximately 4 cm2 per each sample.

Laboratory work

A standardized ELISA protocol will be used for hormons isolation and measurement in hair samples, as described in the article of Bryan et al. 100mg of hair will be weighted and washed with methanol (Omnisolv; VWR, Mississauga, ON, Canada). After drying hair will be milled into the powder. 30mg of the powder will be transferred into the eppendorf with 50 μL of methanol per mg of hair powder.In order to extract steroids and cortisol, samples will be sonicated for 30 min and rotated at 160 rpm in an incubator for 18 h at 50 °C. After centrifuging, supernatant will be aliquotted into separate tubes for progesterone (100 μL), testosterone (50 μL) and cortisol (1000 μL) assays. Commercial kits designed for saliva will be used to measure cortisol, progesterone and testosterone in hair extracts (Salimetrics, Philadelphia, PA, USA).

Statistical analysis

A comparison of physiological and behavioural parameters will be conducted between wolves from locations with different road types (roadless area, roady area, roady area with green bridges). Moreover we will compare the results between different geographical locations (Europe, Asia, North America), 3 distinct populations within each of the geographical regions and between males and females within each population. Heart rate during crossing the bridges or using green bridges will be compared between different animal cohorts. The populations from roadless areas will constitute control groups.

Movement pattern will be analysed with the GPS data transmitted directly to the labs in each geographical region and cleaned for further processing the the main GIS lab. The data will be analysed according to travel distances of the pack, day and night movements and density of movement.

5) Project literature

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Seiler, A. 2001. Ecological effects of roads. A review. Department of Conservation Biology. Swedish University of Agriculture Sciences.

Tennessen, J. B., Parks, S. E., & Langkilde, T. (2014). Traffic noise causes physiological stress and impairs breeding migration behaviour in frogs. *Conservation physiology*, *2*(1), cou032. https://doi.org/10.1093/conphys/cou032

Budget:

Item	1st Year	2nd Year	3rd Year	Total
1.	Direct Costs including:			
A.	Salaries and benefits			1689750PLN
B.	Equipment			1305134PLN
C.	Other direct costs			148500PLN
2.	Indirect Costs			12000PLN
Total Costs (1+2)				3155384PLN

3. Direct Costs including:

A. Salaries and Benefits:

Salaries for main project performers

(Tasks: Planning, preparing, conducting filed trips, managements of field data collections, taking part in field data collections, handling of field data's, analysis of field data (GIS, GPS, Cardiac loggers, wet labs, statistical analysis, participations in conferences, workshops, writing articles)

4500PLN x 36 months x 3 persons = 4,86,000 PLN

4500PLN x 28 months x 2 persons = 2,52,000 PLN

Salaries for Lab manager

(Lab management, administration work, dealing legal issues i.e., permissions for data collections inside protected areas, transportation of biological samples across the international borders)

4500PLN x 12 months x 1 persons = 54000 PLN / 1st year

4500PLN x 12 months x 1 persons = 162000 PLN

Salaries for field assistants

2500PLN + (9% * 2500PLN; insurance) x 30 months x 9 persons = 735750PLN

Salaries for all project performers (fee for task agreement)

4500PLN x 16 months x 18 persons = 1296000

B. Equipment:

GPS collars:

14430PLN x 60 x 5 (Extras) = 937950

Computing and Software:

2,40,184PLN

Batteries:

100PLN x 10 persons = 1000PLN

Flashlights:

200PLN X 10 persons = 2000PLN

Infrared Binoculars (YUKON Night versions):

1500PLN x 10 persons = 15000PLN

GPS: GERMIN ETRIX:

900PLN x 10 persons = 9000PLN

Cardiac Bio loggers:

1000PLN x 60 = 60000PLN

Lab Chemicals:

40000PLN

C. Other Costs:

Daily allowance:

0.90PLN x 5000 km x 10 persons = 45000PLN/year

Conferences – Foreign Conferences:

4500PLN x 3 = 13500PLN/year

2. Publication:

3000PLN x 4 = 12000PLN

Reviews

Aneta Arct - abstract review

This abstract meets the assumptions set for abstracts of research projects. It presents the main objectives and research methods. On this basis, appropriate reviewers can be assigned. However, abstracts have always played another a crucial role in explaining your study quickly and succinctly to reviewers and prompting them to read further. Because nowadays a potential reviewer has a lot of projects to evaluate, writing a compelling abstract is one of the most important things. In my opinion, the first section of the abstract lacks explanation why people should care about this study—why is it significant to your field and perhaps to the wider world? Are the results of this study going to shake up the scientific world? Also the last section of the abstract should include two or three sentences about the potential major finding from and implication and significance of the project. As a general rule, a full stop is not used at the end of a displayed title in scholarly English prose. Despite the disadvantages, after reading the abstract, the project seems to me to be worthy of attention.

Krystyna Nadachowska-Brzyska

1. Assessment of scientific quality of the research project

The general aim of the project is to understand how roads affect physiology and behaviour of wolves' packs. In particular, the authors rise the questions: Do wolves living in areas affected by roads have a high chronic level of stress in comparison to wolves living in roadless areas? Does chronic stress related to road proximity affect the behavior and is it reduced if wolves have access to green bridges? These questions are interesting and important in the light of changing environment and biodiversity threats due to urbanization and habitat fragmentation. The study system is well chosen; wolves populations have been heavily affected by human activity, we do know a lot about wolves populations that allows as to discuss the results in a wider context but at the same time little is known about chromic stress levels in populations living in human proximity (at least to my knowledge).

2. Assessment of potential impact of the research project

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

3. Assessment of feasibility of the research project

The project is very ambitious and needs very careful planning and extensive logistics. In my opinion it can be simplified without loosing its impact, and at the same time it can be improved in terms of sample size and the data collected. Please find my main concerns and suggestions below:

- The project assumes sampling (and monitoring) wolves at 3 different continents but at the same time the number of populations and individuals sampled (and monitored) is very low. What happens if some wolves die before the end of the project? Or get killed? Or cannot be catch at the time of sampling? Statistical power in a presented sampling scheme is very low and can very easily be lowered but situations that are hard to predict. However, many of worlds wolves populations are being radio tracked and monitored for years (e.g Scandinavian wolves). Would it be a good idea to use the existing records?
- The authors plan to measure stress levels in only 4 individuals per population. Apart from the problem of losing an individual (e.g. dying, mentioned above) I wonder what variation in stress hormones levels among different members of the pack we expect? Again it seems for me that analyzing hair from only

4 individuals per population may not reflect true stress levels of a pack. What about noninvasive hair sampling that has proven to be successful (eg. https://doi.org/10.2193/2009-305)? Can it be done here to obtain much larger samples sizes per population including several individuals per population?

- I also wonder if catching wolves several times over a relatively short period of time can alter stress hormones accumulation? Again noninvasive sampling seems easier and safer.
- What about the reproductive success of wolves the authors mentioned in several places in the proposal? I do not really how it will be measured?
- I also wonder how often wolves packs change their territory? Is it possible that they move from a place that is close to reads to a more remote place? Long distance migrations do happen (e.g. Swedish populations were established after a very long distance migration of 1 wolf couple).

While I recognize that for the heart rate monitoring the wolves has to be catch, in my opinion a combination of using the existing monitoring data combined with noninvasive hair sampling can vastly improve sampling scheme and minimize the risks of losing crucial samples.

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

The cost are mostly justified but, again, at least some data can be collected using existing monitoring schemes and non-invasive methods – probably lowering the costs and simplifying the logistics of the project.

4. **Strengths of the proposal**

The project tackles important questions and has a potential to produce high impact results to be appreciated by scientific community as well as conservation and monitoring agencies. It is also rather well written, given a very short time period and the carrier stage of the authors.

5. **Weaknesses of the proposal**

As the weaknesses of the proposal I recognize small sample size and high risk of not obtaining enough data. The logistics of the sampling that includes catching several wolves several times over a relatively short period of time can be simplified.

Siri Devarakonda

1. Assessment of scientific quality of the research project

This is potentially an interesting project aiming to measure the of stress in wolves. Testable hypothesis and measures are given. State of art is generally well described. The project has however two many weaknesses. First, Work Plan is not clearly written, and number of bridges used is not mentioned. More importantly the work plan doesn't contain the outline of one the project goals,i.e., it is missing the test of few hypothesis

Second, I cannot understand how the stress will be specified to the roads when the statement in the line number"156-157" itself states The Specificity of measuring the cortisol in wolves can depend on many factors.

Authors included full description of statistical analysis details which will be conducted, but it looks like they forgot to include the analysis of injuries and its effects on animal behaviour or the cortisol levels

In the research methodology line numbers 147-151 require more details regarding the "GPS collar changes in brown bear and HR measurements".

Line number 153 The body mass measurement is mentioned but not stated in the work plan

Scientist who are not specialist in this topic cannot understand this project.

I suggest the author to include the details about the effect of anesthesia on the cortisol levels. And behavioural aspects of this study.

Additionally the model appears quite complex, with many variables that needs to be measure and compared together.

On the line"181-182" the authors suggest that the wolves behavior and physiology parameters will be studied which is missing in the research methodology.

"125"I Suggest to mention the number of green bridges existing in the home range of the wolves regarding the study ares.

2. Assessment of potential impact of the research project

The project is novel and is likely to have a substantial impact on the conservation field. However, the authors could explain the project in detail about the reproductive success with relation to stress and the current research in the preliminary studies.

My major concern of the project is that I don't see a clear indication of the outputs. from the study.

3. Assessment of feasibility of the research project

It is currently difficult to assess the feasibility of the project. For example, we don't know the occurrence of the road crossing and the other factors affecting the study.

There is no risk analysis in the proposal.

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

I suggest the author to be precise about the budget and specify the needs as they are being repeated multiple times. in line number “287” there are batteries used for 10 persons which needs to be proof read and changed.

Cost calculations need to be explained in detail for each purpose rather than grouping them together.as in “266-269” Salaries for lab manager include the transportation of biological samples.

Budget for the ELISA kits is missing.

Please make a table for the Items

Mention the details of the journals that you propose to publish in.

5. Strengths in of the proposal

Well defined aims and testable hypothesis

Novelty

Well described state of art

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

6. Weaknesses of the proposal

The background of the project is not fully analyzed

Work plan is not clearly outlined

Major problems with statistical analysis

Expected impact and significance is not sufficiently mentioned

Poor literature supporting the proposed methods

English could be improved.

Sylwia Drabik

1. Assessment of scientific quality of the research project.

Project aims at the very relevant topic nowadays as human encroachment in animals' habitats is getting more and more widespread each year. It was previously known that the presence of roads in wild animals' strongholds has a significant impact on their behavior. In the described project authors came up with a novel approach to concentrate also on physiological, noninvasive stress measurements (collecting hair samples) as well as invasive (biologgers and GPS collaring) to further investigate the problem. Moreover, they proposed an interesting experimental group of animals living in roady areas with presence of green bridges to examine their influence on Project has an international context as the samples will be taken from several different locations of grey wolf population in Europe, Asia and North America. Biological data will be collected every 3 months withing the examined period and biolocation – continuously. Thus, study seems to have interdisciplinary, novel perspective on a very important issue of fragmentation of animals' habitats. Moreover, it takes into consideration endangered species – gray wolf which possibly will give us opportunity that after describing results of the project we will have a glimpse of how we can conserve this species. All the hypotheses are well designed and clearly stated, but one part about reproductive success (mentioned in further details underneath) and probable to verify using mentioned techniques.

2. Assessment of potential impact of the research project.

The study is well designed and seems to have a great approach to fill in the knowledge gap not only about animal stress caused by proximity of the roads, its supposed effects on the wolf packs movements and reproductive stress, but also impact of green bridges on the level of chronic stress of animals, their movements and reproductive success. There is a huge probability that this study will bring us closer to design proper mechanisms aiming at conservation of endangered species all over the world.

3. Assessment of feasibility of the research project

Wide range of science techniques are combined in the study which gives it more opportunities to broaden our knowledge about the impact of road on chronic stress levels, movements and reproduction success of wolves. I wonder, however, if there is a possibility to come to clear conclusions while having so small samples (only one or two animals having certain role in the pack). I here I would suggest concentrating on one role or sex of the animal of the pack or analysing more packs per location. However, we need to remember that grey wolf is an endangered species, thus the number of probe is limited independently to PIs. Risk assessment of the study is not stated (eg. That not every wolf is certain to survive long lasting experiment and there is a risk they will die within the period of collecting data). I personally do not like the idea of analysing data from every continent in 3 different labs as it gives a study human bias – I would suggest to mix the data, blindfold them and send randomly to different labs, not depending on the region.

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

List of equipment to be bought is relevant, however, serious calculation mistakes appear within the budget. Eg. Daily allowance is no taken into account and only costs of transportation are stated in this line. The usage of 5 extra collars is not properly explain in the project proposal and gives additional cost of over 70 thousand PLN. There is no cost of computers / servers able to analyse such a big data set neither any hard discs / clouds available to store the data and it is not stated this type of equipment is already present in laboratories.

5. Strengths of the proposal

It is a well-designed, multidisciplinary study with the novel approach (measuring physiological factors in the hair). Moreover, it takes into account green brigdes and their potential role in maintaining animal populations and lowering their around the world as with today's technology we won't be able to stop building roads fragmentating habitats of the endangered animals, threatening them. Thus, if the study showed significant different in lowered stress level or higher reproductive success of the animal depending on the presence of the green bridge, we could possibly have solved a major problem and take further conservation effords. Various novel techniques are planned to be used to measure chronic stress levels, heart rate of the animal and its position and movements. Data will be collected for a long period of time continuously but for biological measurements which will take place every 3 months what gives us 6 timepoints through entire experiment.

6. Weaknesses of the proposal

While having animals anesthetized it would be better to collect also saliva and blood samples for further examination of stress indications and health status. It was proven that a well administered anesthesia does not have impact on animal stress level. I am afraid the group of animals taken into consideration is to small or to various. I can see two solutions to these problems which are already mentioned above. I couldn't find a proper analysis measuring the reproductive success of the gray wolf depending on the presence or absence of roads with or without green bridges. Moreover, a canonic approach to measure reproduction success is counting the number of cubs of offsprings of the animals of interest, thus I reckon 18 months of measurements will not be sufficient to verify this hypothesis stated in the project. I am also wondering about the sense of using HR as an indicator of animal stress while crossing the road. HR is a variable prone to activity patterns chances – that means if the animal runs crossing the road its HR will indicate higher values than when of the one walking.

Final project proposal

Title: Behavioural and physiological changes induced by the road proximity in grey wolf

Authors: Zuzanna Rauk, Pritam Kumar Dey, Monika Hoffmann

Summary:

The negative impact of roads on different species is well documented. Although the behavioural changes are known, they were not tested with physiological measures. In this project, we aim to investigate whether wolf packs exhibit different chronic stress levels depending on whether they live near or far from roads and whether they use green bridges. Moreover, we will analyse if the location affects their behaviour and reproductive activity. We assume that wolf packs living in remote areas have larger packs and territories, higher reproductive activity, and an overall lower stress level. We also anticipate that using green bridges reduces both acute and chronic stress levels in wolves.

In total, we will collar 180 individuals from two geographic regions (Europe, and North America), evaluate their movement patterns, heart rate while crossing the road or using green bridges and assess the accumulation of cortisol and steroids in hair samples. Spatial analysis will be conducted with ArcGIS software and the concentration of the hormones will be analysed with ELISA.

This is an innovative and interdisciplinary study that may provide us with an insight into the relationship between human interference with the environment and wolves' behaviour and physiology. Our project will contribute to the development of conservation biology, as it may help to verify if green bridges are an effective tool to protect grey wolves.

1) Scientific goal of the project

Approximately one million species are at risk of extinction given the accelerated rate of environmental change (IPBES 2019). Across the globe, habitat fragmentation is one of the greatest threats to biodiversity. Fragmentation has altered more than 50% of the Earth's landscapes (Keeley 2019) and the road network is shaping the environment on a global scale (Ibisch et al 2016). Ecosystems are influenced by road infrastructure in a diverse, very complex, and time delayed way. The effects extend far beyond the road itself (Selva et al. 2011). The ecological impact of roads on ecosystems, species, and populations is well documented (Laurance & Balmford 2013, Seiler 2001). Only in the last 20 years have the effects of roads on wildlife gained significant attention and are referred to as "road ecology" (Forman 2003). Direct impacts of road construction include the fragmentation and isolation of landscapes, the physical alteration of sites, dust and salt, heavy metal pollution, changes to the microclimate, noise- and light pollution, behavioural changes, and animal mortality caused by vehicle collision (Kaphegyi et al. 2012). The impact of roads on single species can result in local extinction (Riley et al. 2006). Roadless areas play a significant role in maintaining ecosystem functioning and supporting biodiversity and ecological processes. They may also support species movements, like long-distance dispersal.

In this project, we will try to understand the correlation between the animal stress level due to proximity to roads and their behavioural changes. One of our objectives is to evaluate levels of stress in animals inhabiting the areas with high road density, areas with the green bridges, and roadless areas (here areas at least 50 km away for a road). We will analyse the accumulation of cortisol and steroid hormones in hair samples, body mass and the migration pattern of grey wolf (*Canis lupus*). Due to their widespread distribution and the fact that they usually live in packs, they are suitable to address our objectives efficiently. The broad range of analyses will let us answer 3 research questions and verify following hypotheses:

1. ***Q1. Do wolf packs living in areas affected by roads have a high chronic level of stress in comparison to wolves living in roadless areas? H1.*** *Road proximity increases the chronic stress level in grey wolves.*
2. ***Q2. Does chronic stress related to road proximity affect the behaviour (temporal and spatial movement patterns) and reproductive activity in grey wolves? H2.*** *Wolves living close to the road have smaller movement radius or unusual temporal activity and lesser reproductive activity in comparison to the wolves living in roadless areas.*
3. ***Q3. Do green bridges reduce acute as well as chronic stress levels in grey wolves? H3a.*** *Wolves using green bridges have lower chronic stress level in comparison to wolves living in roady areas but similar or higher chronic stress level in comparison to wolves living in roadless areas* ***H3b****. Heart rate of the wolf is lower while using the bridge in comparison to crossing the road.*

2) Significance of the project

Many studies have shown that road proximity affects wild animals' physiology. Experimentally arranged exposure to human-generated noise induces a physiological stress response in female wood frogs and impaired females travel towards a male breeding chorus in the field, which may negatively affect the population persistence (Tenessen et al. 2014). Noise was also shown to affect small mammals and large carnivores (Iglesias et al. 2012). Grey wolves are known to avoid roads and settlements and breed less successfully in the areas of high road density (Merill 2000). It is however unknown if every day crossing the roads puts the animals under chronic stress, which lowers animals' reproductive activity and increases the risk of population decline. Our study will let us identify a potential relationship between living in roady areas and chronic stress in wolves and make conclusions about the effect of road proximity on wolves' reproductive activity. Green bridges (GB) are the communication facilities that can mitigate the impact of roads on wildlife. It was proved that construction of green bridges restored connectivity in the landscape and helped to recolonize eastern Germany with grey wolf, roe deer, red deer, and wild boar (Plaschke et al., 2020). There is however no data about the

effect of green bridges on wolves' physiological parameters. In the present study we will compare the immediate stress response in wolves crossing the roads and using green bridges, as we assume that using such facilities is less stressful for animals. Moreover, chronic stress indicators will also be analysed in wolves in areas with a high number of GBs, to verify the hypothesis that restored connectivity in the landscape reduces the stress level in wolves. Analysis of the relationship between road crossing and animals' physiology and behaviour is a completely novel approach in wolf biology. This experiment will contribute to the development of conservation biology, as it may potentially verify that green bridges help to protect grey wolves and re-introduce them into traffic-affected areas.

3) Concept and work plan

In this project we will focus on the *Canis lupus* species. We selected two broader geographic locations - North America and Europe for measurements of wolves' behaviour and physiology. Within these locations we will analyse wolves in three regions: roadless areas (at least 50 km away from any roads), roady areas, with highways as part of the wolf's home range and areas with green bridges (at least one bridge is available and used by the wolf pack) in the home range of the wolf population. For each region we will select 10 wolf packs. Per pack we will collar 3 individuals, among them 1 male and 1 female (the breeders of the pack), and 1 juvenile or subadult (in half of the packs we will select male in the other female wolves to have representative data). In total 180 wolves will be examined (see Figure 1). The whole study will be divided into three distinct tasks.

Task 1 - Fieldwork: wolves collaring, cardiac biologgers implantation
In order to obtain data about physiology and behaviour of the wolves, selected individuals will be collared with a GPS tracking collar and implanted with a cardiac biologger. Such devices were successfully used in the research on American black bears' stress response to crossing the road (Ditmer et al. 2018).

Task 2 - Fieldwork: data collection
For the next 18 months collared wolves will be tracked with GPS devices and captured every 6 months for hair samples collection. Additionally, we will collect information about the body mass and length of the animals as indicators of their general health. A constant data collection from GPS devices will provide information about wolves' movement patterns and activity. The combination of data from GPS devices and cardiac biologgers will allow us to obtain information about heart rate (HR) at the moment of approaching and crossing the road and using green bridges, which is a measure of acute stress response (Ditmer et al. 2018). We will collect hair samples to measure cortisol accumulation in hair as an indicator of chronic stress. This approach was proved to reliably reflect chronic stress both in moose and in wolves, in contrast to blood cortisol level which fluctuations depend on multiple factors (Spond et al. 2020, Ditmer et al. 2018). Moreover, we will measure the accumulation of steroid hormones in hair as indicators of population-level reproductive activity (Bryan et al. 2015).

Task 3 - Laboratory work: ELISA experiments, data analysis
The cortisol and steroids accumulation in hair will be measured with ELISA, one of the most popular methods of protein concentration assessment in biological samples. The data about hormones concentration, as well as animals' HR, body mass and length, and movement patterns will subsequently undergo statistical analysis, described in detail in the Methodology part.
The timeline of the whole project is presented in Figure 2.

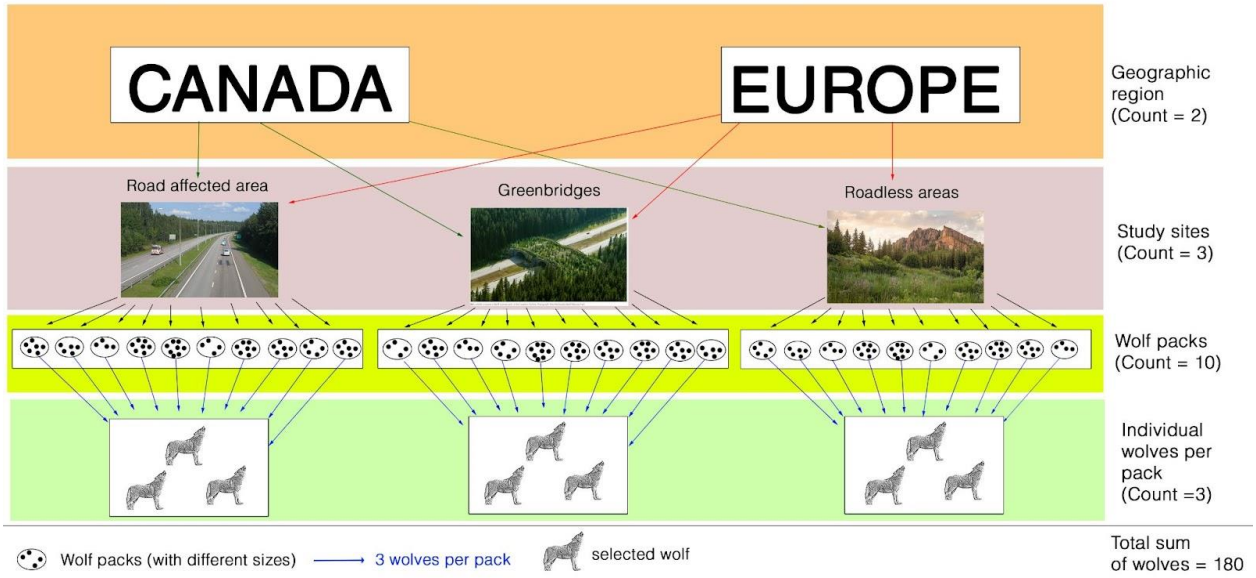

Figure 1. Study Site, Pack number, and wolf selection (Photograph: from left to right, By Migro - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=2598263, © 2022 Guardian News & Media Limited - Ross MacDonald/Banff National Park, Credit: Mason Cummings/The Wilderness Society).

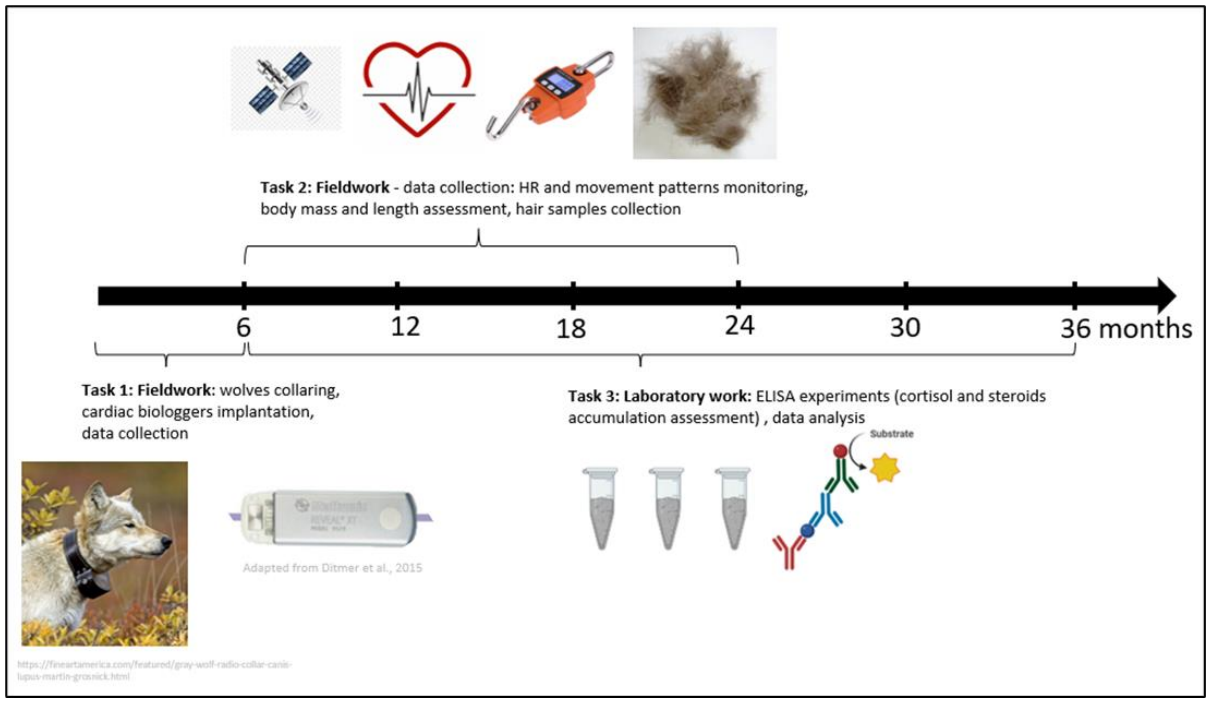

Figure 2. Project timeline with data types that will be collected and analysed.

Risk assessment

One of the major parts of the project is collaring, and implantation of cardiac biologger in wolves. There will always be a chance that collared animals could get shot or die. In that case we could be at risk of not obtaining enough data samples. To address this problem, we will analyse ten wolf packs from each area; with green bridges, with roads, and in roadless areas, and select three wolves from each pack, which will help us obtain sufficient amounts of data. A malfunction of the GPS tracker or cardiac biologger could pose another risk that is secured by a large sample size. In addition, we will collar more than one individual per pack, giving us the ability to easily track the pack and replace the malfunctioning GPS collar or cardiac biologger. Another risk

could be that we are not able to capture the same individuals next time, but we will try to mitigate the problem by coordinating the team together in the air and on the ground and by increasing attempts to capture the same individual per pack for seven days.

4) Research methodology

Field work

The animals will be captured, anaesthetised (6.6 mg/kg ketamine hydrochloride and 2.2 mg/kg xylazine hydrochloride (XYL) administered intramuscularly, Kregger et al. 1987) and outfitted with radio collars as well as cardiac biologgers implemented subcutaneously in a peristernal location. We will use the model that was successfully used in HR measurements in American black bears and contain software that allows linking the discrete events captured by the GPS collar to changes in HR (Medtronic Inc.,Reveal® XT Model 9529, Minneapolis, MN; specifications: 9 cc; 8 × 19 × 62 mm; 15 g, Ditmer et al., 2018). For the next 18 months the measurements of heart rate will be conducted. Every 6 months animals will be captured and anaesthetised for body mass and length measurements (Hook balance (WAGMA Poland)) and hair samples collection. Cortisol accumulation will be measured as an indicator of chronic stress. Steroid hormones accumulation will be analysed as a measure of reproductive activity. Every 6 months 3 hair samples will be collected, one for cortisol analysis and two for testosterone and progesterone. Tufts of hair (20–200 mg) will be cut with scissors from the neck as closely as possible to the root. It was proven that animals chemically sedated in the field typically did not exhibit a cortisol stress response (Champagne et al., 2012), so the result of chronic stress measurements will not be affected by anaesthetic injection.

Laboratory work

A standardised ELISA protocol will be used for hormones isolation and measurement in hair samples, as described in the article of Bryan et al. 2015. Hair samples will be washed with methanol (Omnisolv; VWR, Mississauga, ON, Canada), then dried and milled into the powder. 30 mg of the powder will be transferred into the Eppendorf with 50 µL of methanol per mg of hair powder. In order to extract steroids and cortisol, samples will be sonicated for 30 min and rotated at 160 rpm in an incubator for 18 h at 50 °C. After centrifuging, supernatant will be aliquoted into separate tubes for progesterone (100 µL), testosterone (50 µL) and cortisol (1000 µL) assays. According to the protocol, commercial kits designed for saliva will be used to measure cortisol, progesterone, and testosterone in hair extracts (Salimetrics, Philadelphia, PA, USA).

Statistical analysis

A comparison of physiological and behavioural parameters will be conducted between wolves from regions with different road impact (roadless area, roady area, roady area with green bridges). Moreover, we will compare the results between male and female wolves, within the region of particular road impact and between the regions. Finally, we will compare the data from Europe and North America.

Heart rate during crossing the roads or using green bridges will be compared between different animal cohorts. The populations from roadless areas will constitute as control groups. We will also evaluate the heart rate during approaching the road with or without green bridges to check if their presence changes wolves' anticipation of risk related to crossing the road.

Movement pattern will be analysed with the GPS data transmitted directly to the labs in each geographical region and cleaned for further processing the main GIS lab. The data will be analysed according to travel distances of the pack, day and night movements and motion density. The GPS data gives us information about

the state the wolf is in (e.g., resting mode, fast movement, homing, moderate activity). These data can be correlated with road density.

5) Project literature

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6. Table with budget of the project

	Amount in PLN
Direct costs, including	**8 118 892**
- personnel costs and scholarships	1 548 000
- research equipment/device/software cost	3 435 052
- other direct costs	3 135 840
Indirect costs, including:	**1 786 156**
- indirect costs of OA	162 378
- other indirect costs	1 623 778
Total costs	**9 905 048**

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

Direct Costs include:

A. Salaries and Benefits:

Salaries for main project performers

(Tasks: Planning, preparing, conducting field trips, management of field data collections, taking part in field data collections, handling of field data, analysis of field data (GIS, GPS, Cardiac loggers, wet labs, statistical analysis, participation in conferences, workshops, writing articles))

4500 PLN x 36 months x 5 persons = 810 000 PLN (3 PI & 2 Collaborators)

4500 PLN x 30 months x 2 persons = 270 000 PLN (1 GIS specialist & 1 Statistical Analyst)

Salaries for Lab managers

(Lab management, administration work, dealing legal issues (i.e., permissions for data collections inside protected areas, transportation of biological samples across the international borders))

4500 PLN x 12 months x 1 persons = 54 000 PLN

4500 PLN x 36 months x 1 persons = 162 000 PLN

Salaries for Team

Salaries for all project performers fee for task agreement (Each team will be comprised of one veterinarian, one tranquilizer gun handler, and one field assistant; two teams will be created for two geographical regions; our capture and GPS collaring rate 3 wolves/pack/day; all the calculations have been done accordingly)

500 PLN x 30 days x 2 Veterinarians x 4 times = 120 000 PLN

350 PLN x 30 days x 2 Tranquillizer gun handlers x 4 times = 84 000 PLN

200 PLN x 30 days x 2 Field Assistants x 4 times = 48 000 PLN

B. Equipment:

Computers and Software:

556 912 PLN (workstations including cloud storage and GIS software with cloud storage)

8 000 PLN x 2 (1 Laptop per Team) = 16 000 PLN

1 000 PLN x 4 (2 (10 TB Hard drive) for office & 1 (10 TB Hard drive)/teams = 4000 PLN

GPS collars:

14 013 PLN x 180 wolves = 2 522 340 PLN

Cardiac Bio loggers:

1000 PLN x 180 wolves = 180 000 PLN

GPS: GERMIN ETRIX 65s:

2000 PLN x 4 (2/Team; 2 Teams) = 8 000 PLN

Flashlights:

200 PLN x 4 (2/Team; 2 Teams) = 800 PLN

Infrared Binoculars (YUKON Night versions):

1500 PLN x 4 (2/Team; 2 Teams) = 6 000 PLN

Batteries:

500 PLN x 2 Teams (GPS, flashlights & infrared binoculars for 3 years) = 1 000 PLN

Lab Chemicals:

120 000 PLN

Anaesthetics:

20 000 PLN

C. Other direct costs:

Daily allowances:

(1 Team = Veterinarian + Tranquillizer gun handler + Field Assistant + Project investigator + Collaborator = 5 persons)

Food:

In Canada

130 PLN/day/person (3 meals) x 30days x 5 (Members/Team; 1 Team) x 4 times = 78 000 PLN

In Europe

60 PLN/day/person (3 meals) x 30days x 5 (Members/Team; 1 Team) x 4 times = 36 000 PLN

Accommodation:

In Europe

60 PLN/person x 30 days x 5 (Members/Team; 1 Team) x 4 times = 36 000 PLN

In Canada

200 PLN/Person x 30 days x 5 (Members/Team; 1 Team) x 4 times = 120 000 PLN

Travel cost

Flight for Canada

7000 PLN/person/round trips x 2 Persons (1 PI & 1 Collaborator) x 4 times = 56 000 PLN

Flight for Europe

1500 PLN/person/round trips x 2 Persons (1 PI & 1 Collaborator) x 4 times = 12 000 PLN

Transportation:

Vehicle on rent (4X4, 6 seat) for Field Team 24x7:

In Europe

4500 PLN/month (4x4 SUV, Without Fuel) x 1 team x 4 times = 18 000 PLN

Fuel: 7 PLN/Liter x 8L/Day x 30 days x 4 times = 6 720 PLN

In Canada

8500 PLN/Month (4x4 SUV, Without Fuel) x 1 team x 4 times = 34 000 PLN

Fuel: 7 PLN/Liter x 8L/Day x 900 days x 3 (3 study sites) = 6 720 PLN

Helicopter on rent including fuel and pilot

1860 PLN/hr. x 6hr/day x 60 days (30 days/team) x 4 times (Each sampling time) = 2 678 400 PLN

Conferences:

International Congress for Conservation Biology (ICCB) - ICCB 2025:

Oceania or Canada 10000 PLN (Conference fee/person + accommodation + travel) x 3 Persons = 30 000 PLN

Wolves Across Borders is now rescheduled for May 7-11, 2023:

Sweden 8 000 PLN (Conference fee/person + accommodation + travel) x 3 Persons = 24 000 PLN