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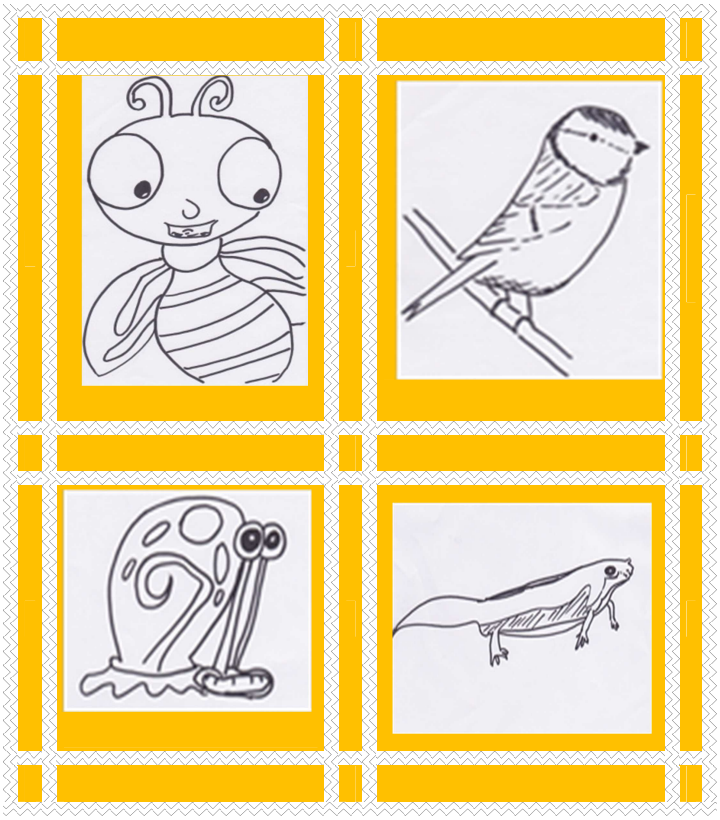

How does length of caudal filament in Lissotriton montandoni males correspond with their reproductive success and the condition of their offspring?

What are the effects caused by herbicides on multiflorous honey quality?

What is the impact of dust pollution on plumage coloration and male attractiveness in blue tits (Cyanistes caeruleus)

Are nanoparticles biomagnifed in terrestrial food chain in relation to particles size and soil concentration?

Course co-financed by the European Union under the European Social Fund

HUMAN CAPITAL
NATIONAL COHESION STRATEGY

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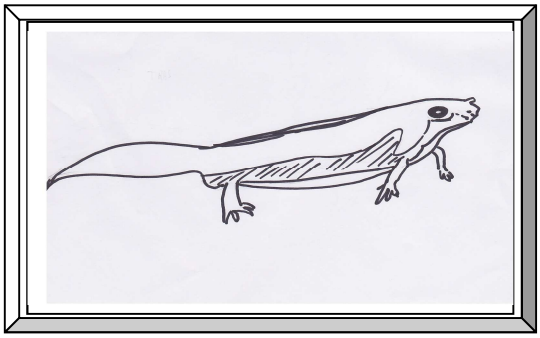
3 RESEARCH TOPICS SUGGESTED BY PARTICIPANTS

- Reasons for mismatched amplexus in the common toad (MB)
- Is tail thread important for the mate choice in newts? (MB)
- Do carnivorous plans eat their pollinators? (MB)
- Causes of the extinction of honey bee populations (MP)
- The impact of electromagnetic radiation on plant growth (MP)
- Interactions of intestinal and environmental microbial strains (MP)
- Crioconservation of avian embryos (AR)
- Communication system in the social parrot (AR)
- Effects of constant light on reproductive cycle in the mice (AR)
- The effects of pesticides on honey quality (AM)
- Comparing diurnal physical activity in different rodent species (AM)
- The study of roe dear foraging habits and environmental preferences (AM)
- The influence of animal road-crossing on amphibians (AP)
- The adjustment of governance system to ecological needs (AP)
- Assessing the threat of invasive species on local biodiversity (AP)
- Extra pair copulations in birds: who is a better liar? (IG)
- Are nanoparticles transferred in the food chain? (IG)
- Can we save crested lark from disappearing from Poland? (IG)
- Does urban pollution affect mate choice and reproduction in the blue tit? (GC)
- Reasons for divorce in monogamous bird species (GC)
- Why is population of bee eater declining in Italy? (GC)
- The influence of leave and root extract from Panax species on rodent behaviour (KW)
- The impact of housing temperature and Prototheca species on development of mastitis in cows (KW)
- The impact of height of grapevines on the fruit sugar content (KW)
- How to protect birds from wind turbines? (EP)
- Controlling soil pollution around landfills (EP)
- Reducing the population of invasive snails (EP)
- Are sand martin populations breeding in industrial heaps affected by pollution? (IG)

4 PROJECTS AND REVIWES

4.1 Does length of caudal filament in *Lissotriton montandoni* males correspond with their reproductive success and the condition of their offspring?



Authors: **Agata Miska & Maciej Bonk**

4.1.1 FIRST VERSION OF THE PROJECT

Project summary:

In many species sexual ornaments are important males' traits influencing both female choice and consequently reproductive success of males. I other word, sexual ornaments are good gene predictor and honest signaling. One of such ornament is caudal filament occurring in two European newts males. Length of the filament may have a signal function to attract female and also can be related to reproductive success. It is already known that newts female prefer males' with longer caudal filament. However there is no information about reproductive success and condition of the offspring in relation to length of the filament in newts. We are going to assess total number of newts' (Lissotriton montandoni) offspring, number of offspring that achieve metamorphosis, and body condition index of metamorphs from males with long and short caudal filament. Additionally we are going to test whether time spend on female decision of mating is related to caudal filament length. We predict that there will be higher amount of offspring from longer caudal filament males', higher number of offspring that achieve metamorphosis, and they will have higher body condition index. We also predict that time spent on mate decision will be shorter when female is exposed to long filament male.

Project description, methodology and expected results

1. *What problem is being proposed and why?*

The aim of the project is to test whether males sexual traits is related with their reproductive success in various development stages of their offspring. The model species in this study will be the Carpathian Newt Lissotriton montandoni. Males of this species has characteristic sexual trait – caudal filament and it was shown that in closely related Palmate Newt (L. helveticus) there is a strong positive relationship between the length of the filament and attractiveness to females (Haerty et al. 2007). Moreover, our model species is suitable for captive breeding (Osikowski and Rafiński 2003).

The key point of the study is mating female with two males: one with shorter caudal filament and one with longer caudal filament. Reproductive success will be measured as (i) total number of offspring (ii) a number of offspring that achieve the metamorphosis. We predict that long filament males will have more offspring and that the offspring of longer filament males will have better survival (i.e. there will be higher ratio of metamorphed offspring to initial larvae number). We also predict that methamorphed individuals will have higher value of body condition index (BCI) when father has longer filament. In other word, we will test whether the length of caudal filament is the honest signaling.

Despite it is generally believed that caudal filament is a sexual trait in newts, it has not been tested yet whether the length of it is related to reproductive success.

Additionally we will test whether time spent on females decision of mating is related to caudal filament length. We predict that time spent on decision will be shorter when female is exposed to long filament male.

2. *What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?*

The role of sexual traits has been a major issue in ecology and evolution as it is concerned to be the honest signaling of males' good genes. As some traits as long tails in birds are energetically expensive, and what is more, making males more detectable to predators males with extraordinary traits should be the better ones as despite having such ornaments survives in nature. Classical experiments and observation has been carried out on birds, mammals and insects (Krebs and Davies 1982, and papers cited therein, Ezenwa and Jolles 2008, Malo et al. 2005). In amphibian several mails traits as concerned to be sexual ornaments (coloration, breeding callas, crests). One of them is caudal filament occurring in two European newts species. This ornament may reflect the condition as this very fragile structure is easily lost for example due to predation. Thus, males which retain long filament are the ones that successfully avoid predation. It has been investigated whether this ornament play any role in breeding behavior. Haerty and coworkers (2007) proved that female prefer males with longer caudal filament. These research investigated the time which female spent in the proximity of males differing in the length of caudal filament. Female spent more time in

the proximity of males with longer filament. However, the time spent with given male may not fully represent whether male is good gene provider.

Our study will continue the idea of the role of caudal filament as a good gene predictor. In comparison to former research on caudal filament in newts we go step further planning to assess reproductive success measured as a total number of offspring. Because in amphibians moment of metamorphosis is the limiting one (Loman 2002), we will also use number of successfully metamorphed individuals as well as their body condition index.

3. What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?

a) Experiment design and statistics

Experiment will be carried out according to following scheme: one female will be mate with two males differing in the length of caudal filament. Each male will be presented to female and after successful mating (spermatophore taking) will be removed and replaced by another male. We plan to arrange at least to mates with each male in reverse order of mating to minimalise fertilizing order effect which is suggested for newts. Then, we aim to collect genetic material from dying larvae and specimens that will metamorph successfully. Such experimental box will be replicated 10x. We will test a null hypothesis of lack of the differences between the number of offspring (dead embryos, dead larvae and mesomorphs together) from males differing in caudal filament length applying ANOVA where the dependent variable will be the total number of offspring (M), investigated fixed factor will be filament length (L; two levels: short (S) and long (L)). Similar model will be applied to investigate filament length relation to only dead embryos and larvae together as a dependent variable and separately for only metamorphs number as a dependent variable. Because the number of metamorphs may be related to initial number of offspring (dead embryos, dead larvae and mesomorphs together) we will apply ANCOVA with initial number off offspring as a covariate. As the caudal filament length may be related to age of males we will conduct additional analysis (ANOVA) applying the age of male as a fixed factor. Analogous ANOVA and ANCOVA model will be applied in case of BCI of metamorphs as a depended variable .

To test whether there is a difference in the time spent on females' decision on mate with two males we will apply ANOVA with filament length as a fixed factor and female as a random factor.

b) Experiment technical details

Newts will be capture during spring migration (March-April). We will collect only specimen couth on land. Such approach is necessary to avoid having already fertilized females in sample (females are fertilized only in water; Juszczyk 1987). Males will be also captured on land before growth of a caudal filament to avoid variability in filament length due to environmental factor (i.e. predation). We will also assess the age of individuals using scelotochronology. This method is commonly used in research on animals with circumannual life cycles (including amphibians) due to a very low margin of error and low financial costs

(Castanet et al. 1993, Sinsch et al. 2007). Males will be kept separately: one mail in one aquarium to avoid aggressive behavior (bites resulting in cutting the filament). Females will be kept in laboratory in two aquariums (60x30x35cm), five females in each of them. Aquaria will be filled with water and kept in standard temperature and photoperiod conditions (see Osikowski and Rafiński 2001). Because filament grows from zero when males get into water we will measure the length of the filament in five randomly chosen males every day until the filament length is fixed. We are going to arrange mating after filament length fixation (10 days without signs of increase in the length). After mating regular observation will be carried out to remove every dead larvae and their alcohol fixation. Obtained metamorphs will be measured and weighted to get the BCI value according to Harris (2008). After measurement every metamorphed specimen will be fixed in alcohol for further microsatellite analysis. We will collect all dead larvae and all metamorphs, then we will randomly choose sample of 30 individuals in each developmental stage per one experimental box. If the number of samples will be lower than 30 we will consider all of the individuals.

To test the time needed for females' decision on mating we will measure the time from beginning of male exposition to female to first sings of breeding behavior (indirect contact). We will not consider the cases where mating is not successful.

Financial support will allow us to organize laboratory condition for the experiment. We have necessary knowledge to captive breeding of model species.

c) Microsatelite analysis

To identify fathers of obtain offspring we will apply three of nine known very polymorphic microsatelite loci (GU574499, GU574498, GU574497; Nadachowska et al. 2010). We are going to have done all molecular analysis in Population Ecology Group in Institute of Environmental Science of Jagiellonian University.

4. What are the expected results of this project?

The results will contribute to our knowledge of role of males sexual traits. In detail, it will brings knew idea of behavioral ecology of Carpathian Newt. We plan to publish at least three articles in refereed scientific journals and attend at least two international conferences.

Literature:

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Loman J. 2002. Rana temporaria metamorph production and population dynamics in the field. Effects of tadpole density, predation and pond drying. Journal for Nature Conservation 10: 95-107.
Malo A.F., Roldan E.R.S., Garde J., Soler A.J., Gomendio M. 2005. Antlers honestly advertise sperm production and quality. Proceedings of the Royal Society B: Biological Sciences 272: 149-157.
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Osikowski A., Rafiński J. 2001. Multiple insemination increases reproductive success of female Montandon's newt (Triturus montandoni, Caudata, Salamandridae). Behavioral Ecology and Sociobiology 49: 145-149.
Sinsch U., Leskovar C., Drobig A., Konig A., Grosse W.R. 2007. Life-history traits in green toad (Bufo viridis) populations: indicators of habitat quality. Canadian Journal of Zoology 85: 665-673.

Project schedule – anticipates tasks

No.	Name and description of task	Expected completion date (mm/yy)	Expected cost (zł)
1	The purchase of necessary equipment and materials	02.2011	20 496
2	Fieldwork in mountains	03.2011	10 400
3	Mating/breeding/transformation	03-09.2011	75 010
4	Molecular analyses	10-12.2011	39 260
5	Assessment of the survival success in methamorphs	01-02.2012	13 910
6	Preparation of manuscripts and conferences presentation, participation in conferences	02-05.2012	45 970
		Total	194 646

Proposed budget

No.	Item	Funds for each budget year (zł)		
		2011	2012	Total
1	Direct costs, including:	105 570	46 200	151 770
	1/ Salaries and benefits	68 500	25 000	93500
	2/ Equipment	8 250	-	8 250
	3/ Other direct costs	28 820	21 200	50020
2	Indirect costs	29 196	13 860	43056
3	Total costs (1+2)	134 766	59 880	194 646

Details of direct cost items:
1/ Solaris and benefits;
Principal investigator person-months; 16 months, 40 000zł
Principal investigator employee; 16 month, 40 000zł
Technical assistant; 5 months, 13 500zł
2/equipment (type, estimated cost, planned month of purchase, justification)
• 42 aquariums 60x30x35 , capacity - 63l; 2 900zł; February 2011; necessary for keeping animals.
• 45 filters HBL-301, 300l/h; 600zł; February 2011; Necessary for keeping the same conditions in each aquarium.
• 45 thermometers ROBIZO; 800zł; February 2011; Necessary for keeping the same conditions in each aquarium.
• 43 aerators HP-100; 800zł; February 2011; Necessary for keeping the same conditions in each aquarium.
• 14 metal shelves 180x80x30; 1050zł; February 2011. Place for aquariums.
• 42 lamps; 800zł; February 2011; Necessary for keeping the same conditions in each aquarium.
• 43 water heaters ATMAN 300W; 1300zł; February 2011; Necessary for keeping the same conditions in each aquarium.
3/Other direct costs (type of expenditure, amount, and relation to project plan)
• Chemical analysis for molecular parental tests (2020 samples); 20 200zł.
• Expenses related to fieldwork in mountains; 3000zł. Collecting wild living newst.
• Office materials; 1 000zł.
• Other services; 500zł.
• Fees and travel costs for two people related to participation in international conferences; 20 000zł.
• Ethanol 96%; 100zł; Necessary to collect samples.
• 5000 eppendofs BIORON; 600zł; February 2011; Necessary to collect and store samples.
• Food for animals; 4200zł.
• Laboratory supply; 420zł; to conduct scelotochronological tests.

4.1.2 REVEWS

- Marta Szulkin

  Review of project: *"Does length of caudal filament in Lissotriton montandoni males correspond with their reproductive success and the condition of their offspring?"*

This is a very good grant application that reads well from the start up till the end. Most importantly, it asks scientifically valid research hypotheses from the field of sexual selection (evolutionary biology), and presents sound ways of addressing the research problems. The application provides an excellent summary, highlighting current knowledge and missing gaps the authors want to provide answers for. I only have minor comments related to this application.

Project summary – very good structure, outlining (a) what is already known, (b) gaps in knowledge, (c) project outline and (d) predictions. Such structure allows the referee to have a good grasp of the project's aims in no time – well done.

Project description

Part 1

Good section.

Line 31: "various" is a bit unclear – be more specific – either name the stages you are interested in, or provide a count estimate (e.g. at 2, 3 developmental stages of the offspring).

Line 35-36 – while this is indeed important information, do mention why it's important – captive breeding allows you to carry out the breeding experiment. It allows you to breed your subject species in captivity, thus fulfilling the criteria for mate breeding experiment that you want to set up.

Line 45-46 – excellent sentence pinpointing the need to link a morphological trait to fitness (i.e. reproductive success) in order to demonstrate its role as a sexual trait, although at the moment it is not tied in with the rest of the text – perhaps re-shifting it to line 36 may be a way round it.

Part 2

Excellent section building up on previous findings and outlining research aims complementing and deepening existing knowledge.

Part 3

A well structured outline of the proposed methodology.

Part A.

No major comments, although I would be inclined to increase the sample size, as testing the reproductive success of 20 males (tied into 10 trials) seems borderline in terms of sample size. It may be worth exploring this option if the budget allows for it.

Lines 81-82 – it is important to double check that caudal filaments grown in captivity are representative of filament length found in the wild. Additionally, what would be the threshold of long / short filaments?

Lines 83 – meaning two males?

Line 95-96 – but what would happen if age does indeed influence caudal filament length? A separate analysis may allow you to pinpoint this problem, but the question still remains as to how to control for such possibility. How about using GLMs (General linear models) which allow you to use more than one explanatory variables instead of Anovas? You could also use GLMMs if you want to include any random effects.

Part B

No comments, very good section.

Part C
Are three markers really enough to determine paternity? It seems like a very low number to me (although such number is indeed used sometimes). You may need to consider (in your budget) a larger number of markers in case your paternity exclusion scores with three markers are not satisfactory.
Proposed Budget
A few comments to be made here.
Project schedule
I cannot find in the equipment outline what can sum up to the very high figure of 75010zl in terms of mating/breeding/transformation, unless it includes salaries? Please clarify. Similarly, figure no 6 appears on the high side, unless (again) it includes salaries.
Salaries
I would not expect the PI to have the same salary as the PI's employee. Moreover, the PI's salary (2500zł/month) should most certainly have a higher salary than the technical assistant (2700zł/month)!
Other
Conference and travel costs: on the high side
Molecular parental testing: you know the fees better than I do, but the costs presented here may be undervalued, particularly if one adds more molecular markers or consumables associated with the lab work.
Overall – a very interesting project with great potential for promising outcomes.

- Agata Pietrzyk
  Review of project: "*Does length of caudal filament in Lissotriton montandoni males correspond with their reproductive success and the condition of their offspring?"*

The proposal is written in a clear, well-organized way and have all the parts needed. Description of the planned project is argumentative and convincing. Scientific background is presented in a persuasive way and it justifies well enough the validity of the project. There are lesser inaccuracies, as in lines 63-64: "Thus, males which retain long filament are the ones that successfully avoid predation" – if it is known, it should be an appropriate citation here, but in the previous sentence there was a presumption ('This ornament may reflect the condition', l.62) so that should be specified and unified. There are some minor spelling and language mistakes (e.g. lines: 12, 30, 58, 60, 65).

Methodology is planned correctly, but there is no information about how many repetition will be taken in the test of differences in the time spent on females' decision on mate with two males.

The budged is a weak point of the project. The costs of salaries comprise almost 50% of the total costs. That would be acceptable if the work of highly qualified specialists was needed. Here, the work is planned to be done by PhD students (sufficiently well-trained and experienced) with a help of technical assistant, so there is no point in reserving such a big part of budget for the salaries.

- Edyta Podmokła

  Review of project: *"Does length of caudal filament in Lissotriton montandoni males correspond with their reproductive success and the condition of their offspring?"*

This project is very good planned. You present everything clearly and logically. Methodology is correct and appropriate for every your question. Statistical analysis are suitable for proposed work. The hole project is correct and I couldn't find any seriously technical mistake. I can see that applicants have a wide knowledge about the amphibians and their biology. Also, you have some experience in work with them and you know very well herpetological literature (144-171). In general, your project is well thought and well planned but I think that the problem is not so important. You want to investigate really narrow field. Although your experiment is planned in details, it is not scientifically significant. I predict that results of your work could be published only in very specialized herpetological journals. Summarizing, I can't give you the money for this investigation in the sum as you asked about.

Other comments:

- In lines 121-122 I don't understand what you want to do exactly. Why do you want to take samples from only 30 individuals and what kind of samples and how?
- Your budget is too high. You want to too much money for salaries and benefits. (185-187)

- Agata Rudolf

  Review of project: *"Does length of caudal filament in Lissotriton montandoni males correspond with their reproductive success and the condition of their offspring?"*

The project is ambitious, but not very innovative. The challenges of the proposed research dosn't seem important to me.

However, the project could be scientifically significant, becouse the authors are applying clever method to investigate the time needed for females decision on mating, after exposition on male with a different filament length. They eliminate the influence of the other factors, which could falsify the results. It may have potential for significant outcomes.

Methodology is not completely clear to me just by reading, but it seems that the shedule is reasoning and logical. Statistical methodology for expected data structure is also correct.

The project seem to be realistic and the costs are not too high for the three year project. Objectives and hypotheses are clearly presented and the cause-and-effects relationship is logical. I also think that proposal research methods would enable to achieve the goals of the project, becouse they seem to be weel thought.

I couldn't find potential scientific or methodological problems that may arise during conducting the planned research, what proves to me once again, that methodology has been estabilished correctly.

The presentation of the project is actually well arranged and concise. However the part of methodology about the experiment shedule is not totally clear.

The title is adequate to the contents of the project, and the language is correct and neat. Even though, the topic and the proposal problems of the project dosn't fell me on the floor, the project arrangement seems to be proper and concise, so I will decide to concede the bailout.

- Iwona Giska
  Review of project: *"Does length of caudal filament in Lissotriton montandoni males correspond with their reproductive success and the condition of their offspring?"*

The aim of the project is to test if length of the caudal filament in Carpathian Newt has any effect on reproductive success of males. The research question has a strong base for the prediction of positive relation between reproductive success and length of the filament and it is supported by literature where information about bigger sexual attractiveness of males with longer filament can be found. Study results would just add Lissotriton montandoni to the list of species that have a sort of sexual ornament indicating high quality genes or will exclude the length of a filament as a morphological feature related to genetical condition. The value of the answer of research question will be just cognitive. If the research would be about breeding, farming species, it would have applied findings, useful for breeders.

The hypothesis and prediction of the study are clear and experiments proposed to test them seem to be suitable as number of offspring and their survival is a good indicator of reproductive success. Authors could add some information on knowledge about relation of sexual ornament and reproductive success of other species that have been well studied regarding sexual ornaments function importance.

Methodology is clearly described and data obtained from the experiment should answer all research questions. I would add the information about the number of females and males that are going to be tested to know the sample size. Authors should add the information if animals will survive the experiment and will set free as it is protected species in Poland. Statistical analysis is well explained. I would just think more about male age as a factor for the analysis. Maybe it would be better to test separately if there is relation between filament length and males age and for proposed project, males at the same age should be used.

Methodological value of the project is high as well planned experiments will clearly answer questions. But nowadays when amphibians populations have serious problems and need protection, often active protection, I am wondering if money should not be spent for more applied studies that in this field would be more usefull.

4.1.3 FINAL VERSION OF THE PROJECT

Project title:

Does length of caudal filament in *Lissotriton montandoni* males correspond with their reproductive success and the condition of their offspring?

Applicants:

Agata Miska, Maciej Bonk

Project summary

In many species sexual ornaments are important males' traits influencing both female choice and consequently reproductive success of males. In other word, sexual ornaments are good gene predictor and honest signaling. One of such ornament is caudal filament occurring

in two European newts males. Length of the filament may have a signal function to attract female and also can be related to reproductive success. It is already known that newts female prefer males with longer caudal filament. However there is no information about reproductive success and condition of the offspring in relation to length of the filament in newts. We are going to assess total number of newts' (*Lissotriton montandoni*) offspring, number of offspring that achieve metamorphosis, and body condition index of metamorphs from males with long and short caudal filament. Additionally, we are going to test whether time spend on female decision of mating is related to caudal filament length. We predict that there will be higher amount of offspring from longer caudal filament males, higher number of offspring that achieve metamorphosis, and they will have higher body condition index. We also predict that time spent on mate decision will be shorter when female is exposed to long filament male.

Project description, methodology and expected results

1. *What problem is being proposed and why?*

The aim of the project is to test whether males' sexual trait is related with their reproductive success in different development stages (i.e. eggs/embryos with larvae and metamorphs) of their offspring. In other word, we will test whether investigated trait is the honest signaling. The model species in this study will be the Carpathian Newt *Lissotriton montandoni*. Males of this species has characteristic sexual trait – caudal filament and it was shown that in closely related Palmate Newt (*L. helveticus*) there is a strong positive relationship between the length of the filament and attractiveness to females (Haerty et al. 2007). Moreover, our model species is suitable for captive breeding (Osikowski and Rafiński 2003), thus allows us to observe breeding behavior and to bring up metamorphs.

The key point of the study is mating female with two males: one with shorter caudal filament and one with longer caudal filament. Reproductive success will be measured as (i) total number of offspring (ii) a number of offspring that achieve the metamorphosis. We predict that long filament males will have more offspring and that the offspring of longer filament males will have better survival (i.e. there will be higher ratio of metamorphed offspring to initial larvae number). We also predict that metamorphed individuals will have higher value of body condition index (BCI) when father has longer filament.

Despite it is generally believed that caudal filament is a sexual trait in newts, it has not been tested yet whether the length of it is related to reproductive success.

Additionally we will test whether time spent on females decision of mating is related to caudal filament length. We predict that time spent on decision will be shorter when female is exposed to longer filament male.

2. *What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?*

The role of sexual traits has been a major issue in ecology and evolution as it is concerned to be the honest signaling of males' good genes. As some traits as long tails in birds are energetically expensive, and what is more, making males more detectable to predators the ones with extraordinary traits should be better as despite having such ornaments survives in nature. Classical experiments and observation has been carried out on birds, mammals and insects (Krebs and Davies 1982, and papers cited therein, Ezenwa and Jolles 2008, Malo et al. 2005). In amphibian several mails traits as concerned to be sexual ornaments (coloration, breeding callas, crests). One of them is caudal filament occurring in two European newts species. This ornament reflects the condition as this very fragile structure is easily lost for example due to predation. Thus, males which retain long filament are the ones that successfully avoid predation. It has been investigated whether this ornament play any role in breeding behavior. Haerty and coworkers (2007) proved that females prefer males with longer caudal filament. These research investigated the time which female spent in the proximity of males differing in the length of caudal filament. Female spent more time in the proximity of males with longer filament. However, the time spent with given male may not fully represent whether male is good gene provider.

Our study will continue the idea of the role of caudal filament as a good gene predictor. In comparison to former research on this structure in newts we go step further planning to assess reproductive success measured as a total number of offspring. Because in amphibians moment of metamorphosis is the limiting one (Loman 2002), we will also use number of successfully metamorphed individuals as well as their body condition index, which should be better predictor of male quality..

3. *What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?*

a) Experiment design and statistics

Experiment will be carried out according to following scheme: one female will be mate with two males differing in the length of caudal filament. We will conceder males a a different when the difference in length of the filament will be equal or higher than 30% of

total filaments lenght. Each male will be presented to female and after successful mating (spermatophore taking) will be removed and replaced by another male. We plan to arrange at least to mates with each male in reverse order of mating to minimalise fertilizing order effect which is suggested for newts. Then, we aim to collect genetic material from dying eggs/embryos, larvae and specimens that will metamorph successfully. Such experimental box will be replicated 15x. We will test a null hypothesis of lack of the differences between the number of offspring (dead eggs/embryos, dead larvae and metamorphs together) from males differing in caudal filament length. We will apply ANOVA where the dependent variable will be the total number of offspring, investigated fixed factor will be filament length (two levels: short and long). Similar model will be applied to investigate filament length relation to only dead embryos and larvae together as a dependent variable and separately for only metamorphs number as a dependent variable. Because the number of metamorphs may be related to initial number of offspring (dead embryos, dead larvae and mesomorphs together) we will apply ANCOVA with initial number off offspring as a covariate. Analogous ANOVA and ANCOVA models will be applied in case of BCI of metamorphs as a depended variable .

To test whether there is a difference in the time spent on females' decision on mate with two males we will apply ANOVA with filament length as a fixed factor and female as a random factor.

b) Experiment technical details

Newts will be captured during spring migration (March-April). We will collect only specimen couth on land. Such approach is necessary to avoid having already fertilized females in sample (females are fertilized only in water; Juszczyk 1987). Males will be also captured on land before growth of a caudal filament to avoid variability in filament length due to environmental factor (i.e. predation). We aim to use only males in one age to avoid potential relation age – filament length. From oversized number of males (50) caught we will chose 30 individuals from the most frequent age. Age analysis will be conducted before placing newts in water. Such order is required as we may obtain to small sample of the same age and in result we will need to capture more males at terrestrial phase. Age will be assessed using skeletochronology. This method is commonly used in research on animals with circumannual life cycles (including temperate amphibians) due to a very low margin of error and low financial costs (Castanet et al. 1993, Sinsch et al. 2007). Males will be kept separately in water environment (one mail in one aquarium) to avoid aggressive behavior (bites resulting in cutting the filament). Females will be kept in laboratory in three aquariums

(60x30x35cm), five females in each of them. Aquaria will be filled with water and kept in standard temperature and photoperiod conditions (see Osikowski and Rafiński 2001). Because filament grows from zero when males get into water we will measure the length of the filament in five randomly chosen males every day until the filament length is fixed. We are going to arrange mating after filament length fixation (10 days without increase in the length). After mating regular observation will be carried out to remove every dead eggs/embryos and larvae which will be fixed in 100% alcohol. Obtained metamorphs will be measured and weighted to get the BCI value according to Harris (2008). After measurement every metamorphed specimen will be fixed in alcohol for further microsatellite analysis. We will collect all dead larvae and all metamorphs, then we will randomly choose sample of 30 individuals in each developmental stage per one experimental box. If the number of samples will be lower than 30 we will consider all of the individuals.

To test the time needed for females' decision on mating we will measure the time from beginning of male exposition to female to first sings of breeding behavior (indirect contact). We will not consider the cases where mating is not successful.

All adult newts will be released into locality of their origin after 10 days from last deposition of egg.

Financial support will allow us to organize laboratory condition for the experiment. We have necessary knowledge to captive breeding of model species.

c) Microsatelite analysis

To identify fathers of obtain offspring we will apply five of nine known very polymorphic microsatelite loci (GenBank: GU574499, GU574498, GU574497, GU574496, GU574495; Nadachowska et al. 2010). We are going to have done all molecular analysis in Population Ecology Group in Institute of Environmental Science of Jagiellonian University.

4. *What are the expected results of this project?*

The results will contribute to our knowledge of role of males sexual traits. In detail, it will brings knew idea of behavioral ecology of Carpathian Newt. We plan to publish at least three articles in refereed scientific journals and attend at least two international conferences.

Literature

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Project schedule – anticipated tasks

No.	Name and description of task	Expected completion date (mm/yy)	Expected cost (zł)
1	The purchase of necessary equipment and materials	02.2011	20 496
2	Fieldwork in mountains	03.2011	10 400
3	Mating/breeding/transformation	03-09.2011	75 010
4	Molecular analyses	10-12.2011	39 260
5	Assessment of the survival success in methamorphs	01-02.2012	13 910
6	Preparation of manuscripts and conferences presentation, participation in conferences	02-05.2012	45 970
		Total	194 646

Proposed budget

No.	Item	Funds for each budget year (zł)		
		2011	2012	Total
1	Direct costs, including:	105 570	46 200	151 770
	1/ Salaries and benefits	68 500	25 000	93500
	2/ Equipment	8 250	-	8 250
	3/ Other direct costs	28 820	21 200	50020
2	Indirect costs	29 196	13 860	43056
3	Total costs (1+2)	134 766	59 880	194 646

Details of direct cost items:
1/ Solaris and benefits;
Principal investigator person-months; 16 months, 40 000zł
Principal investigator employee; 16 month, 40 000zł
Technical assistant; 5 months, 13 500zł
2/equipment (type, estimated cost, planned month of purchase, justification)
55 aquariums 60x30x35 , capacity - 63l; 2 900zł; February 2011; necessary for keeping animals.
45 filters HBL-301, 300l/h; 600zł; February 2011; Necessary for keeping the same conditions in each aquarium.
45 thermometers ROBIZO; 800zł; February 2011; Necessary for keeping the same conditions in each aquarium.
43 aerators HP-100; 800zł; February 2011; Necessary for keeping the same conditions in each aquarium.
14 metal shelves 180x80x30; 1050zł; February 2011. Place for aquariums.
42 lamps; 800zł; February 2011; Necessary for keeping the same conditions in each aquarium.
43 water heaters ATMAN 300W; 1300zł; February 2011; Necessary for keeping the same conditions in each aquarium.
3/Other direct costs (type of expenditure, amount, and relation to project plan)
Chemical analysis for molecular parental tests (2020 samples); 20 200zł.
Expenses related to fieldwork in mountains; 3000zł. Collecting wild living newst.
Office materials; 1 000zł.
Other services; 500zł.
Fees and travel costs for two people related to participation in international conferences; 20 000zł.
2l of Ethanol 96%; 100zł; Necessary to collect samples.
5000 eppendofs BIORON; 600zł; February 2011; Necessary to collect and store samples.
Food for animals; 4200zł.
Laboratory supply; 420zł; to conduct scelotochronological tests.

4.2 The effect of herbicides on multiflorous honey quality.

Authors: **Agata Rudolf & Katarzyna Wężowicz**

4.2.1 FIRST VERSION OF THE PROJECT

Project summary:

The herbicides are wide spread in the agriculture and their influence of the live organisms and food chain has been proved. The herbicides residues can penetrate to the ground water or being spread by wind and transmit to the unpolluted area, and also accumulated in the live organisms. The herbicides were detected in various plant and animal food products such as honey. However, the influence of the herbicide content to honey quality is unknown. Here we show that the herbicide have decreasing influence for such honey quality features as sugar content, water content, diastase number, free acid content and pH, and HMF(hydroxymethylofurfural) content. We found that herbicides can settle on the surface of hives and even penetrate to the tissue of the honeybees. We also found that the herbicides can be storage in the soil for a long time before being completely metabolized by plants. Our results will demonstrate how the presence of herbicide in the honeybees environment will decrease the honey quality, and how fast the content of herbicide in honey will fall down. For example the sugar content is one of the parameter determined the type of honey and also its quality. If the herbicides presence in honey will cause the perturbation of this feature it may cause the difficulties of honey classification and decreased the honey quality. We hope that our results will shed some light on the need to more restrictive honeybees protect.

Project description, methodology and expected results

1. What problem is being proposed and why?

The aim of the project is to test whether using herbicides in agriculture influence the blossom honey quality. The honeybees (*Apis mellifera*) pollinate entomophilen plants by collecting nectar and pollen, which are their nourishment. The effect of honeybees activity is production of valuable for the human food industry products: honey, wax, pollen, royal jelly and propolise.

From ancient times, honey was considered as a food of the gods. Furthermore, it was useful in food conservation, as a component of meals and drinks, and as a legal tender. Honey has healing properties thanks to contents microelements (K, Cl, P, Mg, Ca, Fe, Mn, Co) and vitamins (B group vitamins, pantothenic acid, folic acid, biotin). Moreover, honey consists of carbohydrates (glucose 34 %, fructose 39%), organic acids, ethereal oils, carotenoids, enzymes and acetylcholine.

Nowadays, develop in agriculture causes increasing use of herbicides. The influence of herbicides residues in live organisms seem to be significant.

The key point of the study is to show the difference in quality of honey coming from area in which the different herbicides were used. The determination of organoleptic and physico-chemicals properties of the honey will allow the following hypothesis to be tested; the herbicides content decreased the honey quality. It could be predicted that the features which determinate the quality of honey will be changed and probably abnormalite. The premises for this prediction are based on the facts that harmful chemical substances could penetrate to the honeybees products (Kisała J. and Dżugan M., 2009). However, the negative effects of the herbicides on honey quality has not been shown in any of the previous works.

Moreover, it could be expected that herbicide content in honey will change in time, shows how quickly the herbicides are neutralised.

2. *What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?*

Pesticides become essential elements of chemical protection of plants. They are applied not only to increasing crops, but also to limit the occurrence of weeds in plant-growing. Apart of agriculture the plant protection products are also used in protection of forests, orchards, and lawns. The pesticide residues are found in water, soil, plants, animals and even in human organism. Special attention is dedicated to the problem of ground water protection. The presence of herbicides was found in all kinds of water circulated around the ecosystems- atmospheric precipitation, superficial water and underground water. Active substances being an ingredients of herbicide, could release from crops and penetrate the animals and humans trophic chains. The herbicides could be transfer by wind at neighbouring fields, where their presence is harmful and undesirable.Moreover, they can penetrate to vegetables and animal food products (Wrzosek et al. 2009).

Furthermore, it was corroborated that herbicides can settle in the surface of hives and accumulate in the honeybees tissues. What is more, the presence of herbicides was detected in the honeybees products, especially in honey (Albero et al., 2004).

Nowadays, the consumers pay special attention to the quality of the food products that they are buying. They require the products with the highest standards, often choose the special ecological food stuffs with guaranteed impurities free and healthy properties. The consumers want to be sure that they are buying a high quality products (Mateo et al.1998). That's why it is so important to prevent such animal food products like honey from chemical pollution like presence of herbicides, which may cause the decrease of the quality.

The herbicides could be also detectable in the honey even for a long time of plants exposure for the acts of herbicide. The herbicides content in honey was previous examined but the change of the herbicides level in time has not been shown. Also the problem how the content of the herbicides in honey will influenced the features of quality has not been examined.

3. What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?

The studies will be conducted in Słomniki near Cracow, where the 9 greenhouses (5m x 20 m) will be built. In each greenhouse the seeds of dicotyledones meadow plants will be sow at purchased garden mould in the April. Two herbicides will be used as a decreasing honey quality factor. The experiment will be conducted in three groups. In the first group it will be used herbicide Agil 100 EC (active substance: propachizafop) and in the second group it will be used herbicide Leopard 05 EC (active substance: chizalofop - P -ethyl). The third group will be treated as a control. It is planned to introduce herbicides in the beginning of the June. The next day the 18 hives with honeybees families bringing from unpolluted areas will be placed- 2 hives in each greenhouse. The honeybees are not sensible for the effects of the using herbicides.

The first samples of honey will be collected after 4 weeks from bringing honeybees – 1 sample from each of the hives in each of the 3 greenhouse in each of the 3 groups. Next colleting of sample will be 2 weeks later and the last one 8 weeks after the first samples collecting.

Samples will be examined in the Institute of Environmental Sciences, Jagiellonian University. The organoleptic properties of honey - color, taste, consistency and smell will be evaluated using sensoric methods. Whereas, the physico - chemicals properties of honey will be examined using laboratory methods.

The sugar content (fructose, glucose and saccharose) will be measured using liquid chromatography witch refractometric detector. The water content will be measured using refractometer, diastase number – using photometer, free acids content and pH of honey – using pH – meter and HMF (hydroxymethylofurfural) content – using liquid chromatography.

Herbicide content in honey will be measured using gas chromathography-tandem mass spectrometry (GC-MS/MS) and liquid chromatography-mass spectrometry (LC-MS). These samples of honey will be analysed at Warsaw University in the Department of Chemistry .

Samples gained from the first set of collecting - 4 weeks after herbicide introducing, will be analysed for the herbicides content and the physico- chemicals properties. And the results of the analysis for each physico-chemical features will be separately compared with herbicide content to show the correlation. The differences between two herbicides will be

comparing using the covariance method for each of the features separately, to show the relationship.

Samples of second and third set - after 6 and 8 weeks after herbicide introducing, will be analysed just for herbicide content, and the correlation of that feature in time period will be showed. The differences between two herbicides will be comparing using covariance to show the relationship in the time period.

Each of the obtain covariance will be useful to elucidate different aspect of the problem.

The principal investigators of the project do not have the practical experience with honeybees, that is why it will be necessary to employ a beekeeper. The rest stage of study will be carried out in the laboratory of the University.

4. What are the expected results of this project?

The results of the study will be presented on National Apiarian Conference in Puławy and on the Apiarian Conference in Częstochowa. We plan to publish our findings 2 publications in such journals as Apiary, Environmental Pollution and Journal of Apicultural Science. In the future it is considering to continue the experiment focus on the content of antioxidants and herbicide in honey.

Literature:

Albero B, Sanchez - Brunete C, Tadeo JL. 2004. Analysis of pesticides in honey by solid-phase extraction and gas-chromatography- mass spectrometry. J.Agri.FoodChem 52:5828 – 5835

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Project schedule – anticipated tasks

No.	Name and description of task	Expected completion date (mm/yy)	Expected cost (zł)
1	Setting a plant raising in greenhouses	03-05.2011	191 234
2	Introduction of hives with honey bees and herbicides into greenhouses	06.2011	16 970
3	Collecting honey samples and laboratory work	07-09.2011	30 940
4	Presentation, participation in conferences and report preparation	10-12.2011	2340
		Total	241 484

Proposed budget

Item	Funds for budget (zł)
	2011
1. Direct costs, including:	216 080
1/ Salaries and benefits	20 400
2/ Equipment	131 400
3/ Other direct costs	64 280
2. Indirect costs	25 404
Total costs (1+2)	241 484

Details of direct cost items:
1/Salaries and benefits
Principal Investigator persons- 5 months, 15000 zł
Staff person – months 0 zł
Technicial assistant person- 3 months, 5400 zł
2/ Equipment (type, estimated cost, planned month of purchase, and justification)
Greenhouses, 9 items a'14 000 zł. Cost: 126 000 zł, March 2011. Place of carrying out experiment
Hives, 18 items a'300 zł. Cost: 5 400 zł, June 2011. Place in which honey is collecting.
3/ Other direct costs (type of expenditure, amount, and relation to project plan)
Soil, Cost: 40 500zł. March 2011. Necessary for sow of plants
Seeds, 500g Cost: 280 zł. March 2011. After growing is necessary for enticing honey
Honeybees Cost: 900 zł. June 2011. Necessary for producing honey
Water Cost: 12 000 zł. April – August 2011. Necessary for plant growing

Fuel, Cost: 4 000 zł. April – August 2011. To translocate between Cracow – Słomniki using a car from Institute of Environmental Sciences
Probes analysis in Warsaw, 54 items a'88 zł. Cost: 4 800 zł. July – August 2011. Necessary for estimating amount of herbicides residue in honey samples
Other services. Cost: 800 zł
Travel costs related to participation in conferences (2 conferences, one person taking part in one conference) Cost: 1000 zł

4.2.2 REVEWS

- Marta Szulkin

Review of project: "*The effect of herbicides on multiflorous honey quality"*

The project "the effect of herbicides on blossom honey quality" sets to test the effects of two different herbicides on honey quality. The set hypothesis is relevant to the food industry and human health. The question asked is sound, and large parts of the project description and experimental set-up appear well thought through. However, there are notable weaknesses of the project, related to (a) the general project summary, (b) methodological aspects (sample size, statistical analysis), and (c) cost outline.
Project summary – this section is not of the same quality as the rest of the application – it appears to have been written in a rush. The summary is in fact very confusing, as it presents qualities of an abstract, i.e. of a study that has already been carried out (past tense and statements like "we found that..." and "here we show that...") combined with a project proposal – which is what it should be. A project summary rarely presents evidence of generated work, it focuses instead on what is to be tested, and how the PI wants to carry the project out.

It is important not to state research hypotheses with strong *a priori* judgement – while it is indeed true that one expects pesticides to decrease honey quality, it is not a statement that has been proved. Thus one cannot state "our results will demonstrate how the presence of herbicide [...] will decrease the honey quality", as *we do not yet know* whether that statement is true or false. As a sideline, one-sided arguments in hypothesis testing could be compared to the use of one-tailed tests in statistics.
Project description
Parts 1 and 2 present adequate amount of information, although some sections lack in clarity (i.e. Part 3, statistical analysis).
Part 1
Line 68-69 – good start of section – you state your research hypothesis straight from the start.
Line 73 – this is not a general introduction to a large thesis – it may be best to skip references to ancient times, as it is not directly relevant to the research hypothesis you are set to test.
Line 86-87 – I agree with your statement: it "could" be predicted that honey quality will be altered by pesticides – the use of such wording outlines your expectations, yet does not preclude the possibility that the reality may be different – (see my comment in project summary).
Line 91-94 – this is a good presentation of your experimental set-up, which places it well in the context of current knowledge – the effects of herbicides on honey quality have not been tested yet (which is important information to the funding body), and you additionally introduce a temporal dimension to it. It could be useful to include such statements whereby you would "quantify the effect of pesticides on honey quality on a temporal scale", which brings useful quantitative estimates that readers can use in their own future research.
Part 2

This section outlines quite well knowledge up to date, current problems and current literature. The paragraphs are not very well connected with each other, yet the essential information is there.
Line 113-116: I understand that you want to go one step further that what has been done by Albero et al 2004, who found pesticides in honey – you want to *quantify* the impact of different pesticides at different time scales – this may be worth including in this section.
Part 3
My biggest concern is sample size. In contrast to the studies cited who focus mostly on the chromatography of pesticide-originating chemical elements found in honey (i.e. qualitative analysis), you are asking in the project to what extent one pesticide or the other affects honey quality – thus you need quantitative data. Unfortunately, there are only 6 hives per treatment, which is a really small number if you want to derive quantitative data from it. Although it is true that you have time replicates, you could potentially increase the sample size of 18 hives per treatment (6*3 time sampling), but only by using mixed models and adding "hive" as random effect to avoid pseudoreplication. Additional random effects could be used (e.g., greenhouse), but one needs to be careful not to over-parametrise the model given the low sample size.
Lines 139-142 – may be worth specifying that standard pesticide concentrations.
Lines 166-169 – first of all, is it not better to use an ANOVA? I am not sure how you want to use correlations when one of the variables (type of pesticide?) is not continous.
Lines 165-170 – this section is not clear overall. Be careful to correct for multiple testing (e.g. Bonferroni-type tests)
Lines 171-175 – would it cost that much more to include physic-chemical properties as well? This could result in a better output dataset and potentially more results to report.
Lines 173-175 – This is unclear, and the general statistical framework and types of tests should be rephrased.
Proposed Budget
Greenhouse setting up costs (191 234 zl) appear extremely expensive in the context of the overall budget (241 484 zl), and one wonders whether there are no other cheaper alternatives available. Similarly, soil costs seem very expensive (40 500 zl) – a justification is perhaps needed.
Salaries section – I understand there are two PIs, hence hired for a total duration of 10 months. Thus, why is the PI only mentioned for 5 months (and where is there only one, rather than two of them in the cost outline?). It is therefore likely that this figure ought to be changed from a value of 15000zl to 60000zl.
Additionally, given the high budget (62000 euros!) required for this project, I would strongly urge that the results are communicated worldwide rather than on a national scale only. Moreover, there is still room for improving the set-up cost-wise – since the greenhouses are the most expensive element of the project, the research should perhaps carry on for longer, and present a list of additional research hypotheses than what has been outlined (although I believe this suggestion may be beyond the scope of this evaluation).

- Iwona Giska

  Review of project: *"The effect of herbicides on multiflorous honey quality"*

The aim of the project is to test if use of herbicides influence honey quality. First of all, I would think about research question. It is quite obvious that herbicides will show negative effect on honey quality. Authors want to test it but they do not give any information that could be used to predict positive effect of herbicides. On the other hand, what would be

the outcome if the effect of herbicides is positive? Should we use more herbicides that are known to be toxic? The project does not seem to be very innovative and useful and, forgetting about that, there are some other points that need comments.

Authors mention that pesticides have been already detected in honey (rows 115-116). They say that these pesticides impact on honey quality is unknown (rows 34-36). But for consumers just the absence of herbicides in honey makes its quality low. If herbicides are detected in honey it is not worth testing other features as nobody would like to eat honey that contain a lot of sugar, vitamins or has nice color and taste and at the same time high herbicides content. Analysis of relation between herbicides content and other honey characteristics will just produce meaningless information. In such a case, from methodological site I would propose to check firstly, if there are herbicides detectable in honey. Then, in case if honey is herbicides free, it would be worth performing analysis of other characteristics. So it would be the impact of herbicides usage not herbicides presence in honey.

I would propose to check QSAR database for pesticides to predict behavior of tested herbicides (their active substances) in the environment what could be provide some information about their persistence and uptake by plants and animals (including honeybees) without doing any tests. It would answer the question about herbicides neutralization so there would be no need for the second and third sample set that are planned to be analyzed only to check herbicides concentration in honey. Time and money could be spent for other analyses (more samples, more herbicides) what could improve informativenes of obtained results because the effort that is going to be put in preparing proposed experiment, especially greenhouses building, and its cost is significantly high when you think from the point of view of data obtained after one main sample collection. This data information capacity is quite low. It arises from small number of samples as well from small number of herbicides tested. The choice of these two herbicides could be explained as their active substances are based on the same organic compound, so differences between their properties could be under detection limit.

There is no explanation how sensoric methods are going to be applied and how their results are going to be analysed? They should be applied after chemical methods, in case of toxic effects of herbicides in honey. Concerning applied chemical methods, one of proposed should be enough, as MS has very low limit of detection and quantification. If Authors have special reasons of applying both GC-MS/MS and LC-MS they could explain that. Methods of statistical analysis could be clearified.

From technical point of view, grant form is without reservation. It is clear and well organized. Stronger literature support is advisable. Anyway, the importance of the research question is doubtful and applied values of the project seems to be quite low.

- Giulia Casasole

Review of project: *"The effect of herbicides on multiflorous honey quality"*

The proposed research wants to investigate an interesting problem: if the quality of honey is affected by the use of herbicides.

This research has potential for significant outcomes considering that the topic isn't well known and especially for the interests that consumers have for the quality of the honey. However, there are some weaknesses in this research proposal that I'm going to explain below.

Abstract

The abstract doesn't summarize correctly the contents.
-In lines 37-38-39 it is written that the research project shows that the herbicides have decreasing influence for honey quality features, but this is the prediction that the researchers are going to test and not what the research project shows. The researchers can't know if the herbicides affects or not the quality of the honey: they can only hypothesize that they affect it predicting a decrease in the quality, but they can't assume that it will occur before running the experiment.
-In lines 43-44-45 it is written that the results of the experiments will demonstrate how the presence of herbicide in the honeybees environment will decrease the honey quality, but the research project will only show if the herbicides affects or not the honey quality (changing some physical-chemical features of the honey), but it will not explain the mechanism that leads to it.

Methodology

-In lines 140-141 the herbicides used are mentioned but not the concentration used for each.
-In lines 147-150 it isn't clear the time in which each sample will be collected. It would be better to write 4, 6 and 8 weeks after bringing the bees in the glasshouses because the expression"2 weeks later" could be quite confusing.
-In line 152 it is written that the organoleptic proprieties of the honey will be evaluated, but then there isn't any statistical analysis to verify if there are significant changes in them or not. I think that it could be possible to assign values for each of this characteristic and then analyze them.
-In lines 165 and 171 it is written that the samples will be collected after 4 weeks and 6 weeks the herbicides were introduced, but in the previous lines 147-150 it is written that the samples will be collected after 4-6 weeks the bees were brought in the glasshouse (the bees were put there one day after the herbicides)
-In line 169 it is explained how the data will be analyzed, but it isn't clear what "the differences between the two herbicides" means and which kind of analysis they want to do.
-In line 172 t is written that in the samples collected after 6 and 8 weeks will be evaluated only the herbicide content, but because the features indicating honey quality are water content, sugar content, etc. they should be checked all every time to see if they change, together with the herbicide content.

References

The literature cited is too poor. There are only 4 papers and two of them are from Polish Journals and not International ones.

- Agata Miska

Review of project: *"The effect of herbicides on multiflorous honey quality"*

It is not clear what the topic of this application means, because if author wanted to say that honey will be produced from various species of flowers, then a proper term would be – multiflorous honey.

In general, paper is characterized by informal and poor language. This is the main cause preventing reader from understanding what the applicants wanted to say. Moreover some part of the text can be qualified as popular science writing, not scientific (for ex. 73, 117).

In summary and methodology information (37-42, 135) about outcomes of experiment can be found, so why the applicant is writing grant application when he already know the results?

When some kind of knowledge which is discovered previously is placed in the aplication, it is necessary to put information in brackets who wrote it, otherwise you could be

accused of copyrights (for ex. 104, 106, 127, 146). On the other hand aplicant places literature when it is unnecessary (121) because some facts are obvious.

There is no information why this particular two herbicides were chosen (140-141). Proposed methodology is not clear. Why applicant wants to collect honey samples in 4, 6 and 8 weeks? Is there any literature about similar procedures? There is a possibility that honey quality will change if honey samples will be gathered in different time (the time of plants vegetation is changing during procedure, and that is why the concentration of different components might vary). I suggest to gather samples in the same time, but to start experiment earlier for each group.

In methodology (135) no information about that greenhouses are going to be built can be found, but in project schedule there is no distinction of this task. According to information from task table (215) we can find that the first step is to set plants, but where? In methodology "subchapter" also there are no details about organoleptic tests – for example; who is going to conduct them?

Positive thing is that proposition of future research project appears in the end.

- Edyta Podmokła

Review of project: *"The effect of herbicides on multiflorous honey quality"*

This project seems to be good. The idea is interesting. And hole problem is very important for many consumers. The biggest weakness of this project is that you don't decide what the "quality" means. You take into account several parameters of them but don't want to check how them work together. You mention that herbicides were detected in honey (35, 115) and also that they have decreasing influence for the others component of honey quality (37-39). You could analyze only amount of herbicides in honey and it could be them quality because I believe that there aren't any consumer who want to eat honey with herbicides inside. You could focus on changing this amount of herbicides in honey in relation to the time after introducing herbicides into field (92, 127).

Other comments:

METHODS

You want to test organoleptic properties of honey (152-153), but I can't see the reason for that because you don't use it in your statistical analysis. You should resign from organoleptic tests or you should employ the honey tester and use some scale of organoleptic feature to get some numerical data.

Your description of statistical analysis is not clear (167-170). You should write it more properly. In verse 169 you wrote about differences between two herbicides but it's not clear if it's about content or types of herbicides.

You want to check several components of honey quality and you analyze them separately (168). If you don't fix what is the honey quality you should find a way to analyze them together.

Why do you analyze after 6 and 8 weeks only herbicide content, not every components of honey quality (171-173)?

You don't mention about greenhouse in statistical analysis. Do you want to add it to your analysis as a random factor?

OUTCOMES

Why do you want to present your results only on polish conferences? (187-188)

REFERECES

You should find more articles connected with your project.

- Marcin Plech

Review of project: *"The effect of herbicides on multiflorous honey quality"*

The topic of proposed study has potential to provide data useful for honey manufacturers, providers and consumers. However it is not stressed in the text well enough (lines 117-124), which makes proposed study seem less relevant, however it can be changed if the topic is approached in the text from a different angle. The hypothesis (lines 82-83) could be formulated in a better way (stylistically).

Presented reasoning is logical and can provide some interesting data of broad influence. The title is interesting and catchy and it should be engaging. The abstract reflects the content of the proposal well. The language is correct at large. The study is innovative and should provide good basis for further investigations.

Althought the authors mention several determinants of honey quality (lines 75-79), they intend to examine only few of them, which is not comprehensible. Good analysis should embrace more vital ingredients of honey.

Examination of the speed of herbicide neutralization (lines 92-93) seems one useful analysis, but later on the authors imply that they do not mean to continuously examine the remaining characteristics of honey quality (lines 171-173) along with the herbicide content measurements. It is not good reasoning, for such measurements could give essential information about the changes of composition of honey exposed to herbicides in time. Moreover this type of analyses, if conducted long enough, can also give information on the speed of honey quality recovery after the end of its exposition to herbicides.

Proposed statistical analysis seems correct and can serve for analysis of data produced in this kind of experiments.

The usage of particular herbicides is not well substantiated (lines 140-141), has anyone used them before? Some citation needed. Methodology use seems rather correct, but one can imagine other usage of methods (e.g. usage of commercial, standardized kits for sugars content analysis, suitable for comparative studies), why particular methods were chosen (lines 156-160)? Citations needed. Explanation of procedural steps (147-150) is not clear enough.

The authors are planning to build 9 greenhouses, is this really necessary? It could be much more convenient to build 6 at most and perform more experiments, saving fundings for other expenses. Moreover the authors are planning to gain fundings for 9 months only which might not be enough for conduction of all experiments- therefore different planning is further advised (it is difficult to obtain money for 9 months only). Moreover requested sum is quite big and it could be difficult for anyone to generate this kind of money in such short period for honey research. 9 months would also be to little time to write planned two publications, while still conducting experiments.

- Agata Pietrzyk

Review of project: *"The effect of herbicides on multiflorous honey quality"*

The application has all necessary parts and it is complete from the formal point of view. The aim of the project is well-defined. The influence of the herbicides on the honey quality can be considered as important research question. However, insufficient number of arguments has been given to support that.

In the project description only general information about the composition and features of honey are provided (with a style suitable rather for the popular science texts). The

influence of herbicides on living organisms is only mentioned in one sentence without providing references to the literature. Also in the description of the present state of knowledge there are some information missing. Dispersion of pesticides and consumers' preferences seem to be the main threads in this part whereas it would be more reasonable to write more about the influence of herbicides on plants and animals and accumulation of these substances in the tissues. In the methodology description the reason for choosing these kinds of herbicides should be given as well as the description of 'sensoric methods'.

The budget for this one-year project does not appear as rationally defensible because the costs of the equipment needed are very high and comprise about 55% of total budget. With this kind of initial costs maybe it would be better to plan the repetition of the experiment so that the equipment will be used several times? In this case, the increase in general costs of the project would be relatively small.

In general, the proposal needs to be re-read and supplemented with wider description of essential issues. It should be certainly supported by the references to literature of the subject. In the proposed application the references are poor and they appear in regard to relatively less important information but they are missing in regard to the crucial ones.
The language is a weak point of the project. Because of that, many problems with interpretation occurs. Project summary can be a good example of the language problems as it looks like an abstract of the paper that presents already conducted studies (expressions like: "Here we show that…", "We found that…"). That aspect should be improved.

4.2.3 FINAL VERSION OF THE PROJECT

Project title:

The effect of herbicides on multiflorous honey quality.

Applicants:

Agata Rudolf, Katarzyna Wężowicz

Project summary

The goal of the project is to test whether using herbicides in agriculture influence on the honey quality. The herbicides are widespread in the agriculture and they have an effect on the live organisms and food chains. The herbicides residues are able to penetrate to the ground water or being spread by wind and transmit to the unpolluted area.

The key point of the study is to show if there is any difference in the quality of honey coming from area in which the herbicides were used in comparison with honey samples from herbicides – free area. To verify the hypothesis: the herbicides content affects the honey quality – the investigation of such honey quality features as sugar content, water content, diastase number, free acid content and pH, and HMF(5- hydroxymethylofurfural) content will be conducted. The study will also reveals the herbicides content in time.

Project description, methodology and expected results

1. *What problem is being proposed and why?*

The aim of the project is to test whether using herbicides in agriculture influence on the multiflorous honey quality. Nowadays, develop in agriculture causes increasing use of herbicides. The influence of herbicides residues in live organisms seem to be significant. Essential results of using herbicides are changes in plant cover (Różański, 1998). Herbicides used to inhibit growth of the weeds often kills plants for which secure are being used. Pesticides show also high toxicity to animals and human (Brzóska, 2005). Pesticides residues can penetrate soil and can be used by microorganisms as source of energy and building material, but more often are deadly for them (Różański, 1998). With groundwaters they can translocate to water bodies and affect flora and fauna.

The honeybees (*Apis mellifera*) pollinate entomophilen plants by collecting nectar and pollen, which are their nourishment. The effect of honeybees activity is production of valuable for the human food industry products: honey, wax, pollen, royal jelly and propolise (Turi and Matray, 1999).

The key point of the study is to show if there is any difference in the quality of honey coming from area in which the herbicides were used. The determination of physicochemical properties of the honey will allow test the following hypothesis: the herbicides content affect the honey quality. It could be predicted that the features which determinate the quality of honey will be changed and probably abnormality. The quality standards of honey are now regulated in legal acts like Directive of Council of the European Union No. 2001/110/EC. The premises for this prediction are based on the facts that harmful chemical substances could penetrate to the honeybees products (Kisała J. and Dżugan M., 2009). Blasco et al. founded pesticide residues in 95% of analyzed honey samples collected in Portugal (Blasco et al., 2003). However, the negative effects of the herbicides on honey quality has not been shown in any of the previous works.

Moreover, it could be expected that herbicide content in honey will change in time, shows how quickly the herbicides are neutralized.

2. What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?

Nowadays pesticides become essential for chemical protection of plants. They are applied not only to boost agriculture production, but also to limit the occurrence of weeds in plant cultivation. The presence of pesticides was found in all kinds of water circulated around the ecosystems - atmospheric precipitation, superficial water and underground water. Biologically active substance of pesticide could be released from the crops and penetrate the animals and human trophic chains (Wrzosek et al. 2009). Pesticides with regard of activity are divide into contact and systematic. Contact pesticides cause a scorching of plants. Systemic pesticides are transported in the plant vascular system together with assimilation and other nutrients. Since anthers and nectars are active physiological sinks, there is a possibility of accumulation of those chemicals in pollen grains and nectar which is being collect by bees. However, even though pesticides may not affect bees, residues collected from chemical protected plants can be found in bee products, including honey (Kubik et al.1999).

Honey is the most important product of honey bees activity for human. Honey primarily contains sugar and water. Sugar accounts for 95 – 99% of honey dry matter.

Majority of these are simple sugars – fructose (38,2%) and glucose (31,3%) – which represents 85 – 95 % of total sugars. Also disaccharides such as maltose, sucrose and isomaltose and few oligosacharides were found in honey. Water is the second most important component of honey which content is critical and affects the storage of honey. The final water content depends on numerous environmental factors such as weather, nectar conditions and treatment of honey during extraction and storage. Organic acids constitute 0.57% of honey and include gluconic acid which is a bee product of enzymatic digestion of glucose. The organic acids are responsible for the acidity of honey and contribute largely to its characteristic taste. Minerals are present in honey in very small quantities (0.17%) with potassium as the most abundant. Others are calcium, copper, iron, manganese, and phosphorus. Nitrogenous compounds among which the enzymes originate from salivary secretion of the worker honeybees are also present. They have important role in the formation of honey. The main enzymes present in honey are invertase (saccharose), diastase (amylase) and glucose oxidase. Vitamins C, B (thiamine) and B2 complex like riboflavin, nicotinic acid and B6 panthothenic acid are also found (Olaitan et al. 2007).

Nowadays, the consumers pay special attention to the quality of the food products that they buy. They desire first rate products. The legal standards define qualitative parameters to varied kinds of food stuffs. The consumers want to be sure that they are buying a high quality products (Mateo et al.1998). However, sometimes impurities can penetrate into the product even though the precautions were taken. The appearance of chemical impurities in the environment constitute threat to the quality standards. That is why it is important to get possession of relationship between the possible presence of impurities and the quality of the products. In Poland in 23% households honey is being consumed at least once a week and in 7% everyday (Pałys and Lewandowski, 2009).

All pesticides undergo decomposition in plant tissues. Honey is not a living tissue, but it contains active enzymes that change honey composition during its storage. Also pesticide residues can be decomposed in stored honey. Thrasyvalou (in Kubik et al. 2007) has found that fluvalinate breaks down in honey within about 24 weeks, while bromopropylate persists for over 9 months and malation for 75-95 days (*ibid.*). The content of herbicides in honey was previous examined but the change of the herbicides level in time and effect of the presence of herbicides in honey to the quality of honey has not been yet shown.

3. What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?

The investigations will be conducted in Słomniki near Cracow, where 9 greenhouses (5m x 20 m) will be hired. Purchase of the garden mould is planned in the March and sow of the dicotyledones meadow plants is planned in the April. The experiment will be conducted in three groups. In the first group herbicide named Agil 100 EC (active substance: propachizafop, derivative of arylofenoxypropionian acid) will be used at concentration of 0,8-1,5 l/ha and in the second group herbicide named Focus Ultra 100 EC (active substance: cykloksydym, oheksanodion group) at concentration of 1,5- 3 l/ha. Both of these herbicides inhibits growth of monocotyledons. The third group will be treated as a control.

It is planned to start introducing herbicides in the beginning of the June. The next day, 18 hives with honeybees families coming from unpolluted areas will be placed in greenhouses - 2 hives in each one. The honeybees are not sensible for the acts of herbicides.

After 2, 4 and 6 weeks from bringing honeybees, samples of honey will be collected – 3 samples from each of the hives.

The physicochemical properties of collected samples of honey will be examined using laboratory methods in the Institute of Environmental Sciences at Jagiellonian University.

The sugar content in collected honey samples (fructose, glucose and saccharose) will be measured using liquid chromatography with refractometric detector. The water content will be measured using refractometer, the diastase number – using photometer, free acids content and pH of honey – using pH – meter and titration, and HMF (5 - hydroxymethylofurfural) content – using liquid chromatography.

Herbicide content in honey samples will be measured using gas chromatography - tandem mass spectrometry (GC-MS/MS) in the Faculty of Chemistry at Warsaw University.

The results of herbicides content gained from the first set of samples of honey, that will be collected 2 weeks after herbicide introduction, will be analyzed using single - factor ANOVA. The results gained for each physicochemical features will be analyzed separately using single - factor ANOVA. The relation between each physicochemical quality feature and the herbicide content will be tested applying ANCOVA.

The results of the samples collecting from the second and third set will be analyzed properly in the same way.

The relation of herbicides content in time will be analyzed using two- factor ANOVA, where the first factor is time and the second factor is type of herbicide. Each quality feature will be tested in time period in the same way separately.

Each of the results will be used to elucidate the different aspect of the problem.

The principal investigators of the project have already the practical experience with honeybees. However, it will be necessary to employ a beekeeper to provide appropriate care to the honeybees. The majority of analysis in the project will be carried out in the laboratories of the Jagiellonian University.

4. What are the expected results of this project ?

The results of the study will be presented on National Apiarian Conference in Puławy and the Apiarian Conference in Częstochowa, and the European Conference of Apidology. We plan to publish our findings in 2 publications in such journals as Environmental Pollution and Journal of Apicultural Science. In the future it is considered to continue the experiment and focusing on the content of antioxidants and herbicide in honey.

Literature:

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Project schedule – anticipated tasks

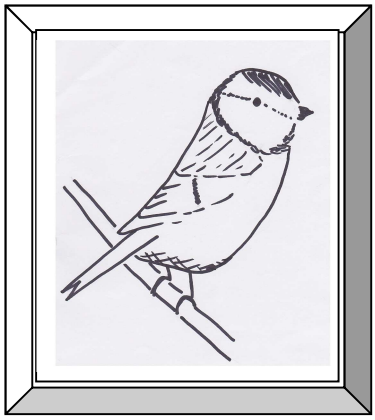
No.	Name and description of task	Expected completion date (mm/yy)	Expected cost (zł)
1	Hiring greenhouses and purchase soil	03.2011	91 790
2	Sowing and rising of dicotyledons in greenhouses	04 – 05.2011	16 484
3	Introduction of hives with honey bees, and herbicides into greenhouses	06.2011	21 825
4	Collecting honey samples and laboratory work	07 - 08.2011	35 360
5	Presentation, participation in conferences and report preparation	09 – 12.2011	21 840
		Total	187 299

Proposed budget

	Item	Funds for budget year (zł)
		2011
1	Direct costs, including:	164 015
	1/ Salaries and benefits	35 400
	2/ Equipment	86 400
	3/ Other direct costs	42 215
2	Indirect costs	23 284
3	Total costs (1+2)	187 299

Details of direct cost items:
1/Salaries and benefits
Principal Investigator person- 10 months, 15000 zł each 2 persons
Staff person – months 0 zł
Technicial assistant person- 3 months, 5400 zł
2/ Equipment (type, estimated cost, planned month of purchase, and justification)
Greenhouses, hiring 9 items for 6 months. Cost:81 000 zł, March 2011. Place of carrying out experiment
Hives, 18 items a'300 zł. Cost: 5 400 zł, June 2011. Place in which honey is collecting.
3/Other direct costs (type of expenditure, amount, and relation to project plan)
Soil, Cost: 4 500zł. March 2011. Necessary for sow of plants
Seeds, 500g Cost: 280 zł. April 2011. After growing is necessary for enticing honey
Honeybees, Cost: 900 zł. June 2011. Necessary for producing honey
Herbicides, Cost: 130 zł. June 2011.
Water Cost: 12 000 zł. April – August 2011. Necessary for plant growing
Fuel, Cost: 4 000 zł. April – August 2011. To translocate between Cracow – Słomniki using a car from Institute of Environmental Sciences
Probes analysis in Warsaw, 162 items. Cost: 11 200 zł. July – August 2011. Necessary for estimating amount of herbicides residue in honey samples
Other services. Cost: 800 zł
Travel costs related to participation in conferences (3 conferences, one person taking part in one conference) Cost: 4000 zł

4.3 Impact of dust pollution on plumage coloration and male attractiveness in blue tits (*Cyanistes caeruleus)*



Authors:
Giulia Casasole,
Agata Pietrzyk,
Edyta Podmokła

4.3.1 FIRST VERSION OF THE PROJECT

Project summary:

Many birds species exhibit colorful plumage that is one of the most common trait involved in mate choice. Females of many species prefer males with more ornamented plumage because it is widely assumed that feathers coloration is a honest signal of body condition. There are some mechanisms that can change feathers coloration after moult, such as bacterial degradation, mechanical damage, expose to ectoparasites, addition of preen waxes and dirt accumulation. Some studies showed that dirt accumulation on feathers may affect plumage coloration and male attractiveness. The project aims to investigate the effect of air pollution (in terms of deposition of dust) on the plumage coloration of a passerine species, blue tit (*Cyanistes caeruleus*).

Blue tit is a dimorphic species that differs between sexes in UV-reflectance in the crown. This plumage trait is used in mate choice: females choose more UV-reflactant males. In the proposed study we want to test three different hypothesis. Firstly, we want to investigate if there is any effect of dust pollution on plumage coloration. Secondly, we want to verify if males from polluted areas are less attractive than males from rural areas. Lastly, we will examine if differences in level of plumage soiling between males from polluted areas affect male attractiveness.

The project will start with the examination of differences in plumage coloration between individuals from two types of areas (rural/urban) using a spectrometer. The following part of the project aims to study females preference using outdoor mate choice aviaries. Manipulation of males plumage will be used in order to add variation in levels of plumage soiling.

We expect that birds from the polluted area are less colorful than birds from the rural areas. We also predict that females from both rural and urban areas choose more often males from the rural areas. Finally, we predict that in polluted areas females choose more often males with less soiled plumage.

Project description, methodology and expected results

1. *What problem is being proposed and why?*

The aim of this project is to investigate whether the air pollution affects the plumage coloration of blue tits (Cyanistes caeruleus) through the deposition of dust particles on the feathers and if this changes in plumage coloration can have an effect on the males attractiveness.

The problem is important as the air pollution (also in a form of dust fall) is a significant factor in urban/industrialized areas and it can have direct or indirect influence on birds. The studies concerning air pollution and the influence of its elements such as heavy metals were conducted but there is not much knowledge about the direct, physical influence on plumage coloration in birds. Considering the fact the mate choice in blue tit is based on plumage coloration we want to address the influence of the dust fall on male attractiveness and consequently female choice.

2. *What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?*

Many birds species exhibit colorful plumage. The intensive coloration is often correlated with the individual condition (Surmacki and Nowakowski 2007). It is widely assumed that the feathers coloration is a honest signal reflecting the body condition of males. For this reason, plumage ornamentation is one of the most common traits involved in mate choice in birds (Hill and McGraw 2006 in Griggio et al. 2010). Females of many species prefer the males that have more ornamented plumage, because they may gain some benefits from pairing with these males.

Blue tit is a passerine species that is markedly sex-dimorphic in the UV-range. Males are more UV-reflectanct on the crown than females. These plumage trait is displayed in agonistic and sexual interactions by horizontal “head-forward” postures and erected nape feathers (Andersson et al. 1998). Female of blue tit tends to choose males that has more UV-reflectant crown (Hunt et al. 1999).

Plumage coloration should be a stable signal because once the coloration is established the colors are separated from any agents presented in a blood stream. However, there are some mechanism of change in feather coloration after moult. The feather colors can change because of bacterial degradation, addition of a preen waxes, mechanical damage, exposure to sunlight, presence of ektoparasites and dirt accumulation. There are some studies that show that dirt accumulation on feathers may affect plumage coloration reducing the reflectance and male attractiveness (Zampiga et al. 2004). Especially a recent study on budgerigar showed that dirt accumulation on plumage affects ultraviolet coloration and this leads to female preference for preened males (Griggio et al., 2010). In urban areas the concentration of the

traffic/industrial dust is higher and some studies show that there is an accumulation of dust particles on the feathers of birds (Nam et al. 2004).
The plumage maintenance is an important part of daily time budget of birds and only individuals in good condition are able to afford to preen sufficiently removing the soiling and dirt from the feathers resulting in temporal trade-off between investment in plumage maintenance and other activities such as foraging and vigilance (Cucco and Malacarne 1997). Considering that this is the present state of the knowledge in this field we decided to focus on the issue of dust influence on plumage coloration to investigate further consequences in male attractiveness and mate choice.

3. *What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?*

Three experiments are planned to be done.
EXPERIMENT 1
The first experiment aims to verify the hypothesis that atmospheric pollution affects color plumage in blue tits.
Two groups, 20 individuals each, will be captured using mistnet in two different urban areas and other two groups (20 individuals each) will be captured in two rural areas.
Measurement of the reflectance of the crown and breast feathers will be done with OceanOptics, Inc. USB 2000 spectrometer and the deuterium-halogen light source (DH-2000). For each individual 5 spectral measurements will be taken from the region of the crown. Then measurements will be averaged for each individual. The color will be quantified using descriptors of reflectance spectra such as brightness, chroma and hue (Griggio et al., 2010).
Altogether we will have 40 measurements for the Blue Tit from the rural area and 40 for the birds from urban populations.
The statistical analysis will be GLMM (general linear mixed model) with sex and area type (urban/rural) as fixed factors and with populations nested in the area type as a random factor.
The predicted outcome is that birds from the polluted area have less colorful plumage than birds from the rural areas.
EXPERIMENT 2
The second experiment aim to verify the hypothesis that males from polluted areas are less attractive than males from rural areas.
20 dyad of males will be formed and in each dyad one male will be from one urban population and the second male from one rural population. 10 females from each kind of area will be chosen. Each female will be randomly matched to one male dyad. All choice trials will be independent and each group (female+dyad) will be used only once.
The experiment will be conducted in outdoor aviary (Fig. 1). The female will be put in the aviary 3 hours before the observation so she could habituate. Each observation will be recorded so we will be able to define the exact time that female spent near each male (Kurvers et al., 2010).
The observations will be done during the period of social pair formation of Blue Tit.

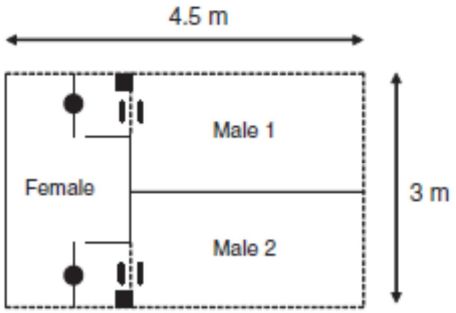


Figure 1. Schematic view of outdoor mate choice
aviaries used for the experiment (Kurvers et al., 2010).

In the statistical analysis we will use differences between time that female spent with male from urban area and male from rural area. We will analyze preferences of each group of females (from urban/rural area) separately using paired t-test. Then we compare preferences of females from both type of areas using unpaired t-test.
The predicted outcome is that females from both origin choose more often males from the countryside that from the urban area.

EXPERIMENT 3

The third experiment aim to verify the hypothesis that in the polluted area male with less soiled plumage are more attractive for the female. In this experiment we want to manipulate the coloration of the plumage of a group of males captured in the rural area in order to imitate the condition that they have in the urban areas.
20 females will be taken from the urban area and 40 males from rural area. All males will be soiled with dust – halt of them at the lower level and half at significantly higher level. All males will have a soft plastic collar that prevent them from preening but allow them to carry out their normal activities (Zampiga et al., 2004).
20 dyad of males will be formed of one male with more soiled plumage and one with less soiled plumage. Each female will be randomly matched to one male dyad. All choice trials will be independent and each group (female+dyad) will be used only once.
The experiment will be conducted in outdoor aviary (Fig. 1) in the same way as written above (Experiment 2.).
The statistical analysis are similar as above (experiment 2). We use differences between time that female spent with male from urban area and male from rural area and analyze female preferences using paired t-test.
The prediction in this experiment is that in polluted areas females choose more often males with less soiled plumage.

4. What are the expected results of this project?

We expect that our results give us the answer to the main research problems, which haven't been explored yet. The study begins new path of experiments on blue tits and give the chance to continue the investigation in the future. Equipment used for this experiment will be also useful in the future experiments.

We want to publish our results concerning each experiment separately.
The title of them could be for example:

- *Impact of the dust pollution on plumage coloration in blue tits,*
- *Males from polluted areas have decreased attractiveness-the example of blue tit,*
- *In the polluted area male with less soiled plumage are more attractive for the female*

Considered journals are: Journal of Avian Biology, Proceedings of the Royal Society B: Biological Sciences and Behavioral Ecology.

We plan to present our results on the Conference of the European Ornithologists' Union. This conferences are held at different places in Europe every two years. They attract several hundred ornithologists from all over Europe and abroad. We also want to show our results on the EGI (Edward Gray Institute) Student Conference. These conferences (which started in 1947) are primarily intended as places for undergraduates, graduate students and very early career postdocs to present their work

Literature:

Andersson S., Ornborg J., Andersson M. 1998. Ultraviolet sexual dimorphism and assortative mating in blue tit. Proc. R. Lond. B 265: 445-450

Cucco M.and Malacarne G. 1997. The effect of supplemental food on time budged and body condition in the black redstart. Ardea 85:211-221

Hunt S., Cuthill I., Bennett A.T.D.,Griffiths. 1999. Preferences for ultraviolet partners in the blue tit. Animal Behaviour 58: 809-815

Hill G.E. and McGraw K.J. 2006. Bird Coloration, vol. 2. Function and evolution, Harvard University Press, Cambridge in: Griggio M., Hoi H., Pilastro A. 2010. Plumage maintenance affects ultraviolet colour and female preference in the budgerigar, Behavioral Processes 84: 739-744

Griggio M., Hoi H., Pilastro A. 2010. Plumage maintenance affects ultraviolet colour and female preference in the budgerigar, Behavioral Processes 84: 739-744

Kurvers R.H.J.M ., Delhey K., Roberts M.L., Peters A. 2010. No consistent female preference for higher crown UV reflectance in Blue Tits Cyanistes caeruleus: a mate choice experiment. Ibis 152: 393–396

Nam Dong-Ha, Lee Doo-Pyo, Koo Tae-Hoe. 2004. Monitoring for lead pollution using feathers of feral pigeons (Columbia livia) from Korea. Environmental Monitoring and Assessment 95: 13-2

Surmacki A. and Nowakowski J.K. 2007. Soil and preen waxes influence the expression of karotenoid-based plumage coloration. Naturwissenschaften 94:829-835

Zampiga, E., Hoi, H., Pilastro, A., 2004. Preening, plumage reflectance and female choice in budgerigars. Ethol. Ecol. Evol. 16: 339-349.

Project schedule – anticipates tasks

No.	**Name and description of task**	Expected completion date (mm/yy)	Expected cost (zł)
1	The purchase of equipment needed, preparing the experimental set	1 – 2.2011	26580
2	Realization of experiments 1 and 2	3-5.2011	4940
3	Data analysis - conducting statistical analysis of the date from the Experiment 1 and 2; preparation of two manuscripts	6-12.2011	5070
4	Realization of Experiment 3	3-5.2012	4940
5	Data analysis - conducting statistical analysis of the date from the Experiment 3; preparation of one manuscript	6-11.2012	5070
6	Report preparation, preparation of the manuscript	11-12.2012	780
7	Conference presentation, participation in the conferences	10.2011-12.2012	9100
		Total	56480

Proposed budget

No.	Item	Funds for each budget year (zł)		
		2011	**2012**	**Total**
1	**Direct costs, including:**	36800	12300	49100
	1/ Salaries and benefits	8500	7500	16000
	2/ Equipment	24500	0	24500
	3/ Other direct costs	3800	4800	8600
2	Indirect costs	3690	3690	7380
3	Total costs (1+2)	40490	15990	**56480**

Details of direct cost items
1/Salaries and benefits
Principal investigator person-moth: 20, 12000 zł
Staff person-moths: 8, 4000zł
2/Equipment:
spectrometer – 8000 zł,
DH-2000 Deuterium Tungsten Halogen Light Sources – 9000 zł,
mistnets – 500 zł
outdoor aviary – 7000 zł
3/Other direct cost:
Food for birds – 1000 zł
Books and journals – 600 zł
Conferences attendance (plane ticket, daily allowance) – 7000 zł

4.3.2 REVIEWS

- Justyna Wolińska
  Review of project: "*Impact of dust pollution on plumage coloration and male attractiveness in blue tits (Cyanistes caeruleus).*"

Summary evaluation:
The project examines the impact of dust pollution on plumage colouration and, consequently, male attractiveness in blue tits. Although this is a well written grant proposal, and the experiments are pretty well designed, the authors did not convince me that their idea is particularly novel. After having read the introductory part, I have the impression that the authors just want to repeat on blue tits, what was previously found on other bird species. Given all the knowledge that we already have from other bird systems about the impact of dust pollution on plumage coloration (and part of this work is acknowledged in the proposal), the authors need to put more effort in highlighting all novel components of their study.

Details are given in my evaluation below and should be considered by the applicants.
Detailed evaluation:
Project summary
Well done.
What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?
"(Hill and McGraw 2006 in Griggio et al. 2010)" – why "in"? I don't understand a link between these two reference sources.
"The feather colors can change because of bacterial degradation, addition of a preen waxes, mechanical damage, exposure to sunlight, presence of ektoparasites and dirt accumulation." – provide references for each mentioned here cause of the feather colour change.
"There are **some** studies that show that dirt accumulation on feathers may affect plumage coloration reducing the reflectance and male attractiveness (Zampiga et al. 2004)." – **some** studies, so provide more references here.
"(…) **some** studies show that there is an accumulation of dust particles on the feathers of birds (Nam et al. 2004)". Similar comment as above. Provide more references.
"The plumage maintenance (…) and vigilance (Cucco and Malcarne 1997)" – The information provided in this paragraph is not really well connected to the rest of the text. And, anyway, it seems a bit irrelevant to the proposal, as you do not plan to do a manipulation of bird condition, do you?

What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?

Experiment 1

"Experiment 1" – I wouldn't call this part "experiment". The experiment often requires some manipulations of experimental conditions, whereas in here you just proposed taking a couple of measurements.

"(…) will be captured in two different urban areas and (…) two rural areas". How will the areas be chosen? For example, will all four of them represent similar climatic conditions? Will they have similar elevation? For example, as the authors acknowledged elsewhere, the sunlight exposure does influence the color of feather.

Will the birds be killed after capturing them? If yes, how? If not, how they will be transported to the laboratory? When and where will they be released after performing the spectrophotometer analyses and experimental tests? By the way – will the same birds be used for the spectrophotometer analyses and all other tests? This should be clarified.

Since you catch these birds anyway (in order to take the spectrophotometer measurements), you might think about taking other measurements, e.g. body mass, age (possible to assess in tits?). In this way you might later get a possibility to control for the potential differences between the groups, by taking into account this type of information in the statistical model.

If you want to test the effect of population in the statistical model, it might not be enough to have just two of them for each area type. Is it visible to extend your study to three populations per area type?

Experiment 2

Change to "The second experiment aim**s** to verify"

"The female will be put in the aviary 3 hours before the observation so she could habituate." – but there is no information given of how many hours will each observation take (i.e. in addition to these 3 hours of habituation).

"Each observation will be recorded" – how? By the observer? Or will any sort of recording equipment be installed?

"Then we compare preferences of females from both type of areas using unpaired t-test." – this is unclear. What values will be compared in this second test? I guess one number needs to be obtained per experimental unit. How will this number be calculated?

Experiment 3

No further comments

What are the expected results of this project ("know-how", patents, methods, equipment), and how will they be disseminated (publications, conference presentations, PhD theses)?

This section is very well written. It shows that the authors are aware of the importance of publishing the results of their study in the scientific journals.

Project schedule

I find it very important to provide such a detail time schedule. I noticed, however, that nothing is being planned in a following two-month period: 1-2.2012. Why is it so?

"Conference presentation, participation in the conferences" As it is presented now, it appears like it will take 14 months to prepare and participate in the conferences. Instead, you should better provide the exact dates of these conferences (i.e. each conference in a single row in that table).

Proposed budget

"Conferences attendance (plane ticket, daily allowance) – 7000 zł" Why doesn't it match the amount of money allocated to conferences in the "Project schedule" table?

- Marcin Plech

Review of project: "*Impact of dust pollution on plumage coloration and male attractiveness in blue tits (Cyanistes caeruleus).*"

Problematics of the project is important for population researchers as it explains and predicts how the range of blue tit populations will change in time as the urbanization of new areas proceeds. Another important aspect is the mating determinants and behavioristic study within the project. However these are not stressed well enough by the authors in the introductory part, neither is it discussed in the description of scientific background and current state of knowledge.

Overall logics and reasoning of the authors is correct and, if properly tackled may produce significant data of predicted standards. The structure and planning of the project are correct and meet the requirements of the call. The title of presented application is well composed and finds its reflection in proposed hypotheses. Proposed experiments address the asked questions, with the exception of small inconsistencies (see paragraphs 4 and 5). The abstract reflects content of the application. Overall language of the proposal is correct and concise (with small exceptions).

The first experiment is well planned, utterly simple and addresses first hypothesis precisely. The chances that it will produce predicted results data are high. Planned samples seem to be representative in size and quality. Statistical analysis is correct.

Second experiment needs some further consideration. Obtained data might not be satisfactory. While collecting specimens for analysis, members of the team should consider conducting some additional tests. It is true that the birds should constitute representative data, as they will be collected from two various locations for each treatment, however it is possible that obtained results might be ambiguous. The group should take into consideration, that mating preference might be caused by some additional, factors, mentioned by the very authors of the projects in the introductory part – page 1, lines 14-16 (e.g. bacterial or parasitic infections, which are not instantly visible). Therefore conduction of some additional tests (e.g. tests for the presence of parasites or microbes) for each specimen is advised, in order to avoid falsely positive/negative results.

The third experiment seems to contain no control. However the reviewer understands that introduction of additional specimen to each examined dyad would be incompatible with the whole structure of performed studies and previous experiments as well as it would require changes in statistical analysis. Nevertheless it should be reconsidered, for it could make data more consistent and stronger.

Among advantafes of the application are the simplicity of proposed experiments, and good understanding of the topics, which can make execution of the project easy and reliable. Nevertheless introduction of a bigger degree of complication could amplify cogency and clout of the project.

The time schedule for conduction of planned experiments and their budget are feasible. Predicted eventual journals for the future publication of obtained data seems reasonable, planned experiments can provide data strong enough to meet the requirements of these journals.

- Maciej Bonk

Review of project: "*Impact of dust pollution on plumage coloration and male attractiveness in blue tits (Cyanistes caeruleus).*"

This proposal presents plans of very interesting studies. The advantage of this project is that for the first time dust pollution will be studied as a factor affecting breeding behavior.

However, my first impression that authors are not aware of the actual importance of their project which is much more interesting than they presented it (see general remarks in the teks below). I assume that it is not so due to lacks of knowledge about the problem but the way of writing.

Having only the manuscript sent I only can imagine what did authors mean. The background of the planed study should be improved as its present form do not suggest its real value.

Several other correction and additional information are mentioned below.

My general opinion on project is good (from 1 to 10 I give to it 6).

68: These plumage trait is displayed…… - this information is unnecessary.

72: I am not realy sure what do you mean. Is it only a case of the model species? In fact, birds changes their coloration even seasonally. This may be true but written enigmatically. Anyway, this also unnecessary information as even changing coloration should be affected with dust. And, finally the issue of your interest is only one part of the year when tits mates. During this time, you could assume no changes.

General remark to first paragraph:

To my opinion you did not highlight the problem enough. However, it is interesting you did not mention why. In deed, there are some general problems you should mention. It is known that due to noise there is a selection against more quite birds. It lead to situation where females prefer louder males. If there is some threshold (assuming no interaction with habitat: rural or urban) of female sensitivity female from cities will not mate with rural males even if put into rural landscape. What is more, immigrating occasionally into cities rural males will not be selected by females. And this lead to wider population effects due to presigotic isolation. We can exclude in most extreme cases speciation. Putting your question in such ecological or evolutionary context make it worth financing.

109: you should describe whether it is plan to be done in field or lab because the measurement may in some degree response to general light condition (i.e. different wavelength in the morning or in the evening, cloudy and sunny weather and so on….).

111: wouldn't it be better to collect more than two replication from each countryside, lets say 3 or more cities and 3 or more rural localities. In my opinion it would be even better when obtain smaller number of birds from each landscape. It is important because the landscape type is the level you are interested in, no individuals (see also Ruxton G.D., Colegrave N. 2003. Experimental design for the life sciences. Oxford University Press pp. 97-99).

138: the prediction is different and shoul be written that the differences will be "–" and significantly different from 0.

147: don't use "significantly" address to other than statistical meaning. At least in scientific papers there is such difference. Anyway, if you are going to test whether you use significantly more soiled males you should write what test it would be.

General remark to methods:

It should be highlighted in the text when birds will be captured and when the experiment will be conduct as it has to be carried out during breeding season.

General remark to 4th paragraph:

Actually the same as general remark to first paragraph.

You may also apply for more general evolutionary or behavioral meetings. According to general remarks your problem is worth presenting in more general than ornithological context.

- Katarzyna Wężowicz

Review of project: "*Impact of dust pollution on plumage coloration and male attractiveness in blue tits (Cyanistes caeruleus).*"

The progress of urbanization and industry influence on increasing the atmospheric pollution. The subject of this project is to study whether the air pollution affects plumage coloration of blue tits through the deposition of dust particles on the feathers and if this change in plumage coloration can have any effect on the males attractiveness.

The research plan, objectives and hypothesis are clearly presented in the project. The choice of the research methods and materials is appropriate, what enable achieving the goals of the project within proposed timescales. The language is correct and concise. The title is adequate to the contents of the project. The abstract summarize the contents. Moreover, the budget is well prepared and has low price. The strong aspect of the project is an ability to continue the investigation in the future with using the purchased equipment.

The reasoning is logical but to experiment 2 should be introduce some modification. In order to verify the hypothesis, all males should be additional examine to exclude the presence of mechanism changing feathers coloration such as ectoparasites. In that way there will be equal chances for males to being chosen. Furthermore the term of social pair formation (128) should be more precisely define. Moreover, using the second time the Latin species name (44) it is allowed to write the Latin generic name in a shorter form.

- Agata Miska

Review of project: "*Impact of dust pollution on plumage coloration and male attractiveness in blue tits (Cyanistes caeruleus).*"

Proposed research address important challenge at the frontiers of the field addressed. Abstract summarize the contents. The project is scientifically significant (mostly because of the specie which was chosen).

Paper is written in formal language, with rare grammar mistakes. The background is made properly, supported with suitable literature. Suggested statistical methods are appropriate for the proposed work and expect data structure.

In subchapter "methodology" it is possible to find some inaccuracy in procedure for all three experiments, because there is no information about time when they will take place. Such Information is mentioned only in table with project schedule (the main point of this section is to inform about budget). Moreover there are no details from where exactly birds are going to be captured (geographical coordinates), and where the experiments will be conducted. It can be just supposition that it might be conduce in Jagiellonian University, Cracow.

I find some difficulties in experiment number two. The problem might be to capture birds from urban areas with mistnet without changing their color plumage. To my knowledge in such kind of actions exist possibility to change amount of dust on bird feathers. I suggest capturing them with plastic gloves.

Budget is well thought out, and there are some advantages for Institute, because after experimental time, all equipment (for ex. spectrometer, halogen light source) probably will be used in other research projects.

All three suggested titles of future papers seem to be interesting and I predict that we would find them in mentioned articles. Listed articles are suitable for publish outcomes from such kind of experiments.

4.3.3 FINAL VERSION OF THE PROJECT

Project title:

The effect of dust pollution on plumage coloration and male attractiveness in blue tits (*Cyanistes caeruleus*).

Applicants:

Giulia Casasole, Agata Pietrzyk, Edyta Podmokła

Project summary

Many bird species exhibit colorful plumage that is one of the most common trait involved in mate choice. Females of many species prefer males with more ornamented plumage because it is widely assumed that feathers coloration is a honest signal of body condition. There are some mechanisms that can change feathers coloration after moult, such as bacterial degradation, mechanical damage, expose to ectoparasites, addition of preen waxes and dirt accumulation. Some studies showed that dirt accumulation on feathers may affect plumage coloration and male attractiveness. The project aims to investigate the effect of air pollution (in terms of deposition of dust) on the plumage coloration of a passerine species, blue tit (*Cyanistes caeruleus*).

Blue tit is a dimorphic species that differs between sexes in UV-reflectance in the crown. This plumage trait is used in mate choice: females choose more UV-reflactant males. In the proposed study we want to test three different hypotheses. Firstly, we want to investigate if there is any effect of dust pollution on plumage coloration. Secondly, we want to verify if males from polluted areas are less attractive than males from rural areas. Lastly, we will examine if differences in level of plumage soiling between males from polluted areas affect male attractiveness.

The project will start with the examination of differences in plumage coloration between individuals from two types of areas (rural/urban) using a spectrometer. The following part of the project aims to study female preference using outdoor mate choice aviaries. Manipulation of male plumage will be used in order to add variation in levels of plumage soiling.

We expect that birds from the polluted area are less colorful than birds from the rural areas. We also predict that females from both rural and urban areas choose more often males from the rural areas. Finally, we predict that in polluted areas females choose more often males with less soiled plumage.

Project description, methodology and expected results

1. *What problem is being proposed and why?*

The aim of this project is to investigate whether the air pollution affects the plumage coloration of blue tits (*Cyanistes caeruleus*) through the deposition of dust particles on the feathers and if these changes in plumage coloration can have an effect on the male attractiveness.

The problem is important as the air pollution (also in a form of dust fall) is a significant factor in urban/industrialized areas and it can have direct or indirect influence on birds.

The studies concerning air pollution and the influence of its elements such as heavy metals were conducted but there is not much knowledge about the direct, physical influence on plumage coloration in birds. Considering the fact the mate choice in blue tit is based on plumage coloration we want to address the influence of the dust fall on male attractiveness and consequently female choice.

1. What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?

Many bird species exhibit colorful plumage. The intensive coloration is often correlated with the individual condition (Surmacki and Nowakowski 2007). It is widely assumed that the feathers coloration is an honest signal reflecting the body condition of males. For this reason, plumage ornamentation is one of the most common traits involved in mate choice in birds (Hill and McGraw 2006). Females of many species prefer the males that have more ornamented plumage, because they may gain some benefits from pairing with these males. Blue tit is a passerine species that is markedly sex-dimorphic in the UV-range. Males are more UV-reflectant on the crown than females. This plumage trait is displayed in agonistic and sexual interactions by horizontal “head-forward” postures and erected nape feathers (Andersson et al. 1998). Female of blue tit tends to choose males that have more UV-reflectant crown (Hunt et al. 1999).

Plumage coloration in birds should be a stable signal because once the coloration is established the colors are separated from any agents presented in a blood stream. However, there are some mechanisms of alteration in feather coloration after moult. The feather colors can change because of bacterial degradation (e.g., Grande et al. 2004), addition of a preen waxes (Piersma et al. 1999), mechanical damage (e.g., Willoughby et al. 2002) , exposure to sunlight (Surmacki, 2008), presence of ectoparasites (e.g., Moreno-Rueda 2005) and dirt accumulation (e.g., Zampiga et al. 2004).

There are some studies that show that dirt accumulation on feathers may affect plumage coloration reducing the reflectance (Surmacki and Nowakowski 2007) and male attractiveness (Zampiga et al. 2004, Lenouvel et al. 2009). Especially a recent study on budgerigar showed that dirt accumulation on plumage affects ultraviolet coloration and this leads to female preference for preened males (Griggio et al., 2010). In urban areas the concentration of the traffic/industrial dust is higher and some studies show that there is an accumulation of dust particles on the feathers of birds (e.g. Nam et al. 2004).

Considering that this is the present state of the knowledge in this field we decided to focus on the issue of dust influence on plumage coloration to investigate further consequences in male attractiveness and mate choice.

2. *What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?*

The research project includes three studies.

I STUDY

The first study aims to verify the hypothesis that atmospheric pollution affects color plumage in blue tits. It will take place in March 2011.

Two groups, 20 individuals each, will be captured using mistnet in three different urban areas (Cracow, Warsaw, Poznan) and other two groups (20 individuals each) will be captured in three different woodland areas (Puszcza Notecka, Puszcza Kozienicka, Puszcza Piska). These areas were chosen randomly and we assume that they are representative of these types of areas in Poland.

Measurement of the reflectance of the crown feathers will be done with OceanOptics, Inc. USB 2000 spectrometer and the deuterium-halogen light source (DH-2000) in the field. For each individual 5 spectral measurements will be taken from the region of the crown and the breast. Then measurements will be averaged for each region in each individual. The color will be quantified using descriptors of reflectance spectra such as brightness, chroma and hue (Griggio et al., 2010).

Altogether we will have 60 measurements for the blue tit from the woodland and 60 for the birds from urban populations.

Before releasing the birds their body mass and tarsus will be measured.

The statistical analysis will be GLMM (general linear mixed model) with sex and area type (urban/woodland) as fixed factors and with populations nested in the area type as a random factor. Body mass and tarsus will be added to the model as a covariate.

The predicted outcome is that birds from the polluted area have less colorful plumage than birds from the woodlands.

II STUDY

The second study aims to verify the hypothesis that males from polluted areas are less attractive than males from woodlands.

40 males and 20 females will be caught with mistnets: half of them from Cracow and the other part from Puszcza Niepolomicka, a woodland near Cracow. The birds will be transported in cages by car to the Institute of Environmental Sciences (Jagiellonian University) where the observations will take place.

20 dyad of male will be formed and in each dyad one male will be from the urban population and the second male from woodland population. 10 females from each kind of area will be chosen. Each female will be randomly matched to one male dyad. All choice trials will be independent and each group (female+dyad) will be used only once.

The experiment will be conducted in outdoor aviary (Fig. 1). The female will be put in the aviary 4 hours before the observation so she could habituate. Each observation will last 3 hours and it will be recorded by a video camera so we will be able to define the exact time that female spent near each male (Kurvers et al., 2010).

The observations will be done during the period of social pair formation of blue tit (from last week of March to the middle of April in 2011).

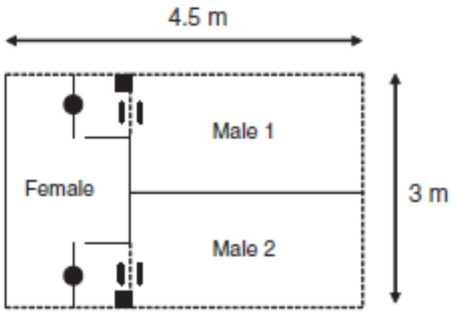


Figure 1. Schematic view of outdoor mate choice aviaries used for the experiment (Kurvers et al., 2010).

After the experiment birds will be released in the same area they come from.
In the statistical analysis we will use differences between times that female spent with male from urban area and male from woodland area. We will analyze preferences of each group of females (from urban/woodland) separately using paired t-test. Then we will compare preferences of females from both types of areas using independent sample t-test.
The predicted outcome is that females from both origins choose more often males from the woodland that from the urban area.

III STUDY

The third study is an experiment that aims to verify the hypothesis that in the polluted area males with less soiled plumage are more attractive for females. In this experiment we want to manipulate the coloration of the plumage of a group of males captured in Puszcza Niepolomicka, a woodland near Cracow, in order to imitate the condition that birds can have in the urban areas.
20 females will be taken from a city park in Cracow and 40 males from Puszcza Niepolomicka, a woodland near Cracow. All males will be soiled with dust – halt of them at a lower level and half at a higher level. All males will have a soft plastic collar that prevent them from preening but allow them to carry out their normal activities (Zampiga et al., 2004). 20 dyad of male will be formed of one male with more soiled plumage and one with less soiled plumage. Each female will be randomly matched to one male dyad. All choice trials will be independent and each group (female+dyad) will be used only once.
The experiment will be conducted in outdoor aviary (Fig. 1) at the Institute of Environmental Science, Jagiellonian University, in the same way as written above (Part 2 of the project.).
The observations will be done during the period of social pair formation of blue tit (from last week of March to the middle of April in 2012). After the experiment the birds will be released in the same area they come from.
The statistical analyses are similar as above (Part 2 of the research project). We use differences between time that female spent with male from urban area and male from woodland and analyze female preferences using paired t-test.

The prediction in this experiment is that in polluted areas females choose more often males with less soiled plumage.

3. What are the expected results of this project?

We expect that our results give us the answer to the main research problems, which have not been explored yet. The study begins new path of experiments on blue tits and give the chance to continue the investigation in the future. Equipment used for this experiment will be also useful in the future experiments.
We want to publish our results concerning each experiment separately.
The title of them could be for example:
Impact of the dust pollution on plumage coloration in blue tits,
Males from polluted areas have decreased attractiveness-the example of blue tit,
In the polluted area male with less soiled plumage are more attractive for the female

Considered journals are: Journal of Avian Biology, Proceedings of the Royal Society B: Biological Sciences and Behavioral Ecology.

We plan to present our results on the Conference of the European Ornithologists' Union. These conferences are held at different places in Europe every two years. They attract several hundred ornithologists from all over Europe and abroad. We also want to show our results on the EGI (Edward Gray Institute) Student Conference. These conferences (which started in 1947) are primarily intended as places for undergraduates, graduate students and very early career postdocs to present their work.

Literature:

Andersson S., Ornborg J., Andersson M. 1998. Ultraviolet sexual dimorphism and assortative mating in blue tit. Proc. R. Lond. B 265: 445-450
Cucco M.and Malacarne G. 1997. The effect of supplemental food on time budged and body condition in the black redstart. Ardea 85:211-221
Hunt S., Cuthill I., Bennett A.T.D.,Griffiths. 1999. Preferences for ultraviolet partners in the blue tit. Animal Behaviour 58: 809-815
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Grande JM, Negro JJ, Torres MJ (2004) The evolution of bird plumage colouration: a role for feather-degrading bacteria? Ardeola 51:375–383
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Project schedule – anticipates tasks

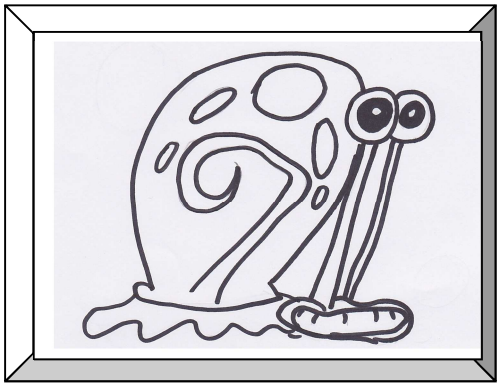
No.	**Name and description of task**	Expected completion date (mm/yy)
1	The purchase of equipment needed, preparing the experimental set	1 – 2.2011
2	Realization of experiments 1 and 2	3-5.2011
3	Data analysis - conducting statistical analysis of the date from the Experiment 1 and 2; preparation of two manuscripts	6-12.2011
4	Realization of Experiment 3	3-5.2012
5	Data analysis - conducting statistical analysis of the date from the Experiment 3; preparation of one manuscript	6-11.2012
6	Report preparation, preparation of the manuscript	11-12.2012
7	Conference presentation, participation in the conferences	10.2011-12.2012
		Total

Proposed budget

No.	Item	Funds for each budget year (zł)		
		2011	**2012**	**Total**
1	**Direct costs, including:**	39300	13300	52600
	1/ Salaries and benefits	8500	7500	16000
	2/ Equipment	24500	0	24500
	3/ Other direct costs	6300	5800	12100
2	Indirect costs	4440	3990	8430
3	Total costs (1+2)	43740	17290	**61030**

Details of direct cost items
1/Salaries and benefits
Principal investigator person-moth: 20, 12000 zł
Staff person-moths: 8, 4000zł
2/Equipment:
spectrometer – 8000 zł,
DH-2000 Deuterium Tungsten Halogen Light Sources – 9000 zł,
mistnets – 500 zł
outdoor aviary – 7000 zł
3/Other direct cost:
Food for birds – 1000 zł
Books and journals – 600 zł
Conferences attendance (plane ticket, daily allowance) – 7000 zł
Travels within the country (daily allowance) – 3500 zł

4.4 Are nanoparticles biomagnifed in terrestrial food chain in relation to particles size and soil concentration?



Authors: **Iwona Giska & Marcin Plech**

4.4.1 FIRST VERSION OF THE PROJECT

Project summary:

Nanotechnology constitutes a new, rapidly developing industrial sector. As more and more nanoparticles (NPs) are used in our daily life, they are released into the natural environment in increasing amounts. While toxicity of NPs to individual species is considerably well known, there is a lack of information on their actual impact on functioning of whole ecosystems. It has been suggested, that some NPs can be bioaccumulated and furthermore biomagnified in trophic chains, however no convincing data supporting this hypothesis has been produced to date. The main objective of proposed study is to test biomagnification of NPs on a model of simple food chain consisting of terrestrial plant species *Sinapis alba* as a producer and a garden snail *Helix aspersa* as a primary consumer. *Sinapis alba* will be grown on artificial soil with the addition of nano-Al_2O_3, nano-Ag and nano-ZnO diversified in size and in various concentrations. Nextly its leaves will be fed to snails. Biomagnification occurrence will be verified on the basis of chemical analysis of NPs concentrations in snail and plant tissues. Biomagnification factor can possibly be dependent on NPs size and their soil concentration. The results of this project can contribute to the improvement of NPs Environmental Risk Assessment.

Project description, methodology and expected results

1*. What problem is being proposed and why?*

The proposed project aims at testing if metal nanoparticles: Ag, ZnO, TiO_2, are biomagnified in a terrestrial food chain. It is known that nanoparticles are accumulated by some plant and animal species and show toxic effect on them. As reliable Environmental Risk Assessment requires information not only about chemicals' toxicity and concentration in the environment, there is a strong need of studying nanoparticles behavior in food chain that can affect whole ecosystem functioning. This kind of research in terrestrial environment has not yet been conducted.

The study model includes artificial soil spiked at different concentrations with NPs differing in size, white mustard plant *(Sinapis alba)* cultivated on this soil and garden snail *(Helix aspersa)* that is fed with the plant's leaves. *Sinapis alba* plant can accumulate high amounts of heavy metals. *Helix aspersa* snail is commonly used in laboratory ecotoxicological experiments and it plays significant role in chemicals transfer between producers and predators.

Concentration of NPs in plants and snails tissues will be measured using ICP-MS analysis. On the basis of obtained results biomagnification factor (BMF) will be calculated as the ratio of nanoparticles concentration in snail divided by their concentration in plants. It will answer the main research question if nanoparticles are biomagnified in food chain, as it is assumed that biomagnification occurs when BMF>1. The influence of particles size and their soil content on the biomagnification will be determined. Additionally, the impact of NPs size and their soil content on accumulation by plants will be checked as well. To prove that metals are present in plant and snail tissues as nanoparticles not ions form, electron transmission microscopy will be used.

Moreover, to test if there are differences in food chain behavior between NPs and their bulk equivalents, experiments will be conducted also using soil spiked with Ag, Ti, Zn salts. It will let us know if nanoparticles can affect organisms differently than metals ion forms.

2. What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?

Nanoparticles production is a new, rapidly developing branch of technology and it is beginning to exert effects on various industrial sectors (Nowack & Buchelli, 2007). Huge potential of this technology and its broad applicability brought about worldwide increase in investment in nanotechnology research and development. It is predicted that in the coming decade the production of nanomaterials is going to grow 30 times (Ma et al. 2010, Mueller & Nowack 2008, Nowack & Buchelli 2007). NPs of metals tested in proposed study are used in production of: medical and comestible articles (nano-Ag, nano-ZnO), paints, coatings (nano-Al_2O_3) and sunscreen comsetics (nano- Al_2O_3, nano-ZnO) (Mueller & Nowack 2008).

As the consumption of nanomaterials grows, increasing release of NPs to the environment is expected, posing further threats to the environment. It has already been observed that NPs are accumulated by different species (vertebrates, invertebrates, plants) and

have toxic effects on them (Oberdörster et al. 2005, Holbrook 2008). It has been suggested that some NPs undergo biomagnification in food chains (Nowack & Buchelli 2007). According to Conell (1989) biomagnification is the transfer of chemicals exclusively from food to an organism resulting in higher concentration in the organism than in the source. In mammals toxic effect was observed on an organism level (inflammation, fibrosis), and on cellular level (increased reactive oxygen species production and antioxidant activities) (Oberdörster et al. 2005). Invertebrate species, like *Tetrahymena pyriforrmis* (ciliates) can uptake both: metal ions and NPs from the environment (Holbrook 2008). Helix aspersa snails have been often used in laboratory ecotoxicological experiments and accumulation of some heavy metals in their tissues was observed (Laskowski & Hopkin, 1991, Scheifler et al. 2002), causing impairments in growth and decrease in reproduction success. Therefore there is a risk they might accumulate metal NPs as well. In plants many physiological processes can be impaired, including growth (biomass reduction), root elongation, its morphology (deformation of root cap) and seeds germination (Lin and Xing., 2008). On the other hand some NPs have been shown to have positive effects on plants growth, e.g. nano- TiO2 was reported to improve photosynthesis and growth of spinach (Yang et al. 2006).

Although the NPs effects on particular organisms have already been described, very little is known about their behavior in more complexed systems, such as food chains. Studies of such kind could answer question of the actual adverse impact of nanomaterials on the level of ecosystem. Only two studies

The only experiments of NPs trophic transfer conducted so far were very simplistic and concerned exclusively aquatic organisms (Holbrook 2008, Nowack & Buchelli 2007). First of mentioned studies employed plankton and zooplankton (Holbrook 2008). The second one concerned transfer between zooplankton and fish (Zhu et al. 2010). Both studies confirm that bioaccumulation occurs. Although biomagnification was not observed in aquatic invertebrates, it can not be ruled out for terrestrial species (Holbrook 2008. Zhou et al. 2010). Moreover different kinds of nanoparticles than used in the above mentioned experiments might behave differently.

Therefore testing if nanoparticles of Ag, ZnO, Al_2O_3, are biomagnified in a terrestrial food chain is crucial for profound understanding of NPs ecotoxicological importance. Demonstration whether observed processes are correlated with the soil content of NPs and their size is also relevant.

3.What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?

The research will be conducted at Jagiellonian University, Institute of Environmental Sciences, at Ecotoxicology and Stress Ecology Department where climate chambers for plants and animals growth are available. Realization of the project will take three years.

Standard artificial OECD soil will be prepared and spiked with different size ($\leq$ 20nm, $\leq$50nm, $\leq$100nm) nanoparticles (Ag, Al_2O_3,ZnO) and their metal equivalent bulk salts ($AgNO_3$, $Al(NO_3)_3$, $ZnSO_4$) to reach four different concentrations of each compound (1, 10, 100, 1000 mg/kg). Chemicals will be added to soil as water solutions (bulk salts) or suspensions (nanoparticles suspensions will be sonicated). Soil without addition any toxicants will be used as a control.

Sinapis alba plants will be cultivated in containers with different soil types (different NPs size and concentration). After four weeks of cultivation, plants will be cut, divided into roots, stems and leaves. Fresh leaves will be used as a food source for snails. Roots and stems will be freeze-dried and stored till chemical analysis. Some leaves will be frozen and stored for TEM analysis.

Adult *Helix aspersa* snails with similar mass will be held in plastic boxes, one snail in each box. Five boxes per each NPs size - concentration combination will be prepared. The experiment will last eight weeks. Leaves will be given to snails twice a week, the same mass of leaves to each snail. At the same time boxes will be cleaned. Remains of not eaten leaves will be collected, dried and weighed. On the basis of this mass differences in consumption rate between snails from different soil treatments will be assessed. At the end of the experiment snails will be starved, killed by deep freezing and divided into soft tissues and shell. One snail from each treatment will be frozen for later TEM analysis.

Metal content in all samples, including soil, plants and snails, will be analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) that was already used for nanoparticles analysis *(Zhu et al. 2010).* Before the ICP-MS analysis, samples will be digested in concentrated acids. Plants and snails tissues for TEM analysis will be fixed, dehydrated and polymerized. Micrographs of ultrathin sections will be taken. TEM analysis has already been successfully used for visualization of NPs in plant and animals *(Corredor et al. 2009).*

Using obtained data about metals concentrations in plants and snails, biomagnification factor (BMF) will be calculated by dividing concentration in snail by concentration in plants *(Conell 1989).* Calculations will be made for each nanoparticles size – concentration combination and for bulk salts. There will be an evidence of biomagnification if BMF>1. Relation between BMF values and NPs size and their soil concentration will be analyzed using two-way ANOVA analysis. The same analysis will be conducted for relation between nanoparticles plant accumulation and their soil content.

The investigators of the project have practical experience in most of the methods and techniques enlisted above, with the exception of TEM usage.

Literature:

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Project schedule – anticipated tasks

No.	Name and description of task	Expected completion date (mm/yy)	Expected cost (zł)
1	ICP-MS and TEM analysis of samples with Ag	January 2012	24000
2	ICP-MS and TEM analysis of samples with Zn/ZnO	January 2013	18000
3	ICP-MS and TEM analysis of samples with Al/Al2O3	January 2014	18000
		Total	

Proposed budget

No.	Item	Funds for each budget year (zł)			
		2011	2012	2013	Total
1	Direct costs, including:	134000	78000	78000	290000
	1/ Salaries and benefits	60000	60000	60000	180000
	2/ Equipment	50000			
	3/ Other direct costs	24000	18000	18000	60000
2	Indirect costs	25200	23400	23400	72000
3	Total costs (1+2)	159200	101400	101400	362000

4.4.2 REVEWS

- Justyna Wolińska

  Review of project: *"Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and soil concentration?"*

Summary evaluation:

The project examines one of the emerging problems in ecotoxicology: if nanoparticles (NPs) are being biomagnified in the food chain. Although the biomagnification of NPs has been already studied in aquatic ecosystems, this might be the first study, to the best knowledge of the projects' authors, which investigates terrestrial food webs.

The proposed topic is very innovative and, if the project was properly carried out, its outcome might significantly advance our knowledge about the environmental risks concerning rapid increase in production of nanoparticles which are then being released to natural environment. The proposed methodology seems to be appropriate as it is mainly based on a modification of previously established techniques. My biggest concern, however, is about the experimental design itself. I am afraid the authors of the project did not spend enough time / effort on that part of the grant proposal, and did not take into account some important principles about properly designed experimental work. If the experiment is poorly designed, for example if it involves too few replicates, this drastically reduces a chance of this otherwise innovative and important project, to be successful at the end. Thus, I can not recommend funding of this project in its presented now. Instead, I recommend a resubmission. In the revised version, however, the authors should provide detailed experimental design, including some thoughts about the statistical power of their experiment, and improving the description of statistical methods they plan to apply in order to analyse the data.

Details are given in my evaluation below and should be considered by the applicants.

Detailed evaluation:

Project title

"in relation to (…) soil concentration" - I think this should be replaced with: "in relation to their concentration in the soil". Similar wording (i.e. "soil concentration") appears several times in other parts of the proposal and should be corrected accordingly.

Project summary

This is one of the best parts of that grant proposal. Well done!

What problems are being proposed and why?

I don't understand why the list of nanoparticles to be tested does not correspond with the list given in the abstract (i.e. there is no TiO_2 in the abstract).

Replace "soil content" with "concentration in the soil" (see my comment above)

"(…) tissues as nanoparticles not ions form" – I don't understand. What is that supposed to mean?

What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?

"Invertebrate species, like *Tetrahymena pyriforrmis* (ciliates)" – ciliated do not belong to invertebrates.

"Helix aspersa snails" – species names should be written in italics

"In plants many physiological processes can be impaired" – by what? Unclear.

"Only two studies" – ?? this sentence does not have an end. Delete and join with the following paragraph.

"(…) plankton and zooplankton" - ? I guess the authors meant "phytoplankton and zooplankton"

"Both studies confirm that bioaccumulation occurs. Although biomagnification was not observed in aquatic invertebrates, it can not be ruled out for terrestrial species (Holbrook 2008. Zhou et al. 2010)". – But these two sentences contradict each other! In any case, the references should be placed before "in can not be ruled out (…)", because they refer to the reported findings, and not to the speculations, I guess.

"soil content of NPs" – replace with "concentration of NPs in the soil".

"is also relevant" – relevant to what? Explain.

What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?

"to soil as water solutions (…)" – replace with "to soil either as water solutions"

"*Sinapis alba* plants will be cultivated in containers with different soil types (different NPs size and concentration)." I though the plants will also be cultivated on soil with different bulk salts concentration as well as in a control soil treatment. This is not clear any more, as it is written here. In addition, some important information is missing: 1) where these plants will be cultivated? (greenhouse, climate chambers?), 2) under what conditions? (temperature, light, humidity), 3) what kind of "plants" will be used? Starting from seeds / seedlings? 4) How many individual plants per container? How many containers per treatment?

"Roots and stems will be freeze-dried and stored till chemical analysis. Some leaves will be frozen and stored for TEM analysis". I don't understand the reasoning here. Why should you perform the chemical analysis on roots and steams, whereas only leaves are being used for feeding? Thus, leaves should be analyzed instead.

Where those snails will be taken from? Field collections or laboratory cultures? If field collections, maybe those snails are already "contaminated" by NPs or, alternatively – pre-adapted? Shouldn't you control not only for a mass but also for age of the snails? Don't we expect differences in feeding rates and, maybe, in biomagnification rates, between young and old individuals?

"Adult *Helix aspersa* snails with similar mass will be held in plastic boxes, one snail in each box." Where these snails will be kept (in the climate chambers)? Under what conditions (i.e. temperature, light, humidity)? How big the boxes will be?
"Five boxes per each NPs size - concentration combination will be prepared." – how about bulk salts and control? What will be the total number of experimental units? What I calculated it's 200. Is that right? (3 NPs types x 3 size ranges x 4 concentrations x 5 replicates) + (3 bulk salts x 4 concentrations x 5 replicates) + (1 control x 5 replicates). This kind of information strictly belongs to the experimental design; so it certainly needs to be provided in a grant proposal.
"At the end of the experiment snails will be starved" – why? For how long?
"One snail from each treatment will be frozen for later TEM analysis." – does that mean that 40 snails in total will be analysed by TEM (see my calculations above: 27 NPs treatments, 12 bulk salts treatments plus 1 control). You also wrote that plant tissues will be analysed as well. Do you thus plan to analyse 40 plant samples as well? This might be pretty expensive (i.e. 40 + 40 samples). Is it really needed? Since you only want to prove that "metals are present in plant and snail tissues as nanoparticles", maybe it is enough to test some but not all conditions? (e.g. only the snails/plants from the highest concentration treatment).
"Relation between BMF values and NPs size and their soil concentration will be analyzed using two-way ANOVA analysis." I would say that this is a three-way ANOVA instead. There are three different treatment types in this experiment: NPs type, NPs size and NPs concentration. If you have only 4 replicates in such a complex ANOVA test, the statistical power might be too low. Moreover, what happens if some of the snails die during the course of the experiment?
Will "bulk salts" also be analyzed statistically?
It is unclear if and how the control treatment will be used in the analyses.
"The investigators of the project have practical experience in most of the methods and techniques enlisted above, with the exception of TEM usage." – so how is that problem going to be solved? You have to write here, where the TEM analyses will be conducted, and who is going to help you with that. Are there any external collaborators on that project?
References
Species names should be italicized.
Project schedule
When the experiment is going to be conducted?
Will that really take ~2.5 years to analyze the samples from a single experiment?
When the data analyses will be performed? Publication be written? Is there any time needed for the establishment of the experimental/measurement methods? (All these steps should be taken into account in the time schedule).
Proposed budget
What kind of equipment needs to be bought?
Provide details of direct cost items.
Final remarks
I noticed the following section is missing: "What are the expected results of this project ("know-how", patents, methods, equipment), and how will they be disseminated (publications, conference presentations, PhD theses)?" It is very important to provide the expected outcome of the project, especially in terms of the number of publication.

- Maciej Bonk

Review of project: *"Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and soil concentration?"*

Authors would like to study potentially very important issue in ecotoxicology and environmental protection. I concern these research as very innovative.
Unfortunately, several problem arise while planning such experiments. One is the decision what value tells us about biomagnification occurrence. Actually, neither hypothesis (both biological and statistical) nor prediction is not presented.

In general, text is confusing due to inconsequences and mixing keyword meaning or use these words in impropriate places. I suggest improvement in statistical analysis and review one more time the methodology presented.
I give to it note: 4 (from 1 to 10).
No **keywords** are attached to the proposal!!!
Proposal is not complete!!!
General remark: it is much better to write that there is no study on biomagniffication rather than cite papers about bioaccumulation instead!!!
6: the title. Never use **centered** format if editor doesn't ask about it!
16: **"**…more and more……." sounds like tv program about global ecological disaster**.** Be distatnced to your communication!
17: not only natural!
19: "…of information on their actual impact on functioning of **whole** ecosystems…" don't use such strong expressions. Especially when you study only the food chain, and only rat of that chain.
20: I think that you should not use "bioaccumulation" word. To my knowledge, if the biomagnification occurs it may be temporary (in fact higher level of substances may occur in higher than lower row of food chain only for a moment) and thus, not related to bioaccumulation which is at leas more permanent
37: here again, as 20. Bioaccumulation doesn't implicate biomagnification.
38: you should at least put in bracket citation where I could find what the Environmental Risk Assessment is.
50: BMF, you should mention at least one paper about accuracy of this method in biomagnifications assessment.
55: ok, I understand that other method than microscopy wont differ between ions and NPs. So how are you going to separate ions concentration from NPs concentration. Only one way to avoid this problem is to measure initial concentration of ions in plant and snail tissue before NPs treatment. It is quite easy in plant, but if you need a lot tissue of snail, and probably you will need that, you will kill your snails before the experiment - nonsense! However, you may breed snails of one cohort in standard condition. Several or better, more snails should be taken to ions measurement before the experiment. You have to assume that sample of snails for only ions measurement is representative for all population you have. But the experiment will be less elegance.
56: a little bit confusing.
79: ciliates are not Invertebrates, however it is not systematic unit it is good to use "invertebrates" for animals, ciliates are simply ciliates.
80-86: I am not concerned whether ions accumulation implicate NPs accumulation, moreover, you still confuse these two words with different meaning.
89: but writing in that way you suggest that there are some onformation. YOU SHOULD CITE IT!!!

90: too advanced conclusion – you will only asses whether there is bioaccumulation not impact of bioaccumulation on snails. Off course, it is great idea to find out if impact occurs, but these are plans for further studies.
96: so who is the author of second experiment; Nowack & Buchelli 2007 or Zhu et al. 2010???
97: see 80-86, 37 and "general remark" at the top.
111: where there any pilot study or literature data suggesting concentration proposed?
112: so as a water solution or suspensions? Maybe both, but when, which and why?
122: How are you going to feed snails for two weeks as plant will be cut at once, you should mention that they will be stored and in what condition. Moreover, does storing affect NPs concentration???
138- : what would be the model like? What are your factors and null hypothesis? What is the hypothesis that predict whether the bioaccumulation occurs???

- Agata Rudolf
  Review of project: *"Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and soil concentration?"*

The proposal is address to the ambitious topic and dedicated to important challenges at the frontiers of its field. The project may be scientifically significant.
However, in the "objectives" it is not clearly shown what is going to be done. From the start the reader get too much information about the methods, and becouse of that the full context and the aim of the project is becoming less visible.

Next in verse 80- 82 the authors said that it was observed that accumulation of some heavy metals in Helix aspersa snails causing impairments in growth and decrease in reproduction success. I have a suggestion to check that issue too, in the same project, as another point of view on the problem. However, I am wonderring if that issue could perturbate the results becouse of decrease of snails weight compared with the nanoparticles content in the snail tissues. The same with increasing effect of TiO2 on plants growth. The effict was found in spinach, but it could be, that this effect occures also in white mustard plant.
In verse 90 the sentence seems to be pull of, it doesn't have the end...
Next thing is that the authors are not saying from which part of the snail the samples would bee taken. That could be important, becouse some more nanoparticles could be accumulated for example in the liver of the snail.
The experiment shedule is in principle clear and more than less visible, just by reading the methodology.

There is no expected results of the project. Do the authors want to disseminate results of they work? Where they would do that? In some publications? Maybe they want to go for some conference presentations, or use the results as their PhD theses?
I guess the budget is also not finished, and also the total cost of project schedule and the final proposed budget is not equal.

The title: Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and soil concentration- suggest that authors want to examine the soil concentration, not nanoparticles concentration. I think they should say: "Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and concentration in soil."

What is improrant the project may have potential for significant outcomes. Unfortunately the authors didn't write about those potential of outcomes... However, the shedule is logical and realistic. The project seems to be reasoning and methodologically correct.

- Katarzyna Wężowicz
  Review of project: *"Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and soil concentration?"*

Project is innovative due to touching upon a new question of influencing nanoparticles on functioning of the trophic chains. The nanoparticles production increases rapidly what in connection with its broad applicability posing threats to the environment. That is why the translocation and accumulation of nanoparticles in the environment are so important. The aspect of biomagnification nanoparticles in food chain have not been raised in detail yet, which highlights the importance of this experiment.

Project is written logically. The objectives and hypothesis are clearly presented. The choice of the research methods is appropriate for the project. The language is correct and concise. The abstract precise summarize the contents.

The weak aspect of the project is a lack of information about amount of soil controls (116).Unclear is also conditions of plant and snail raising. What is more, there is no information about way of dissemination of the project results. In addition to this, the budget is unclear and incorrectly estimated. Moreover, I advise to consider if there is a necessity to conduct research for 3 years. Unclear is also sentence, which begins at line (93). Incorrect is beginning the sentence with Latin species name (24,118). Furthermore, using the second time the Latin species name (45) it is allowed to write the Latin generic name in a shorter form.

- Giulia Casasole
  Review of project: *"Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and soil concentration?"*

I have really appreciated this grant proposal because I think that is an innovative project that can shed some light on a process that may also affect through the food chain human beings life.

I consider the title adequate to the contents and the language correct and clear.

I think that the abstracts summarize the content, the background is well presented and shows the need to investigate this research topic because it hasn't been studied for terrestrial species and the objectives and the hypothesis are clear and testable.

Even if I think that the project has potential for significant outcomes I couldn't find in the grant proposal any information about that. I don't know if the applicants have the purpose to publish their results or not in International papers (and in details which), to present them in International conferences, etc.

I would also add some observations about some weaknesses that I have found in the grant proposal.

-In the objectives (line 37) it is written that the proposed project aims at testing the following metal nanoparticles: Ag, ZnO, TiO_2, but in the abstract (line 25) and then in the methodology (line 113) it is told that is used Al203 instead of TiO2.

-In the methodology it isn't clear if the metal bulk salts ($AgNO_3$, $Al(NO_3)_3$, $ZnSO_4$) are added in the containers in which there are also the equivalent nanoparticles or in different ones (lines 112-116) and they aren't mentioned in lines 118-119 where it is written about the different soil types in which Sinapis alba will be cultivated.

(-In the part concerning the present state of the knowledge in the field in the line 94 there is an interrupted sentence.)

4.4.3 FINAL VERSION OF THE PROJECT

Project title:

Are nanoparticles biomagnified in terrestrial food chain in relation to particles size and their concentration in the soil?

Applicants:

Iwona Giska, Marcin Plech

Project summary

Nanotechnology constitutes a new, rapidly developing industrial sector. As more and more nanoparticles (NPs) are used in our daily life, they are released into the environment in increasing amounts. While toxicity of NPs to individual species is considerably well known, there is a lack of information on their actual impact on functioning of ecosystems. It has been suggested, that some NPs can be bioaccumulated and furthermore biomagnified in trophic chains, however no convincing data supporting this hypothesis has been produced to date. The main objective of proposed study is to test biomagnification of NPs on a model of simple food chain consisting of terrestrial plant species *S. alba* as a producer and a garden snail *Helix aspersa* as a primary consumer. Plant *S. alba* will be grown on artificial soil with the addition of nano-Ag, nano-Al_2O_3 and nano-ZnO diversified in size and in various concentrations. Nextly its leaves will be fed to snails. Biomagnification occurrence will be verified on the basis of chemical analysis of NPs concentrations in snail and plant tissues. Biomagnification factor can possibly be dependent on NPs size and their concentration in soil. The results of this project can contribute to the improvement of Environmental Risk Assessment of NPs.

Keywords:
nanoparticles, biomagnification, food chain, *Helix aspersa*, *Sinapis alba*

Project description, methodology and expected results

1. What problem is being proposed and why?

The proposed project aims at testing if metal nanoparticles: Ag, Al_2O_3, ZnO are biomagnified in a terrestrial food chain. It is known that nanoparticles are accumulated by some plant and animal species and show toxic effect on them. As reliable Environmental Risk Assessment (*Directive 93/67/EEC*) requires information not only about chemicals' toxicity and concentration in the environment, there is a strong need of studying nanoparticles behavior in food chain that can affect whole ecosystem functioning. This kind of research in terrestrial environment has not yet been conducted.

The study model includes artificial soil spiked at different concentrations with NPs differing in size, *Sinapis alba* plant cultivated on this soil and snail *Helix aspersa* that is fed

with the plant's leaves. *S. alba* plant can accumulate high amounts of heavy metals. *H. aspersa* snail is commonly used in laboratory ecotoxicological experiments and it plays significant role in chemicals transfer between producers and predators.

Concentration of NPs in plants and snails tissues will be measured using ICP-MS analysis. On the basis of obtained results biomagnification factor (BMF) will be calculated as the ratio of nanoparticles concentration in snail divided by their concentration in plants. It will answer the main research question if nanoparticles are biomagnified in food chain, as it is assumed that biomagnification occurs when BMF>1. The influence of particles size and their concentration in soil on the biomagnification will be determined. It is predicted that BMF values will be higher in case of smaller particles size and their higher concentration in the soil. It is also assumed that biomagnification will be different depending on NPs type (Ag-NPs, Al_2O_3-NPs or ZnO-NPs). Additionally, the impact of NPs size and their concentration in soil on accumulation of NPs by plants will be checked as well. To prove that NPs are present in plant and snail tissues transmission electron microscopy (TEM) will be used.

Moreover, to test if there are differences in food chain behavior between NPs and their bulk equivalents, experiments will be conducted also using soil spiked with Ag, Al, Zn salts. It is expected that BMF will vary between NPs and their salts equivalents. The results will let us know if nanoparticles can affect organisms differently than ion forms of metals.

2. What is the present state of knowledge in the field, and to what extent does this project verify it? How will the project advance discovery and understanding in its field or across fields? Is this a new or a continued problem?

Nanoparticles production is a new, rapidly developing branch of technology and it is beginning to exert effects on various industrial sectors (Nowack & Buchelli, 2007). Huge potential of this technology and its broad applicability brought about worldwide increase in investment in nanotechnology research and development. It is predicted that in the coming decade the production of nanomaterials is going to grow 30 times (Ma et al. 2010, Mueller & Nowack 2008, Nowack & Buchelli 2007). NPs of metals tested in proposed study are used in production of: medical and comestible articles (nano-Ag, nano-ZnO), paints, coatings (nano-Al_2O_3) and sunscreen comsetics (nano- Al_2O_3, nano-ZnO) (Mueller & Nowack 2008).

As the consumption of nanomaterials grows, increasing release of NPs to the environment is expected, posing further threats to the environment. It has already been observed that NPs are accumulated by different species (vertebrates, invertebrates, plants) and have toxic effects on them (Oberdörster et al. 2005, Holbrook 2008). It has been suggested that some NPs undergo biomagnification in food chains (Nowack & Buchelli 2007). According to Conell (1989) biomagnification is the transfer of chemicals exclusively from food to an organism resulting in higher concentration in the organism than in the source. In mammals toxic effect was observed on an organism level (inflammation, fibrosis), and on cellular level (increased reactive oxygen species production and antioxidant activities) (Oberdörster et al. 2005). Species like *Tetrahymena pyriforrmis* (ciliates) can uptake both: metal ions and NPs from the environment (Holbrook 2008). *H.aspersa* snails have been often used in laboratory ecotoxicological experiments and accumulation of some heavy metals in their tissues was observed (Laskowski & Hopkin, 1991, Scheifler et al. 2002), causing impairments in growth and decrease in reproduction success. Therefore there is a risk they

might accumulate metal NPs as well. In plants many physiological processes, including growth (biomass reduction), root elongation, morphology (deformation of root cap) and seeds germination, can be impaired by the presence of NPs (Lin and Xing., 2008). On the other hand some NPs have been shown to have positive effects on plants growth, e.g. nano-TiO_2 was reported to improve photosynthesis and growth of spinach (Yang et al. 2006).

Although the NPs effects on particular organisms have already been described, very little is known about their behavior in more complexed systems, such as food chains. Studies of such kind could answer question of the actual adverse impact of nanomaterials on the level of ecosystem.

The only experiments of NPs trophic transfer conducted so far were very simplistic and concerned exclusively aquatic organisms (Holbrook 2008, Zhu et al. 2010). First of mentioned studies employed phytoplankton and zooplankton (Holbrook 2008). The second one concerned transfer between zooplankton and fish (Zhu et al. 2010). Both studies confirm that uptake of NPs occurs but biomagnification was not observed in aquatic invertebrates (Holbrook 2008., Zhou et al. 2010). Nevertheless a different scenario for terrestrial species cannot be ruled out. Moreover other kinds of nanoparticles than used in the above mentioned experiments might behave differently.

Therefore testing if nanoparticles of Ag, ZnO, Al_2O_3, are biomagnified in a terrestrial food chain is crucial for profound understanding of NPs ecotoxicological importance. Demonstration whether observed processes are correlated with the concentration of NPs in the soil and their size is also relevant for the description of NPs fate in the environment.

3.What is the proposed methodology? How will it solve the problem? What equipment will be used? Does the applicant have the required equipment skills and access?

The research will be conducted at Jagiellonian University, Institute of Environmental Sciences, at Ecotoxicology and Stress Ecology Department, where climate chambers for plants and animals growth are available. Realization of the project will take three years.

Standard artificial OECD soil will be prepared. First part of the soil will be spiked with NPs (Ag, Al_2O_3,ZnO) of different size ($\leq$ 20nm, $\leq$50nm, $\leq$100nm) at different concentrations of each compound (1, 10, 100, 1000 mg/kg). Second part of the soil will be spiked with bulk salts containing metal equivalent of NPs ($AgNO_3$, $Al(NO_3)_3$, $ZnSO_4$) at two concentrations (10, 1000mg/kg). Chemicals will be added to soil as water solutions (bulk salts) or suspensions (nanoparticles). Suspensions will be sonicated in order to destroy aggregates of NPs. Soil without addition of any toxicants will be used as a control.

S. alba plants will be cultivated in containers containing either soil with NPs (different NPs diversified in size and concentration) or bulk salts, or control soil. Cultivation (from seeds), will be performed in phytotron at constant light, temperature and humidity. One container for each soil treatment will be prepared. After four weeks plants will be cut. Fresh leaves will be used as food for snails. Some leaves will be frozen and stored for TEM analysis.

Adult *H. aspersa* snails will be bought. Snails with similar mass will be chosen for the experiment. They will be weighed at the beginning and at the end of the experiment to check if their mass does not change during the execution of the experiment. Snails will be held in

plastic boxes, ten snails in each box. One box for each soil type will be prepared, including all NPs size-concentration combinations, bulk salts and control. Boxes will be kept in climate chamber at constant temperature and humidity. The experiment will last eight weeks. Leaves will be given to snails twice a week, the same mass of leaves to each box. At the same time boxes will be cleaned. Remains of not eaten leaves will be collected, dried and weighed. On the basis of this mass differences in consumption rate between groups of snails from different soil treatments will be assessed. At the end of the experiment snails will be starved for 48 hours to let them empty their guts. They will be killed by deep freezing and divided into soft tissues and shell. One snail from each treatment will be frozen and stored for TEM analysis.

Metal concentration in all samples, including soil, plants (leaves) and snails (divided into soft body and shell), will be analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) that was already used for nanoparticles analysis *(Zhu et al. 2010).* Before the ICP-MS analysis, samples will be dried, homogenized and digested in concentrated acids. Digestion will be done at Ecotoxicology Department, the Institute of Environmental Sciences and special digestion oven will be bought for this purpose. For ICP-MS analysis samples will be sent to Chemistry Department of Warsaw University.

Using obtained data about metals concentrations in plants and snails, biomagnification factor (BMF) will be calculated by dividing concentration in snail by concentration in plants *(Conell 1989).* Calculations will be made for each nanoparticles size – concentration combination and for bulk salts. There will be an evidence of biomagnification if BMF>1.

On the basis of ICP-MS results, snails and plants (that have been frozen earlier) with the highest metals concentration will be chosen for TEM analysis that has already been successfully used for visualization of NPs in plant and animals *(Corredor et al. 2009).* Plants and snails tissues will be fixed, dehydrated and polymerized. Micrographs of ultrathin sections will be taken.

Relation between BMF values and NPs size and their concentration in soil will be analyzed using two-way ANOVA analysis (two factors - size of NPs and their concentration in the soil, dependent variable – BMF). After three years, when all experiments are finished, three-way ANOVA will be performed to check how the relation between BMF and size of NPs, NPs concentration in the soil and NPs type (Ag-NPs, Al_2O_3-NPs, ZnO-NPs) looks like (three factors: size of NPs, concentration of NPs in the soil, type of NPs, dependent variable - BMF). This final analysis will be done with the assumption that all experimental conditions during all parts of the experiment stay the same. This assumption will be checked by analysis of control samples. To check if NPs are biomagnified in different way than metals from their salts equivalents, two-way ANOVA with metal form (NPs or salt) and the concentration in the soil as factors and BMF value as dependent variable will be performed.

The investigators of the project have practical experience in most of the methods and techniques enlisted above, with the exception of TEM usage. Samples for TEM analysis will be analyzed at Microscope Laboratory, Faculty of Biology, Warsaw University.

5. What are the expected results of this project?

The results of this project will fill the gap in the knowledge about environmental fate of nanoparticles. It is necessary for high quality Environmental Risk Assessment. Findings of

this study will be disseminated in numerous ways. We will present results of the study at SETAC Europe Annual Meeting, where we will give oral presentation. We plan to publish at least three papers in such journals as Environmental Pollution, Toxicological Science, Ecotoxicology or Environmental Science & Technology.

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Project schedule – anticipated tasks

No.	Name and description of task	Expected completion date (mm/yy)	Expected cost (zł)
1	Experiment with the first metal (e.g. Ag),	VIII-IX 2011	90000
2	ICP-MS and TEM analysis of samples with Ag	XII 2011	101200
3	Experiment with the second metal (e.g.Zn/ZnO)	VII-IX 2012	40000
4	ICP-MS and TEM analysis of samples with Zn/ZnO	XII 2012	61400
5	Conference and manuscripts	V-VI 2013	15000
6	Experiment with the third metal (Al/Al_2O_3)	VIII 2013	20000
7	ICP-MS and TEM analysis of samples with Al/Al_2O_3	XI-XII 2013	56000
8	Conference, articles and report preparation	I-II 2014	10400
		Total	394000

Proposed budget

No.	Item	Funds for each budget year (zł)			
		2011	2012	2013	Total
1	Direct costs, including:	134000	78000	78000	290000
	1/ Salaries and benefits	60000	60000	60000	180000
	2/ Equipment	82000			
	3/ Other direct costs	24000	18000	18000	60000
2	Indirect costs	25200	23400	23400	72000
3	Total costs (1+2)	159200	101400	101400	394000

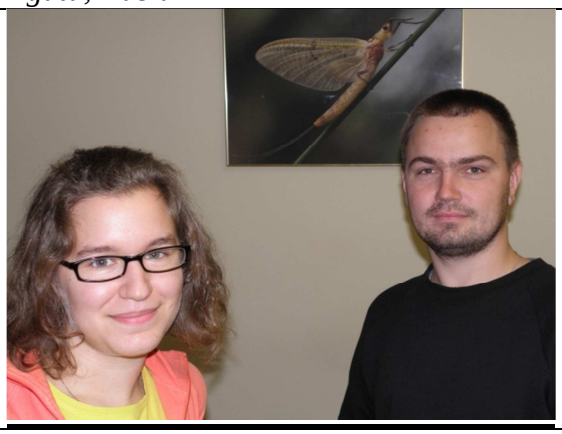
Details of direct cost items:
1/Salaries and benefits:
Principal Investigator person-months - 36
Staff person-months – 3
2/Equipment:
sonicator (2000zł, January 2011), freezedryer (30000zł, May 2011), microwave digestion oven (50000zł)

5 PHOTOS

Iwona, Marcin

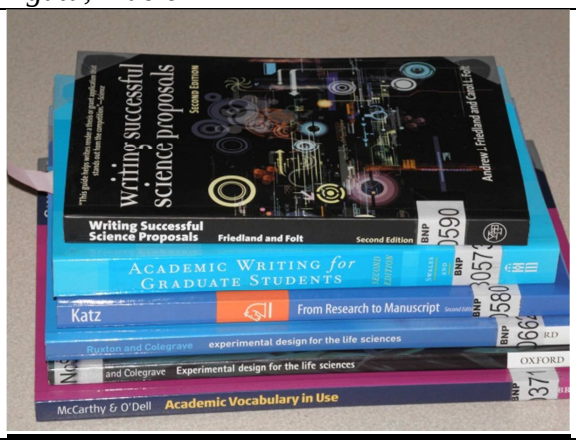
Agata, Kasia

Agata, Edyta, Gulia



Agata, Maciek

Prof.dr hab. M. Cichoń



Photos mede by: Joanna Rutkowska