WARSZTATY METODOLOGICZNE Z BIOLOGII EWOLUCYJNEJ
15-20 września 2014, Ochotnica Górna

2/2014

nejczer

Nejczer 2 (2014)
Redaktor numeru: Wioleta Kocerba-Soroka, Terézia Horváthová
Ilustracja na okładce: Terézia Horváthová
Zdjęcia: dr hab. Joanna Rutkowska

1. UCZESTNICY WARSZTATÓW

Andrzej Antoł
andrzej.antol@uj.edu.pl
Zespół Ewolucji Strategii Życiowych, Instytut Nauk o Środowisku UJ

Terézia Horváthová
terezia.horvathova@uj.edu.pl
Zespół Ewolucji Strategii Życiowych, Instytut Nauk o Środowisku UJ

Natalia Derus
natalia.derus@uj.edu.pl
Zespół Ewolucji Strategii Życiowych, Instytut Nauk o Środowisku UJ

Wioleta Kocerba-Soroka
wioleta.kocerba@uj.edu.pl
Zespół Ekologii Mikroorganizmów Wodnych, Instytut Nauk o Środowisku UJ

Aleksandra Piontek
ola.piontek@uj.edu.pl
Zespół Ochrony Przyrody, Badań łowieckich i Edukacji Środowiskowej, Instytut Nauk o Środowisku UJ

Mateusz Sobczyk
mateusz.sobczyk@uj.edu.pl
Zespół Ekologii Mikroorganizmów Wodnych, Instytut Nauk o Środowisku UJ

Wojciech Tokarz
wojciech.tokarz@uj.edu.pl
Zespół Ochrony Przyrody, Badań łowieckich i Edukacji Środowiskowej, Instytut Nauk o Środowisku UJ

Beniamin Wacławik
beniamin.waclawik@uj.edu.pl
Zakład Entomologii, Instytut Zoologii UJ

Adam Krupski
akrupski@miiz.waw.pl
Polska Akademia Nauk, Muzeum i Instytut Zoologii

2. PROWADZĄCY I RECENZENCI

Organizator i Prowadząca Warsztaty:
Dr hab. Joanna Rutkowska
joanna.rutkowska@uj.edu.pl
Zespół Ekologii Populacyjnej, Instytut Nauk o Środowisku UJ

Recenzenci zewnętrzni:
Prof. dr hab. Adam Łomnicki
adam.lomnicki@uj.edu.pl
Instytut Nauk o Środowisku UJ

Prof. dr hab. Mariusz Cichoń
mariusz.cichon@uj.edu.pl
Zespół Ekologii Populacyjnej , Instytut Nauk o Środowisku UJ

Dr Aleksandra Walczyńska
aleksandra.walczynska@uj.edu.pl
Zespół Ewolucji Strategii Życiowych, Instytut Nauk o Środowisku UJ

3. ZAPROPONOWANE TEMATY BADAŃ

1. Variation in body size of Gammarids along the stream (W KS)
2. Variability of biodiversity index of fauna along the Jaszcze stream (W KS)
3. Relationship between weight of houses of caddisfly larvae and strength of water stream (W KS)
4. Levels of mites infestation in Carabidae beetles from different habitats (disturbed and undisturbed) (ND)
5. Trade-off between stone size and number in caddislfy larvae houses (ND)
6. Insects biodiversity on grazed meadows near and far to the cows faeces (ND)
7. Induced defence strategies in the springtails (AA)
8. Thermal preference and tolerance of grasshopper in the altitude gradient (AA)
9. How speed of water current affects survival of Gammarids (AA)
10. Relationship between altitude and body size in small mammals from meadow habitats (WT)
11. Benefits of meadow grazing for richness of insects (WT)
12. Do food preferences of small insectivores mammals affect their densities in different habitats (WT)
13. Food preferences of Carabidae beetles for different meat types (AP)
14. The influence of cleaning detergents for foraging of small rodents (AP)
15. Do ants prefer the bright surface? (AP)
16. The cost of building big houses in Trichoptera (TH)
17. The effect of temperature on performance in grasshoppers (TH)
18. Do wood-feeding arthropods prefer diet enriched with fungi (TH)
19. Do stonefly nymphs are good indicators of petrol pollution in aquatic ecosystems (MS)
20. Influence of moss exposition on invertebrates species richness (MS)
21. Diurnal changes in insect activity on the meadows (MS)
22. Plant species composition and abundance of litter invertebrates (BW)
23. Biodiversity of invertebrates in relation to distance to the forest (BW)
24. Effect of stone shape on density of Trichoptera houses (BW)
25. Diurnal changes in crickets time budget (AK)
26. Biodiversity of fungi on fallen trees in different microhabitats (AK)
27. Attractiveness of cow dung for invertebrates in different microhabitats (AK)

WYBRANE TEMATY

1. Trade-off between stone size and number in caddislfy larvae houses (ND)
2. The effect of temperature on performance in grasshoppers (TH)
3. Do stonefly nymphs are good indicators of petrol pollution in aquatic ecosystems (MS)

OSTATECZNIE WYKONANE PROJEKTY

1. Optimazation of cases building strategy in caddisfly larvae (Trichoptera, Insecta)
2. The effect of the temperature on the movement performance in grasshopper species
3. Usefulness of mayflies (Ephemeroptera, Insecta) as bioindicators of fuel contamination of water

4. GRUPY I WYBRANE TEMATY

Terézia Horváthová, Wojtek Tokarz, Mateusz Sobczyk

The effect of the temperature on the movement performance in grasshopper species

Adam Krupski, Wiola Kocerba-Soroka, Natalia Derus

Optimazation of cases building strategy in caddisfly larvae (Trichoptera, Insecta)

Andrzej Antoł, Benjamin Wacławik, Ola Piontek

Usefulness of mayflies (Ephemeroptera, Insecta) as bioindicators of fuel contamination of water

5. PROJEKTY I RECENZJE

5.1. The effect of acclimation temperature on the movement performance of grasshoppers

Authors: Terézia Horváthová, Mateusz Sobczyk, Wojciech Tokarz

5.1.1. Project proposal

Summary

It is generally known that the temperature can have a strong effect on different morphological, physiological and behavioral traits in various ectothermic species. For example, cold temperature may slow down the growth, metabolic rate or physical performance. The relationship between the temperature and performance is often described by inverted U-shaped curve with performance being optimal in intermediate temperature range. The optimal temperature can differ between- as well as within-species, e.g. individuals from tropical regions generally show higher optimal temperature. Grasshoppers are the group of insect species with very wide distribution range, however little is known about the temperature range optimal for their activity. Grasshoppers are suitable model species for answering this question because their predatory defense ability depends on easily measurable trait that is jumping performance. This trait is one of the most important factors influencing the individual survival because it enhances the probability of successful predator avoidance.

In our project, we will examine the influence of temperature on jumping performance of grasshoppers to determine to which extent temperature influences the grasshoppers diurnal activity and thus the ability of predator avoidance.

Aim / Hypothesis

The main aim of the project is to examine the effect of the temperature on jumping performance in grasshoppers. We state two general hypotheses:

H1: Jumping performance of grasshoppers is enhanced at higher temperature.
H2: The jump length depends on the body mass to hind leg length ratio.

Methods

Grasshoppers of one species will be collected in meadow near the research station in Ochotnica Górna, Poland. Individuals (N=45) will be equally divided into three temperature treatment groups (10, 23 and 30°C) and kept for two hours for the acclimation. After acclimation, we will measure the jump performance by placing the grasshoppers in a tunnel of 300 cm long, 5 cm wide and 120 cm high. To stimulate the jump, grasshoppers will be gently touched with the brush. The length of first three jumps will be measured with a 30 cm ruler and the average will be calculated. Each individual will have one minute to perform the jumps. Total number of jumps during the session will be noted. All grasshoppers will be used only in one performance test. After the jump session, individuals will be weighed and the length of the hind leg (always right) will be measured. The data will be analysed with ANCOVA (hind leg length to body mass ratio as covariate).

Impact of results

Results of our project can bring answer to important questions about the temperature dependent predator avoidance success, such as: what is the optimal temperature range for grasshopper activity and to what extent temperature affects the diurnal activity of insects, through influencing their physiological activity and probability of predator avoidance.

Large differences in jumping performance between low and high environment temperatures, measured in jump length and frequency, would suggest that low temperature can limit the activity of insects not only by influencing the physiological rates, but also indirectly, by lowering the probability of predator avoidance.

5.1.2. First version of report

Summary

It is generally known that the temperature can have a strong effect on different morphological, physiological and behavioral traits in ectotherms. The optimal temperature for performance traits is assumed to match with temperatures that individuals experience in their habitat. However how is the thermal performance influenced by sudden changes of temperature is at present poorly understood. We used grasshopper species to examine how the acclimation in cold and warm temperature affects the movement performance in natural conditions (outside temperature). Our results showed that grasshoppers performed poorer if they were acclimated in cold temperature. The enhances performance at highest temperature suggest that grasshoppers are able to cope with sudden temperature increase.

Introduction

It is generally known that the temperature influences various morphological, physiological or behavioural traits in different ectothermic species, including life-history traits, metabolism, locomotion or the colour pattern (Angilletta 2009). Because ambient temperature varies in time and space at different scales, individual performance is continually challenged to maintain homeostasis. The relationship between temperature and performance has been characterized by inverted U-shaped curve, where performance gradually increases with temperature until reaching an optimal value (optimal temperature), and then steeply decreases towards higher temperatures (Castañeda, et al. 2004) Angilletta 2009. If performance is directly related to fitness, the coadaptation of optimal temperature and the temperature preferred by the individual is expected to evolve (Huey and Bennett 1987). Thus the animal performance is expected to being optimized at the temperatures that animals encounter in their microenvironment. However, the often discussed question of the global warming, which is assumed to cause rapid changes in environmental temperature, is important to consider when studying the thermal performance in ectothermic species. How ectotherms cope with sudden changes of the ambient temperature remains an open question, as well as what consequences can it bring for their occurrence in different regions of the world.

Our study was aimed to examine what is the effect of the acclimation temperature on the movement performance of grasshoppers, which is a suitable model in the studies on the factors influencing motoric abilities. Grasshoppers are a group of species which use a common mechanism of movement, which consists of sequential jumps driven by rapid energy release during the contraction of hind femur muscles. The accumulation of energy, which is essential for performing this movements, is assumed to be dependent on the ambient temperature, however this hypothesis has not been, especially in the context of rapid temperature changes in the environment. We tested how the influence of rapid change in temperature, both increase and decrease, affect the individuals movement abilities, which can reflect their success in predator avoidance as well as the effectiveness of feeding.

We tested following hypotheses: i) sudden increase of temperature affects the movement performance of grasshoppers stronger than the decrease, ii) the movement performance is dependent on the size of motoric organs to body mass ratio.

Material and Methods

During the first day of the research (17 September 2014) 27 grasshopper individuals of different species and sizes were collected from a steep mountain meadow in Ochotnica Górna, in Gorce mountains, Poland. The individuals were divided into 3 groups (n=9) and placed in controlled temperature conditions of three levels: 10°C (inside a fridge), 20° (in room temperature) and 30°C (next to a heater). Each individual was used in a physical test, which was designed to measure its movement and jumping performance.

The jump test consisted of placing the individual in the starting point on the experimental arena and stimulating it to jump. Each session was finished when the individual performed a total number of 10 jumps or when the session time exceeded 15 minutes. The arena was situated in the yard of the UJ Research Station in Ochotnica Górna, in shaded conditions at the temperature The stimulation included gentle touching the back of the insect with a small brush, by two researchers. Values measured during the test were: the length of single jumps (1), walk distance (2), total time in which the individual performed the test (finishing with the last landing) (3). For some of the individuals also the time from start to first jump was recorded. After each session the femur length and body mass of the individuals were measured, together with the temperature of the environment (arena site). Femur length to body mass ratio was calculated, in order to show the proportion of the motoric system size to the body mass which has to be moved during the jump. The ratio was used to determine if there is also an influence of individual size vs. motoric abilities (size of the most important element of the individuals' motoric system) proportion on the main tested effect, that is influence of temperature on movement performance.

After the performance test sessions, the speed results for each individual were calculated and compared between the groups. The data was analyzed with statistical tests ANOVA, GLM (temperature as fixed factor, researcher as random factor, ambient temperature during the test as covariate) in Statistica v.10 and SPSS 17.0 computer programs.

Results

The temperature of acclimation had effect on grasshoppers speed ($F_{2,22}$ = 7,367, p=0.004) (Fig.1., Tab 1.). Animals which were kept in 10°C had significantly lower speed that those from 20°C and 30°C groups. The difference between the groups kept in temperatures 20°C and 30°C did not occurred. Ambient temperature and the researcher (person who stimulated individual insects) had significant effect on speed value. Ambient temperature (covariate) ($F_{1,22}$ = 7,538, p=0.12) and stimulator (random effect)($F_{1,22}$ = 12,260, p=0.002). Grasshoppers stimulated by WT achieved higher speed than insects stimulated by TH. Correlation between body mass and femur length (as motoric organs size) was significant (R^2 =0.378 p=0.001). Body mass did not have effect on speed performance (p=0.73) so was excluded from the GLM model. Femur length to body mass ratio also did not have influence on grasshoppers speed (p=0.59).

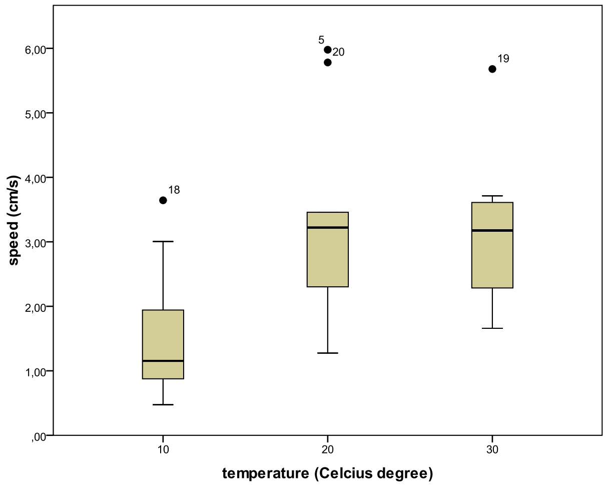


Fig.1 Results of speed value achieved by grasshopper acclimation in different temperature. Whiskers show 95% confident interval, bold horizontal lines show medians.

Table 1. GLM analysis results for speed and temperature relation – full model; dependent variable = speed (V) value, temperature = fix factor, stimulator = random factor, ambient temperature (arena) = covariate

Source	df	F	p
temperature	2	0,771	0,565
stimulator	1	0,346	0,616
ambient temperature (arena)	1	10,866	0,005
stimulator * ambient temperature (arena)	1	1,797	0,200
temperature * stimulator	2	1,693	0,217
temperature * abient temperature (arena)	2	2,042	0,164
temperature * stimulator * abient temperature	2	1,869	0,188

Table 2. GLM analysis results for speed and temperature relation – reduced model; dependent variable = speed (V) value, temperature = fix factor, stimulator = random factor, ambient temperature = covariate

Source	df	F	p
temperature	2	7,367	0,004
stimulator	1	12,260	0,002
ambient temperature (arena)	1	7,538	0,012

Discussion

Nowadays, an important challenge facing ecologists is to understand how climate change will affect the species distribution and their persistence. For example, it has been shown that dozens of lizard species will become extinct within next 50 years due to increase of ambient temperature (Sinervo et al. 2010). Our results showed that the acclimation temperature had a significant effect on the movement performance in grasshopper species. Grasshoppers acclimated at cold temperature performed poorer compared to individuals from warmer temperatures. Furthermore, grasshoppers from the highest temperature showed enhanced movement, suggesting the ability to cope with sudden temperature increase. This implies that grasshoppers might be able to successfully escape from predators and thus survive even if they encounter sudden environmental change. Grasshopper movement performance is strongly connected with survival as it enables the individual to escape from the predator. We might assume that the trait which is strongly correlated with fitness will show greater plastic response to changing conditions in order to increase the chances to survive. No difference between twenty and thirty degrees shows that the temperature range for optimal performance is wide in this group of insect species, and support the above hypothesis. The strong effect of acclimation in cold temperature is an interesting result, as insects may often encounter such temperature during the day. We would assume that ten degrees should not have such strong effect on their performance. This question thus need further investigations by conducting experiment with additional temperature treatments and larger number of species and individuals.

Importance

This small-scale preliminary study gives a contribution to the understanding of the question what is the insect individual response to rapid temperature changes, which could be caused inter alia by ongoing climate change. Results of our project can also help to answer important questions about the temperature dependent predator avoidance success, such as what is the real effect of the temperature based fluctuations in the diurnal activity period length on the survival and fitness of insects.

References

Angilletta, M. J. 2009. Thermal Adaptation: A Theoretical and Empirical Synthesis. Oxford University Press, Oxford , 304 pp.

Castaneda, L. E., M. A. Lardies and F. Bozinovic. 2004 Adaptive latitudinal shifts in the thermal physiology of a terrestrial isopod. Evolutionary Ecology Research 6(4):579-593.

Huey, R. B and A. F. Bennett. Optimal Performance Temperatures of Lizards. Evolution, 41: 1098-1 115.

Sinervo B. et al. 2010. Erosion of lizard diversity by climate change and altered thermal niches. Science, 328:894-9.

5.1.3. Reviews

Prof. dr hab. Mariusz Cichoń

The study aims at investigating an effect of temperature change on jumping speed of grashoppers. The main result is as follows: individuals kept at cold temperature shows lower jumping speed than other two groups while the other two groups did not differ. This is a nice trial study and interesting question, but the whole report is not entirely clear to me.

I hardly understand all this "climate change" contexts. What in fact this study has to do with climate change, except that both the current study and climate change deals somehow with temperature. But in a bit different context.

There are some methodological flaws. The reader is not informed for how long insects were kept in low or high temperature and if the tests were performed immediately after removing them from refrigerator. This definitely can not be named acclimation what the authors did. Acclimation means that whole physiology is affected for good. What you did is just simulation of sudden cold or warm spells. In fact, the effect of cold temperature is easy to explain if the individual were tested shortly after transferring them from the cold temperature. It just needs time to increase metabolism in ectotherms. In that sense this result may seem to be trivial.

The authors say they use different unknown species differing in body size. Do they make sure that the groups did not differ in body size and species composition? Otherwise all what they observed may reflect non-random distribution of subjects.

Quite many variables were measured but the authors use only jump speed. It is all unclear. First why they mention these other measures of performance if they do not say a word of whether they use these variables in any analyses. Second, we are left curious how the jump speed was measured if individuals did a number of jumps during the trial. Did you use a mean or use only one somehow selected jump? How did you measure jump speed since the duration of a single speed was presumably not measured? Instead total time was measured, but I guess within this time window some other behaviors occurred.

I do not understand the analyses. The method section and table captions mention GLM, but the figure presents medians suggesting non-parametric test. Did you check the variables for normality? Moreover the figure caption mention 95% confidence intervals, but why are they non-symmetrical? Did you perform some strange transformations? Not all symbols at this figure are explained. What are dots with numbers next to them? The authors seem to suggest that low temp group differs from other groups, but this is not supported by any post-hoc test. There must be a mistake reporting p-value for the covariate. If you report on correlation r2 is not a valid statistics, at it does not inform directly on correlation, also you need to report df here. It is also not enough to report p-values only even if p>0,05, you must present statistics. It is strange to see two models with interactions and without interactions. You must explain what you did and how you performed the model selection.

The discussion is far too general and do not deal directly with the results.

Dr Aleksandra Walczyńska

The report focuses on the locomotor performance of grasshoppers acclimated to low or high temperature. Additionally, the results are related to the locomotor efficiency calculated as the ratio of femur size to body mass. The results show the significant effect of temperature of acclimation and of the person stimulating the studied individuals to make the movements (jumps). All other effects (body size, ambient temperature and 'femur size/body mass') were found not significant.

The temperature-dependent performance of animals in general is a widely studied, but still poorly understood topic of research. Therefore, the presented report gives a valuable insight into this issue. The study is well planned and carefully realized. Yet, several points demand better explanation and more thorough interpretation.

Introduction:

1. The authors claim that animals were acclimated to low or high temperature. Yet, they do not report any details on for how long they were kept in such conditions, or at which part of a day, in relation to the time when they were exposed to tests. It is therefore not certain whether the rearing conditions fulfilled the assumptions of "acclimation".
2. The authors report that animals were tested at ambient conditions. The temperature fluctuated and it was (very rightly) used as a covariate in a statistical analysis. Yet, there is not a single word in the report on the real values of this factor (mean, range, SD, etc.).
3. I do not see the rationale behind the first hypothesis: why should *sudden increase of temperature affect the movement performance of grasshoppers stronger than the decrease*?

Methods:

1. It is unclear neither how many individuals were in each test group (n=9 is not in accordance with dfs given in the Results section) nor how authors dealt with the sample consisting of different species.
2. The authors do not mention how constant were the rearing conditions. My guess is that only the low temperature was more or less stable.
3. Despite the detailed description of locomotor performance metrics measured (and very nicely planned), only one measure is used in further analysis and it is not clear whether the dependent variable analysed is a compilation of all those, or only one chosen (how?).
4. In a description of statistical analyses there is a lack of information on how the 'femur size/body mass' ratio was tested.

Results:

1. Why is the significance of acclimation temperature effect referred to Table 1 (GLM model with covariate interactions) while the significance of covariate itself and of a stimulator is referred to the model after removal of these interactions? After the positive feedback on fulfilling the assumptions of using a covariate in a model (not significant interactions in a full model), all the main effects should be tested in a model with interactions removed.
2. The results of a model with body mass included should be shown and in my opinion this covariate should not be removed from the model, or at least the qualitative consequences of its removal in relation to other effects tested should be discussed.
3. The p-value of the lack of significant effect of 'femur size/body mass' ratio is not understandable, because no analysis including this factor was described.

Discussion

1. It is not understandable why the authors write that *grasshoppers from the highest temperature showed enhanced movement, suggesting the ability to cope with sudden temperature increase*, when, according to the results section (and Fig.1), there is no difference between 20°C (control?) and 30°C treatment.

2. There is a lack of discussing of the interesting and important point on the authors' finding that the personal abilities of investigators may significantly affect the tests!
3. It would be nice now to refer to the hypotheses formulated in Introduction.

Importance:

1. How could *insect individual response to rapid temperature changes* contribute in the understanding of the response to climate change? Aren't they of different scales?
2. How the speed of jumping of a grasshopper may be a measure of the *temperature dependent predator avoidance success*?

General conclusions:

Strongest point: the neat and thorough experimental plan.

Weakest point: the temptation to overblow the results of a specific, local case study. Such results are important and interesting BECAUSE they are specific and local, and the only thing they need is a wise and simple interpretation.

<u>Benjamin Wacławik</u>

Project *The effect of acclimation temperature on the movement performance of grasshoppers* concerns very interesting problem of influence of rapid temperature change on ectothermic animals. Maybe idea is not really novel- it is not difficult to find papers from 70s concerning this problem, however this problem is still not well studied.

As the object of research, authors had chosen grasshopper, which had its pros and cons. As an insect, it is really interesting object for research. Knowing insects' abundance, variety of forms and also multiple habitats occupied by them, we can see this research as a beginning of discovering processes which are very important for whole environment. On the other hand, as authors mentioned, influence of ambient temperature on grasshopper performance has been poorly studied. Maybe if we want to study performance in very specific conditions (after strong acclimation) we should first get to know in which way particular factor overall influences the performance (in other words, what is general effect on temperature on grasshopper jumps). Experiment was well planned and performed. It is really important point that authors remembered about finding and evaluating factors that could disturb the results (ambient temperature, body mass etc).

Their results are quite interesting. Although, it would be better if they take into account another important factor- temperature that animal meet during his life cycle. Studying insects active only in a half of September give us only a small part of whole picture. It is quite possible that grasshopper from July would act totally different. I know that during workshop it was impossible ☺ but this is a real problem that authors should somehow refer to.

I am also not convinced to their idea of connecting their results with global warming problem. Of course, common thing is reaction of organism to the temperature, but I think that is all. Such rapid changes of temperature are not highly connected with fast but still much slower processes occurring as a result of global climate change. Even if we talk about really drastic changes, like some oscillations that occurred during last 700 thousand years, it is still not the case that they studied in their research. Thus, the project is interesting, but claims of authors are totally not convincing to me. However if they somehow reformulate their conclusions and possible impact of their work it would be a great paper for "Nature".

<u>Adam Krupski</u>

Abstract is writtten briefly and explain background of studies. Main aim of research and results are also present as well as importance of studies. Introduction is expanded version of first part of abstract. There is more details about background of studies confirmed by references. However first sentence about generally know meaning of temprature influence has not enough references – just one. Authors should give examples from liturature about these different factors, because it is not clear if Angilletta published about all of them in his article (2009) or just about last one – colour pattern. Aims of study and hyptheses are clearly formulated. Methods are written in understable way with good explanation of different factors. There is only one exeception – femur lenght. There is no explanation why only that body measurment were done, and not for example body weight. Result are well formulated, with simple graph and not-to-compliated tables. In fig. 1 description should be explanation what means black dots? In tables statistically significant values should be bolded – that would be helpful. In discussion authors explain meaning their results and give posibble reasons. Also wider context is marked. However lack of references is a big disadventage of this article – only one reference in discussion (study on lizards, not grasshopers). Secondly discussion is not nested in literature, no exaples from other insect families.

References – only 4 for whole article, each of them with different formatting.

<u>Aleksandra Piontek</u>

I like the introduction – it enhances the bigger problem and explains the reasons behind conducting such experiment. You didn't mention the Latin name of studied taxonomic group which may cause difficulties in future for people searching for your paper. The hypothesises are clearly stated, however, the introduction provided didn't suggest the second hypothesis will be tested in the study. The reasons behind testing the relationship between the size of motoric organs to body mass ratio and grasshoppers performance are revealed later on in methods but I think one of the two main hypothesises of the study should be somehow introduced.
Materials and methods are well described. Maybe even too well – is it important to mention how many researchers were stimulating the grasshoppers? Why not all individuals had time from start to first jump recorded and what does this result contribute? I would mention the unit of the grasshoppers' speed in methods because it is not clear.
Why there is no attempt to explain why the grasshoppers stimulated by WT achieved higher speed than insects stimulated by TH?
In the graph I would change the x axis name to "acclimation temperature" because now it is a bit misleading.
In discussion I think the two sentences about escaping the predators mean more or less the same and one of them is needless. Also there is nothing in discussion about the second tested hypothesis. There are only four references cited.
From small mistakes – two sentences (in introduction "The accumulation of energy, which is essential for performing this movements..." and in methods "The arena was situated in the yard...") are unfinished.

<u>Wioleta Kocerba-Soroka</u>

Title, summary and introduction are accurate and suitable for general readers. Title and summary accurately describe the contents of the article. At the end of introduction are the hypotheses which are clearly stated.

There is a lack of the keywords

The main clams of this report is clear and understandable. This is also important research in relation to climate changes especially global warming, but this is not novel idea of using grasshoppers in artificial condition (different temperature, moisture ect) which is reflected in reports from the last years. However it seems that object of the study is good chosen, because of their wide abundance in environment. It is also easy to find in the natural environment. It is also the reason why sample size should be bigger.

I worry about the fact that to the experiment used several species of grasshoppers, which can have different sensitivity to change of temperature. Division of the organisms to small and large group should be done before the experiment, because the differences in jump length might be connection with body mass. Unclear is choosing to different person for stimulation. It was the statistically differences between this two person but not explained what was the difference between them (one person touched more gentle?)

Statistic methods are chosen well and clear and the data was correctly analyzed.

References have only few positions but well chosen.

Andrzej Antoł

The formal parts of report: summary, introduction, materials and methods and discussion are written properly in terms of required form. One error is lack of affiliations, also separation the section "importance" is redundant in my opinion. It should be in discussion.

The research conducted by the authors are interesting and I'd say important. I know that climat changes is trendy topic and some scientists don't like the overuse of that in research. I don't think so. We know with large confidence that climat is changing, and proper investigating of that process is really important. Also in the level of jumping performance of grasshoopers.

The experimental setup is proper, the measured variables are chosen correctly. What I'd doubt about is the sample size, measuring 9 individuals is too less. Even if obtained results are significant, we can see the influence of stimulating person. In full model (I still don't understand why from full model was excluded femur lenght and body size?) we can see significant effect of ambient temperature only. Than in reduced model we see significance of stimulator, temperature and ambient temperature. Maybe the significance of stimulator can be explained by that that WT had for example bigger animals (randomly)? I'd doubt about the statistics and also I suggest to describe the analysis more precisely.

The issue of small sample size, used tempertures and species is noticed by the authors in the last sentence, so I don't have to repeat it.

I also suggest to search more in the literature. The topic of thermal performance is widely studied and writing the report with only 4 positions is I'd say too less. I miss the direct refer to the hypotheses from introduction, especially when authors rejected both of them. It should be in the first paragraph of discussion.

In general the report is writen in proper English, quite clear and understandable. On the other hand it can be a bit longer and show more wide perspective, especially in discussion and introduction part. The research are interesting and important, but not enough revealing to Nature.

Natalia Derus

Research conducted by this group includes in title a promise to test the influence of acclimation temperature on jump performance in grasshoppers. However, the hypotheses are not accurate to the title – "sudden increase of temperature affects the movement performance of grasshoppers stronger than the decrease" and "the movement performance is dependent on the size of motoric organs to body mass ratio". By the title I expected examination of acclimation effect, not the effect of rapid temperature changes after acclimation.

Introduction gives some theoretical background but seems to be unsufficient to justify the importance of this study. What is more, you explain the validity of the experiments (acclimation to one temperature and providing tests in another) by 'global warming, which is assumed to cause rapid changes in environmental temperature'. I agree that climate change might be good 'big theory' to refer to (it is big and important), but rather not in this particular case. Grasshoppers lives shortly, so the global temperature changes – even very rapid – still will be experienced by the consecutive generations rather than during individuals life. In this context studying grasshoppers acclimation to one temperature and then examining their performance in dramatically different thermal conditions can not be a method of evaluation their future chances. To do so I would expect between-generations studies. This research might be connected to, for example, the metabolic theory of ecology (you examined body size and temperature influence). Also, it is vague what exactly 'accumulation of energy, which is essential for performing this movements, is assumed to be dependent on the ambient temperature' means. Maybe there was a good moment to introduce some information about metabolism?

As you noticed, the stimulator had an effect on grasshopper performance - probably you should conduct the experiments again with only one researcher stimulating the grasshoppers.

Another thing is the way you wrote. I know you were hurry (like me in this moment), but it can not be an excuse (I am afraid scientific papers usually are written in the rush).For example sentence 'The arena was situated in the yard of the UJ Research Station in Ochotnica Górna, in shaded conditions at the temperature' probably do not contain the ending, and 'Ambient temperature (covariate) ($F_{1,22}$ = 7,538, p=0.12) and stimulator (random effect)($F_{1,22}$ = 12,260, p=0.002).' should contain several more words such as 'had a significant effect'. I spot surprisingly high level of bloviating ('water pouring' and 'buttery butter'), for example 'This implies that grasshoppers might be able to successfully escape from predators and thus survive even if they encounter sudden environmental change. Grasshopper movement performance is strongly connected with survival as it enables the individual to escape from the predator.' My comment relates not to the used words or language but the repeated information.

For such, as you claim, important topic there should be more references. Also, basing on the current list of articles you refer to, I assume that you have not explore the theoretical background and similar (already done) studies deep and enough. What is more, two of the four cited publications concerns vertebrates which can react differ on temperatures even if they are ectothermic like grasshoppers.

After all, I am impressed by your persistence and imagination essential to run such experiment. I appreciate the fact that you have noticed the importance of stimulating person. Creative people who also can spot unexpected factors are a new hope for science and humankind preparing to face the climate change.

5.1.4. Final version of report

The effect of the temperature on the movement performance in grasshopper species

Terézia Horváthová, Mateusz Sobczyk, Wojciech Tokarz

Summary

It is generally known that the temperature can have a strong effect on different morphological, physiological and behavioral traits in ectotherms. The optimal temperature for performance traits is assumed to match with temperatures that individuals experience in their habitat. We used grasshopper species to examine how individuals perform in three different temperatures: 10, 20 and 30°C. The measure of the performance was the speed of the movement which is an important trait for escaping from the predators. Our study showed that grasshoppers performed poorer at 10°C which may be explained by the negative effect of cold temperature on metabolism. The speed of the movement did not differ between higher temperatures suggesting that the optimal temperature for this performance trait has a wide range in grasshoppers from the studied locality.

Introduction

Performance and the fitness of ectotherms are profoundly affected by the environmental temperature (Angilletta 2009). Because environmental temperature varies temporally and spatially, animals are continually challenged to maintain their homeostasis. The relationship between temperature and individual performance has been described by the inverted U-shape curve, where performance gradually increases with temperature until reaching an optimal value (optimal temperature), and then declines near the upper critical or lethal temperature (Castañeda, et al. 2004, Angilletta 2009). It has been suggested that animals, which encounter high ambient temperatures in nature have evolved higher optimal temperatures (Huey and Bennett 1987). Thus, the optimal temperature is expected to vary across different species as wells as individuals of the same species inhabiting different climatic conditions. Such patterns have been shown in lizards, fruitflies, isopods and even plants (Huey and Bennett 1987, Castaneda et al. 2004, Angilletta 2009, Angert et al. 2011).

Although the optimal temperature strongly correlates with the environmental temperature experienced by animals, several studies showed that the performance can be maximized at range of ambient temperatures (Huey and Bennett 1987, Kingsolver and Woods 1997). Moreover, such optimal temperature range may differ between the traits. For example, behavioral traits such as sprint speed or jumping rate usually show wider temperature range (Hertz et al. 1983) than traits connected with physiology (i.e. respiration and digestion, Kingsolver and Woods, 1997), which implies that the thermal performance should be considered to be trait- rather than taxa-specific. However, why behavior shows greater optimal temperature range is at present poorly understood.

The aim of our study was to examine how the temperature affects the movement speed in grasshoppers from different climatic conditions. Grasshoppers use jumping to escape from the predators and movement performance is thus an important trait for their survival. However, at which temperature is this performance optimized is generally not known. We tested the following hypotheses: i) the movement speed increases with the temperature and ii) the movement speed depends not only on the temperature but also on the relative size of femur. We assumed that insects with higher femur length to body mass ratio have higher movement performance.

Material and Methods

The experiment was conducted on 17 September 2014. Firstly, 27 grasshopper individuals of different species and sizes were collected from a steep mountain meadow in Ochotnica Górna, Gorce mountains, Poland. The individuals were randomly divided into 3 groups (each with n=9) and placed in controlled stable temperature conditions: 10°C (fridge), 20°C (room temperature) and 30°C (next to a heater), for the minimum of one hour before starting the experiment. Each individual was used in a movement performance test only once.

Animals were taken out from the experimental conditions in random order and tested for the movement performance in outside conditions (always in shadow, ambient temperature varied from 11 to 19°C during the experiment). The movement performance test consisted of placing the individual in the starting point on the experimental arena and stimulating it to jump. Each session was finished when the individual performed a total number of 10 jumps in maximum time of 15 minutes. The stimulation included gentle touching of the grasshopper back with a small brush (performed by WT and TH). Values measured during the test were: the length of single jumps (1), the walk distance (2), total time in which the individual performed the test (3). After each session, the femur length, body mass of the individuals and ambient temperature at arena site was measured. For the measure of the motoric organ size, femur length to body mass ratio was calculated. The ratio was used to determine if there is also an influence of individual motoric abilities (size of the most important element of the individual motoric system) on the main tested effect, that is influence of temperature on the movement performance.

After the movement performance test sessions, the speed results for each individual were calculated by dividing the total distance of jumps and walk by the total time of the session and then compared between the groups. The data were analyzed with general linear mixed model (GLMM) () with temperature as fixed factor; the experimenter as random factor and two covariates: ambient temperature and femur length-body mass ratio in SPSS 17.0 program. After the full model was tested, we removed sequentially the insignificant interactions.

Results

Our data fulfilled general linear model assumptions: normal distribution and homogeneity of variance. Body mass distribution between the temperatures groups did not differ (Levene test $F = 2.846$, $p = 0.077$). The temperature had significant effect on grasshoppers movement speed ($F_{2,22} = 6.946$, $p = 0.005$; Fig.1, Tab.1). Animals which were kept in 10°C had significantly lower speed that those from 20°C and 30°C groups (post-hoc Tuckey test, 10°C vs 20°C $p = 0.011$ and 10°C vs 30°C $p = 0.028$). The groups 20°C and 30°C did not differ ($p = 0.909$). Ambient temperature and the person who stimulated individual insects had also significant effect on movement speed (Tab. 1). Grasshoppers stimulated by WT achieved higher speed than insects stimulated by TH (Fig. 2). Femur length to body mass ratio did not correlate with movement speed ($F_{1, 25} = 1.105$, $p = 0.303$) so we excluded it from the analysis.

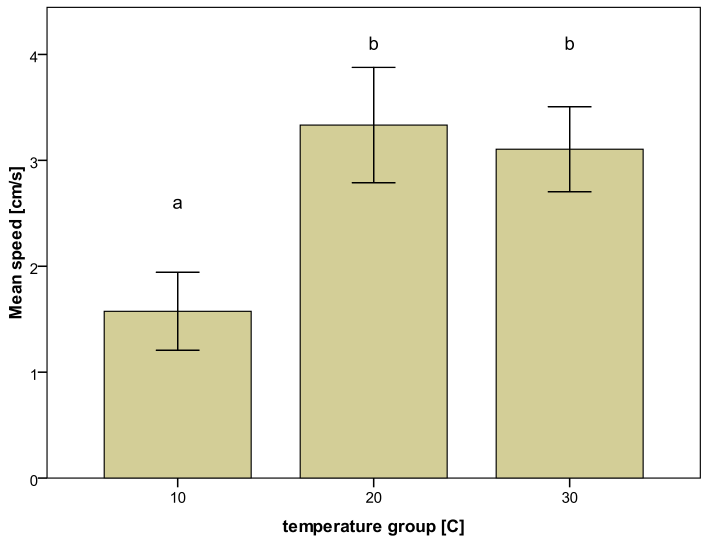


Figure 1. The effect of the temperature on the movement speed of .... (mean±SE). Letters....

Table 1. General linear model results for the relationship between grasshoppers movement speed and temperature (temperature = fix factor, stimulator = random factor, ambient temperature = covariate) (N = 27)

Source	df	F	p
temperature	2	6.946	**0.005**
stimulator	1	11.141	**0.003**
ambient temperature	1	6.628	**0.017**
error	22		

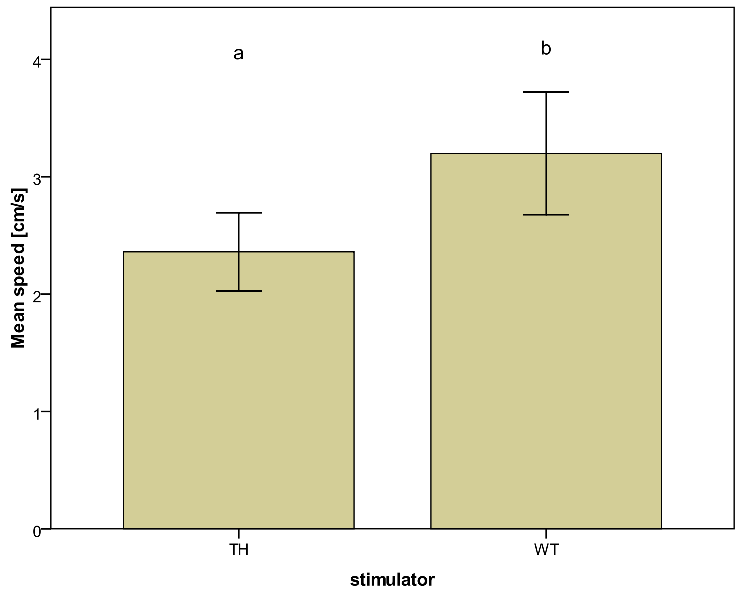


Figure 2. The effect of stimulator on the movement speed (mean±SE).

Discussion

Our results showed that the temperature had a significant effect on the movement speed in grasshopper species. Grasshoppers from 10°C moved slower than individuals from 20 and 30°C. Individuals from higher temperatures did not differ in their movement speed.

The performance of ectotherms is known to be strongly affected by the environmental temperature and the performance is enhanced with increasing temperature (Kingsolver and Woods 1997, Forsman 1999, Angilletta 2009). The temperature at which performance reaches the optimum usually differs between individuals that inhabit cold and warm environment (Angert et al. 2011). Our results showed that movement speed of grasshoppers from studied locality is enhanced when animals are kept at higher temperatures. This suggests that optimal temperature for speed movement ranges between 20 and 30°C. For example, grasshopper species *Tetrix bipunctata* and *Tetrix subulata* from Swedish populations showed the highest jumping performance at 30°C, the jump length and moved distance was however decreased at lower temperatures (15, 20, 25°C, Forsman 1999). The enhanced performance (e.i. growth, digestion and sprint speed) at thirty degrees has been also described in different ectothermic species such as fruitfly, moth or lizards (Huey and Bennet 1987, Huey and Kingsolver 1993, Kingsolver and Woods 1997), but as the temperature encountered in the field often exceeds this value, this is not a surprising result. Our grasshoppers from Poland locality is characterized by much broader optimal temperature range compared to other studied insect species. Twenty degrees is in most studied cases retarded the performance of the individuals and the optimal temperature ranged between 30 and 35°C (Huey and Kingsolver 1993, Kingsolver and Woods 1997, Lachenicht et al. 2010). However, as the behavioral traits show wider optimal temperature range compared to traits connected with physiology (Hertz et al. 1983), this may partly explain why grasshoppers moved faster at 20 as well as 30°C. Another likely explanation is that in our study we used several different grasshopper which may differ in their optimal temperature. The effect of the ambient temperature on the movement speed was most likely caused by different measurements time (the experiment started at 2pm and finished at 6pm).

The movement speed of grasshoppers is assumed to depend not only on environmental factors (temperature) but also on the morphology of the animal (Snelling et al. 2013). Our results showed that the relative length of femur does not affect the movement speed. We assume, that the femur length is an appropriate measure of the motoric system abilities in grasshoppers and several morphometric measurements should be taken into account. The combination of different measurements corrected for body mass then can be used as the single measure of the motor abilities.

The surprising results was the significant effect of the stimulator on the movement speed. However, as we did not find the significant interaction between the stimulator and the temperature, we claim that the results were not biased by this factor.

To conclude, the movement speed of grasshoppers in our studied locality was similar at wide temperature range (from 20 to 30°C).

Acknowledgements

We would like to thank all our course colleagues and Joasia Rutkowska, who gave us inspiration and support on all stages of our work.

References

Angert, A .L., Sheth S. N. and Paul J. R. 2011 Incorporating population-level variation in thermal performance into predictions of geographic range shifts. Integrative Comparative Biology 51:733-750.

Angilletta, M. J. 2009 Thermal Adaptation: A Theoretical and Empirical Synthesis. Oxford University Press, Oxford, 304 pp.

Castaneda, L. E., Lardies, M. A. and Bozinovic, F. 2004 Adaptive latitudinal shifts in the thermal physiology of a terrestrial isopod. Evolutionary Ecology Research 6(4):579-593.

Hertz, P. E., Huey, R. B., Nevo, E. 1983 Homage to Santa Anita: thermal sensitivity of sprint speed in agamid lizards. Evolution 37:1075-1084

Huey, R. B and Bennett, A. F. 1987 Optimal performance temperatures of lizards. Evolution 41:1098-1 115.

Kingsolver, J. G. and Woods, H. A. 1997 Thermal sensitivity of growth and feeding in Manduca sexta caterpillars. Physiological Zoology 70:631-638.

Lachenicht, M. W., Clusella-Trullas, S., Boardman, L., Le Roux C. and Terblanche, J. S. 2010 Effects of acclimation temperature on thermal tolerance, locomotion performance and respiratory metabolism in Acheta domesticus L. (Orthoptera: Gryllidae). Journal of Insect Physiology 56: 822–830.

Snelling, E. P., Becker, C. L. and Seymour, R. S. 2013 The Effects of temperature and body mass on jump performance of the locust Locusta migratoria. PLoS ONE 8(8): e72471.

5.2. Are stonefly (Plecoptera) nymphs good indicators of petrol pollution in aquatic ecosystems?

Authors: Andrzej Antoł, Aleksandra Piontek, Beniamin Wacławik

5.2.1 Project proposal

Summary

The growing petroleum pollution of waters requires the resonable bioindicator to allow fast and precise recognition of that pollution. We would like to test the usefulness of stoneflies larvae for that purpose. In two experiments we will test the mortality of stoneflies in different petrol concentrations and compare the result with caddisflies and mayflies. This research will drive us to better understanding of usefulness of stoneflies as bioindicators.

Aim / hypothesis

The growing number of cars and other mechanical vehicles is one of the biggest threats to environment. Air contamination is not the only problem; also water is highly polluted by leaks. Biggest catastrophes like Exon Valdez in 1989 paid public attention to that issue. But we should be aware that the problem of petrol contamination of water concerns not only huge accidents in the sea. Each day million tons of the oil and its products is released into the lakes and running waters. Thus, polluted watercourses are present in almost all water habitats around the world. In many cases they are not detected and cleaned because of difficulties with fast and precise estimation of contamination and unawareness of local people. One of the first steps to be done is to find the proper indicator for petroleum pollution.

The use of bioindicators is a valuable method used in monitoring as it reflects the overall environment condition. For measurements of water pollution the macroinvertebrates are often used bioindicators as they represent the wide range of different pollution tolerance and can be easily collected.

The aim of our project is to check if the stoneflies (Plecoptera) are good petrol contamination indicators. Stoneflies nymphs are quite vulnerable to pollution in general (they are placed in 6 – 9 position in polish Biological Monitoring Working Party score) and are also sensitive to petrol pollution. These reasons support the idea to check if stoneflies would be the proper petroleum indicator.

Our hypothesis is that stoneflies larvae are vulnerable to petrol contamination of water more than other two groups commonly used in water pollution control- larvae of caddisflies (Trichoptera) and mayflies (Ephemeroptera).

Methods

All larvae will be collected in Jaszcze mountain stream in Ochotnica Górna (Poland) using scoop and "by hand" method. First experiment concerns vulnerability of stoneflies to petrolcontamination. Collected larvae will be put into small containers with watercontaminated with oil in three concentrations (3%, 6% and 9%) for 5 hours. Survival of larvae after 5 hours will be measured. All larvae will be kept for further assignment to family.

Second experiment will be conducted to compare vulnerability of stoneflies and other two studied groups- caddisflies and mayflies. Insects from latter two groups will be put in cups with particular amount of oil concentration for 5 hours. This amount will be calculated using LC50 value. From three concentrations used in first experiment will be chosen the one, in which proportion of dead stoneflies after experiment is closest to 50 %. In both experiments we plan to have 5 replications per group. The proportion of animals that survived will be compared with one-way ANOVA test.

Impact of results

The results will provide valuable additional information about the influence of petrol pollution on water macroinvertebrates. The gathered data will improve the use of stoneflies as a bioindicators of water pollution. The comparison of the petrol pollution tolerance between different taxonomic groups will help to create the range of pollution in which stoneflies may be the best.

5.2.2. First version of report

Usefulness of mayflies (Ephemeroptera, Insecta) as bioindicators of fuel contamination of water

Abstract

Search for fuel polluted water boindicators that can be used fast and easily is one of most important problems concerning environmental conservation. Mayfly (Ephemeroptera) larvae, which are common, easy to find and also vulnerable to pollution, are good candidates to be such indicators. In this study sensibility of mayfly larvae from Jaszcze stream to different concentrations of diesel petrol was tested. To detect size-dependent effect larvae were divided into two groups: large and small. 197 specimens were tested. Study showed high vulnerability of these larvae and significant size-dependent difference (higher mortality of small ones). Mayfly larvae can be used as proper bioindicators of fuel water pollution without assigning them to species.

Introduction

Anthropogenic water pollution is one of the biggest environmental problems (Fleeger et al. 2003). Pollution coming from fuel leaks –both results of big catastrophes like Exon Valdez in 1989 and also everyday human activity is significant part of this problem. Oil pollution has big influence on biota living in fresh and sea water, but sometimes because of lack of proper bioindicators it is hard to detect. Water macroinvertrebrates, for example many beetles (Coleoptera), crustaceans (Crustacea) or mayfly (Ephemeroptera), caddisfly (Trichoptera) and stonefly (Plecoptera) larvae are useful bioindicators of water pollution. However, to obtain proper information about contamination, level of their vulnerability to particular substances must be known.

In mayfly live cycle, dominant stadium is nymph, which may live for some years, when the adult often lives for one day. Moreover, these nymphs are usually very abundant and are one of dominant taxonomic groups in running water benthos. Mayflies are vulnerable to natural and anthropogenic contaminations (Wesner et al., 2014), thus, they are widely used as bioindicators (Kłonowska-Olejnik et al. 2012, Kłonowska-Olejnik & Skalski, 2014). Abundance of mayfly larvae is part of EPT index (Ephmeroptera + Plecoptera + Trichoptera) which is considered as proper way to measure level of environment degradation (Hickey & Clements 1998). Being quite sensitive to industrial pollution, populations of mayfly nymphs has lower densities in part of streams below places when this kind of leaks appeared (Schloesser et al. 1991).

To detect such destructive and common pollution as fuel pollution, the best way is to find bioindicators that might be used fast and easily. Mayflies larvae are good candidates to be used as petroleum contaminaton bioindicators, because of their abundance in environment, accessibility during long part of year (Landa 1968) and vulnerability to oil pollution (Russell et al. 1979).

The easiest way to measure contamination is to check out abundance of all mayfly larvae, without assigning them to particular families. But one important problem can be met here. Because mayflies moult and swarm more than once a year and also different species and populations moult in different periods (Landa 1968), larvae of many ages and sizes appear in one habitat. Many experiments showed that small stream invertrebrates are more sensitive to contamination than large ones (Kiffney and Clements 1996) and this is also connected with life cycle stadium (McCahon et al. 1989).

The aim of our study is to check the vulnerability of mayfly larvae to petroleum pollution in different concentrations in relation to their body size. To test that we prepared an experiment where we tested mayflies of two categories of body size (big and small) in 6 concentrations of contaminant (0,25%, 0,5%, 1%, 3%, 6% and 15%).

Materials and methods

The animals were collected in Jaszcze mountain stream in Ochotnica Górna south Poland, Gorce mountain range. Animals were collected by hand, from stones. After the collection the mayflies were separated from other similar taxonomic groups using the binocular. After that they were arbitrairly divided into two body size categories: big and small. The range of body size allowed us to distinguish them in the categories quite precisely, the big animals were few times larger than the small.

Into each of 16 plastic vessels there was put 40 ml of stream water. Then, diesel fuel was added in concentration of: 3%, 9% and 15%, which is: 29 ml/l, 90,9 ml/l and 150,7 ml/l respectively. In control group there was pure water without any addings.

In each treatment we prepared 4 replicates. To each box aprr. 4 mayflies were added (accurate numbers for each body size are shown in tab.1).

Table 1. Sample sizes in the experiment

Size of animals	N
Big	58
Small	65
Small – second experiment	64

After that the mortality of individuals was recorded every one hour. The water was blowed each 30 minites using short plastic pipe to provide the oxygen. After 5 hours experiment was stopped.

The next day new trail was done only on small larvae and using lower fuel concentrations (0,25%, 0,5% and 1%, which is: 2,5 ml/l, 5 ml/l and 10 ml/l).

From the data of survivalability we counted LC10, LC25, LC50 which are the lowest concentrations of toxicant in whch 10, 25 and 50% of animals are dead after certain amount of time respectively (Robidoux et al. 1999). According to Warne and van Dam (2008) we skipped use of the LOEC (Lowest Observed Effect Concentration) and NOEK (No Observed Effect Concentration) approach.

The survavibility was analyzed with Cox hazard model with size as qualitative and concentration as quantitative variable.

Results

The high mortality caused by presence of diesel fuel was observed already after the first hour of experiment.

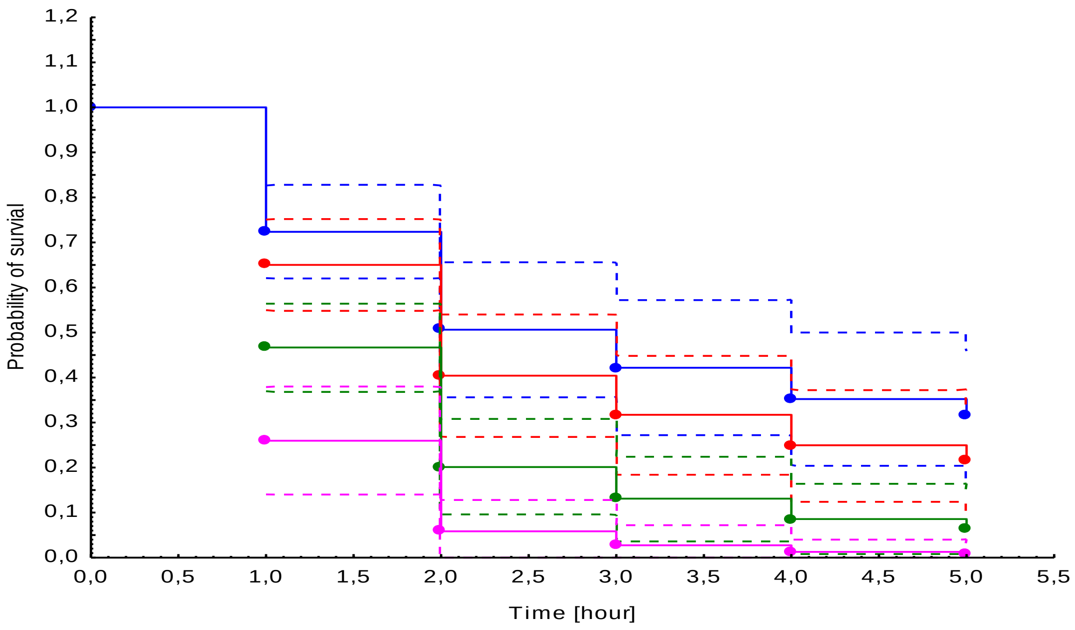
The values of LC 10, LC 25, LC 50 obtained in the first experiment after the first hour of exposition are shown in tab.2.

Table 2. Values of lower concentration need to kill the 10, 25 and 50% of animals respectively obained after the one hour of exposition.

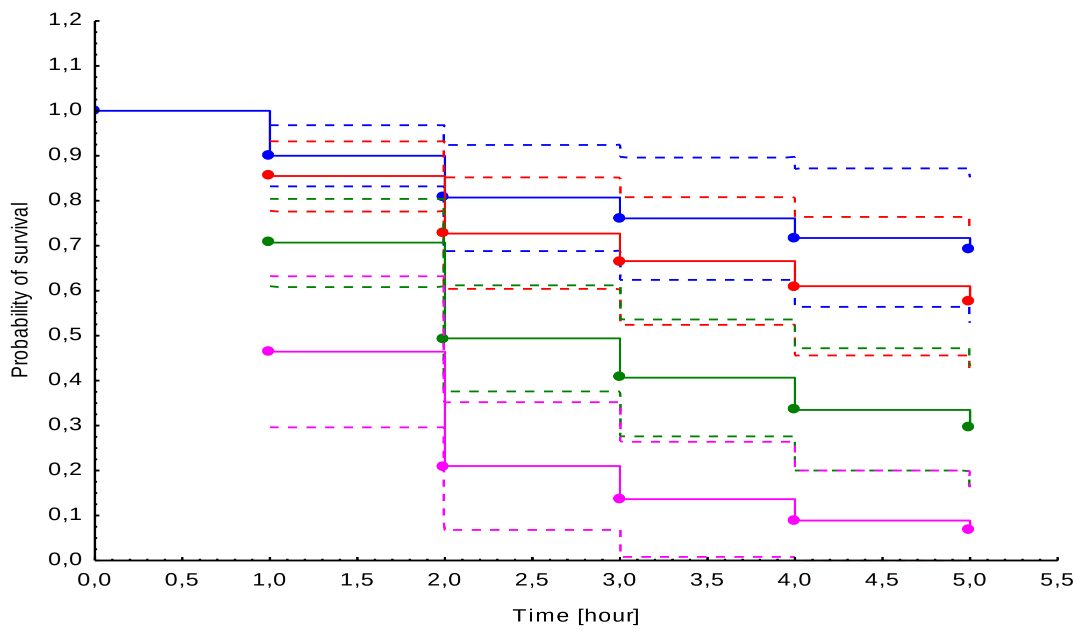
SIZE	LC 10	LC 25	LC 50
BIG	< 29ml/l	< 29ml/l	>150,7 ml/l
SMALL	< 29ml/l	< 29ml/l	< 29ml/l

The analysis of Cox model showed the significant influence of size (Waldχ=26,13, p<0,0001) and concentration (Waldχ=13,09, p=0,003). The survival decreased in the gradient of diesel fuel concentration and was higher among big animals (graph 1, graph 2).

In the second experiment we also observed high mortality after one hour among small animals and LC50 was below the lowest concentration (2,5 ml/l).



Graph 1. Function of survival in time for the small animals. Colour of line shows the concentration of diesel fuel in the treatment: blue line – 0 ml/l, red line – 29 ml/l of, green line – 90,9 ml/l, pink line – 150,7 ml/l. Dashed line show 95% CI.



Graph 2. Function of survival in time for the big animals. Colour of line shows the concentration of diesel fuel in the treatment: blue line – 0 ml/l, red line – 29 ml/l of, green line – 90,9 ml/l, pink line – 150,7 ml/l. Dashed lines show 95% CI.

Discussion

All larvae in our study were vulnerable to oil pollution. For the highest contamination value (150.7 ml/l) almost no individual survived for more than 2 hours (graph 1, graph 2). The LC_{10} values for 1 hour exposure were lower than 29 ml/l for both small and big larvae. This confirms the value of mayflies as a bioindicators of petroleum pollution. Studies on mayflies in natural conditions had shown the strong negative effect of oil spill on local populations of mayflies. Oil pollution of sediment was probably main reason of disappearing of *Hexagenia* mayflies from the Great Lakes (Schloesser et al. 1991). Also after spill of oil in Asher Creek, Missouri mayflies were not detected in the water for 9 months (Ort et al. 1995).

The small larvae were much more vulnerable to oil contamination than big ones. The LC_{50} value for 1 hour exposure was below 2,5 ml/l. Both fish and invertebrates are known to exhibit size-dependent sensitivity to contaminants (Kiffney & Clements 1996). Kiffney and Clements (1994) found that small larvae of *Drunella grandis* were more sensitive to exposure on Cd, Cu and Zn than bigger larvae from the same population.

We observed higher mortality of small larvae also in control group (over 60% mortality after 5 hours). The mayfly larvae are vulnerable not only to anthropogenic pollution but also natural occurring factors like temperature changes (which directly influence the water oxygen level) (Bridgeman et al. 2006). Thus we think that higher mortality of small larvae in our study may be also the result of different temperature and oxygen level in comparison to their natural environment.

The long recovery time is connected to larvae mortality caused by direct exposure to oil but also lower rates of colorization of oil-contaminated substrates (Ort et al. 2005). Rosenberg et al. (1980) found lower numbers and diversity of species of mayfly occupying the stones contaminated with oil even after four months of colonization. We tested only the short-term influence of petroleum contamination on larvae survival but we assume that even small contamination may reduce the density and diversity of mayfly populations in long time after the leak spill.

Our result supports the hypothesis that mayflies can be used as good petrol contamination indicator, because they are vulnerable to even small amounts of contaminant. The diverse response to contaminant according to their body size would allow to indicate the fuel diesel contamination just by estimating the amount of small animals. Also without assigning the species we found similar response to contamination what shows that mayflies can be treated as a group, which facilitates the bioindication. Small sample size and short time of experiment as well as lack of oxygen and temperature control strongly biases our result. Further studies are necessary.

Acknowledgments

We would like to thank all participants of Evolutionary ecology – methodological workshop in Ochotnica Górna for the discussion and good advices and Mr Marian Krzyśko for donating the diesel fuel which we used in our research.

References

1. Bridgeman, T. B., Schloesser, D. W., & Krause, A. E. (2006). Recruitment of Hexagenia mayfly nymphs in western Lake Erie linked to environmental variability. *Ecological Applications*, *16*(2), 601-611.
2. Fleeger J.W., Carman K.R., Nisbet R.M. (2003) Indirect effects of contaminants in aquatic ecosystems *The Science of the Total Environment 317* 207–233.
3. Hickey C.W., Clements W.H. 1998 Effects of heavy metals on benthic macroinvertebrate communities in New Zealand streams *Environmental Toxicology and Chemistry*, *Vol. 17, No. 11*, 2338–2346.
4. Kiffney, P. M., & Clements, W. H. (1994). Effects of heavy metals on a macroinvertebrate assemblage from a Rocky Mountain stream in experimental microcosms. *Journal of the North American Benthological Society*, 511-523.
5. Kiffney, P. M., & Clements, W. H. (1996). Size dependent response of macroinvertebrates to metals in experimental streams. *Environmental Toxicology and Chemistry*, *15*(8), 1352-1356.
6. Kłonowska-Olejnik M., Skalski T. (2014) The effect of environmental factors on the mayfly communities of headwater streams in the Pieniny Mountains (West Carpathians) *Biologia 69/4 Section Zoology*: 498—507,
7. Kłonowska-Olejnik, M., Stoch, T., & Skalski, T. (2012) Zgrupowania jętek (Ephemeroptera) w górnej części zlewni Koniny w Gorczańskim Parku Narodowym (Karpaty Zachodnie). *Ochrona Beskidów Zachodnich, 4*: 57–68.
8. Landa V. (1968) *Developmental cycles* of Central European *Ephemeropte*ra an their interrelations. *Acta Entomologica Bohemoslovaca* 65: 276-284
9. McCahon C.P., Whiles A.J., Pascoe D., 1989 The toxicity of cadmium to different larval instars of the trichopteran larvae *Agapetus fuscipes Curtis* and the importance of life cycle information to the design of toxicity tests. *Hydrobiologia Volume 185, Issue 2*, 153-162
10. Ort, M. P., Finger, S. E., & Jones, J. R. (1995). Toxicity of crude oil to the mayfly, *Hexagenia bilineata* (Ephemeroptera: Ephemeridae).*Environmental Pollution*, *90*(1), 105-110.
11. Robidoux, P. Y., Hawari, J., Thiboutot, S., Ampleman, G., & Sunahara, G. I. (1999). Acute Toxicity of 2, 4, 6-Trinitrotoluene in Earthworm (*Eisenia andrei*). *Ecotoxicology and environmental safety*, *44*(3), 311-321.
12. Russel, P. P., Resh, V. H., & Flynn T. S. (1979). A continuous flow bioassay technique for assessing the toxicity of oil-shale-related effluents: Preliminary results with two species of

caddisfly larvae. *Oil Shale Symposium. Sampling, Analysis and Quality Assurance. March 197,* 416-431
13. Schloesser, D. W., Edsall, T. A., Manny, B. A., & Nichols, S. J. (1991). Distribution of Hexagenia nymphs and visible oil in sediments of the Upper Great Lakes Connecting Channels. *Hydrobiologia*, *219*(1), 345-352.
14. Warne, M. S. J., & van Dam, R. (2008). NOEC and LOEC data should no longer be generated or used. *Australasian Journal of Ecotoxicology*, *14*(1), 1.

5.2.3. Reviews

Prof. dr hab. Adam Łomnicki

I have not corrected the English of this report except for some few obvious mistakes ('colorization' instead of 'colonization').

Many of the information from literature are not immediately related to this studies, and even if they are related they are not always placed in proper places. For example, the first two sentences in the Abstract are too general and they contain nothing of what was really done.

If large animals were several times larger than the small ones, there should be very easy to inform the readers what is the size (in mm) which divide these two groups. The fact that it was arbitrarily divided does not exclude possibility to give measurements which would allow others to repeat these experiments. This is a basic requirement of any scientific method description.

There were three experimental groups: small and large animals in the first experiment and small only in the second experiment. Why in the second experiment there was no large animals? It was not explained. There are only two figures (graph 1 and graph 2) showing the survival. Was the second experiment excluded from the graphs or small animals for both first and the second experiment were combined? It is not clear. By the way, reports usually have only tables and figures. Graphs are included into figures, this is a general rule used by most of scientific journals.

Some parts of the report are not quite clear and they required additional explanations. First, six different concentrations given in percentage differ slightly from those expressed in ml/l. This should be thoroughly explained what from are these small differences. An even if the real concentrations differ from the assumed percentage, for a reader it would be much easier to follow the results of the experiments if the authors use the assumed percentage and not detailed amounts of fuel per water. Second, Cox model and the symbol Wald χ are not widely used in ecology and therefore few words of explanations of this model and this symbol should be given. By the way, the information brought about with this symbol brings about less information than the analysis of variance which would allow us to see how much of variance is due to concentration and how much to the size of the animals.

Prof. dr hab. Mariusz Cichoń

This study aims at finding out whether mayflies are sensitive to petroleum water contamination. In fact, this was confirmed at least in the range of petroleum concentrations used in this study. Importantly the authors show big animals to be less sensitive than small onces. The authors idea was however to find a good bioindicator of petroleum water contamination, and they argue that mayflies may constitute a good candidate while not considering other potential candidates. As the authors properly noticed, mayflies are good indicators of water pollution in general, but not specifically to petroleum contamination. So, they disappearance do indicate water pollution, but the origin of pollution can not be judged. Please notice that even mayflies from control group did not survive well. They are just sensitive even if kept in unpolluted water. In fact, petroleum constitute such strong pollutant that hardly any organism can survive contamination. Thus, one can not conclude relaying on the results obtained in this study that mayflies are good indicators of

petroleum contamination, as many other organisms may work equally well! So, the most important weakness of this study is its motivation. This logic is just difficult to buy.
The report does not refer to any data on possible level of petroleum contamination in freshwater streams. The concentrations used in this study seems to be chosen *ad hoc*. The results confirm that these concentrations were not chosen very accurately. Even in the second experiment the concentratons seems to be too high. Such concentrations may occur only locally at the place of petroleum leak. This is generally a difficult issue as the concentration is highly changing in running waters.
The experimental design is difficult to follow. It is said that 16 vials were used with 2 treatment groups and 2 body size categories and 4 replicates in each treatment (body size categories?), and 3 concentrations in the experimental groups. So, how all this was distributed among these 16 vials? The table 1 with number of big and small flies is odd and do not provide much information. Just waste of space. This information, if needed at all, may take just a single line in the text. This is also true for table 2.

The statistical analyses are poorly described. In fact, nothing is said about data structure and its analyses. The experimental design is quite complicated with 3 unbalanced levels. Then, Wald statistics is referred, but one can not judge what was actually a subject of this analysis.

The results of the second experiment is reported extremely shortly. Just one sentence in the Results section. What was then the purpose of this experiment if the authors provide hardly any results?

It is strange to me that LC values are calculated based on such small sample size. Calculating percentages from 4 individuals is not very informative, particularly if most animal died within first hour of observation. This is just not reliable.

The authors should more carefully use English words, as sometimes they misunderstood the meaning,

Adam Krupski

Authors of this research ask questions about mayflies as bioindicators of fuel contaminations of water. In introduction they explain in a simple way problem of water pollution and catastrophic effects of fuel contamination. Also idea of bioindicators and their usage is written clearly with many species as examples. However in some parts of introduction more references would be more helpful, especially for people interested in this subject. Last sentence of introduction is unnecessary, because this information is later repeated in methodology and also there is more suitable. Methodology and results are written clearly and briefly. Animals were divided into two groups by body size, but data with more details would be more useful. Graphs are simple and easy to understand. Authors in discussion explain possible reasons of their results – why small mayflies are more vulnerable to fuel contamination than bigger individuals. Ideas are supported by references. Moreover other possible explanations of results are provided. Disadvantages of this research as short time of experiment and lack of better apparatus are mentioned, however concept for future studies and upgrades of methodology in case of better funding would be useful.

In references formatting sometimes is different – small details.

Wioleta Kocerba-Soroka

Title, abstract and introduction are suitable for general readers. At the end of introduction is a clear aim of this study which is to check the vulnerability of mayfly larvae to petroleum pollution in different concentrations in relation to their body size.

There is a lack of the keywords

The sentence in line 28: “In mayfly live cycle, dominant stadium is nymph, which may live for some years, when the adult often lives for one day” needs citation.

The choice of the study object is justified, because is generally known that Ephemeroptera is bioindicators and only a candidate to be a indicators of water pollution like a authors wrote. The experiment should be conduct on one species, because the differences between vulnerability to pollution could be depends on the species. On the other hand this study proved that pollution of water can reduce not only a number but also diversity in mayfly population.

It is really good idea to divided the specimens to two group in relation to body mass before the experiment start. The choice of range of petrol concentration was arbitrarily. It should be done after checked the real concentration, which occur in the natural environments, which the mayfly larvae can live or in available publications. LOEC and NOEC methods, which was used by authors were very common in ecotoxicology research and were accurate for this study too.

Statistic methods are chosen well and clear and the data was correctly analyzed.

References is complete and well chosen, but it should not be a numbered. When authors want to use numbered position should also use the number in the text in the order of citation.

Wojciech Tokarz

The work is of the general interest mostly of the persons dealing with practical aspects of ecotoxicology in use, rather than to scientists with broader interests in global trends and phenomena.

The title and abstract accurately describe the content of the article, the hypothesis is stated quite clearly, and the methods used to test it are generally well designed. Methods are described precisely enough to be replicated, but they do not give the possibility to obtain results showing the precise contamination vulnerability of the bioindicator candidate species group.

The article is definitely novel and should be treated as pilot study for more complex experiments aiming to determine the precise levels of petrol concentration in water which would be used to indicate the contamination. It is although controversial to state that the this specific group of organisms (mayflies) are better than other ones to be used as bioindicators and therefore such a project (if there only was more time of course) should be started with a search for the most accurate species, showing the less fluctuating vulnerability level.

The possible usage of mayflies as bioindicators of petrol pollution would require precise information about the concentration level which is toxic for those organisms, as well as determining the variance of the vulnerability in a larger group of individuals from many different places. What is more, if someone would expand the study and perform it another time in more complex configuration, he should concider if the bioindication method is worth of it – there also is a possibility to use electronic devices to measure the contamination precisely and relatively cheap, without concerns like: “is it the petrol what causes the lack of mayflies in the river, or are there other reasons?”.

The results are analysed with proper statistical tools and show accurately the most important information. Discussion, although well prepared does not fully persuade the reader to the idea of mayflies as good bioindicators. References are full and diverse, what increases the meritorical level of the paper.

The article is generally very well written, good job ☺

Natalia Derus

Dear Authors,

My heart is bleeding, but I have to follow some rules. Because one of these categories: "submissions that deal only: a) with the levels of pollutants in environmental matrixes, b) **with toxicity testing**; or c) with human health issues." can be applied to your research I have to reject your submission ("The [Ecotoxicology] Journal will not consider submissions that deal only (…)". However, I received a request from dr. Joanna Rutkowska (Jagiellonian University in Cracow) to give you some comments and advice on your report. I hope it will help you to improve your manuscript and allow you to publish it elsewhere (for example in Nejczer).

At first – the abstract. Number and levels of concentrations used by you in order to check larvae sensitivity would be more interesting and tempting details than certain sample size. Also the information about experimental conditions (natural or laboratory) should be included in this part of report.

You gave broader view on the oil pollution problem, but some further details about the scale of this issue would justify importance of your research better. The explanation for using that specific taxa is quite broadly described, however there are several places where you started repeating your arguments and information ("Mayflies are vulnerable to natural and anthropogenic contaminations…"; "[mayflies] vulnerability to oil pollution"; "quite sensitive to industrial pollution"). In the end of introduction it is not 100% clear what substance you will use to examine larvae vulnerability – you wrote 'check the vulnerability of mayfly larvae to petroleum pollution' and in the next sentence '6 concentrations of contaminant', but one can assume that maybe you will use for example a single specific component of the oil.

Methods of material collecting are clearly described. Dividing the mayflies larvae to two groups of sizes was arbitral and this method might be inadequate as you argumented that individuals size might be important predictor of vulnerability to contamination and is one of examined traits. You could, at least, put the millimeter paper under the urine container with collected individuals. Even if in your study it was easy to distinguish between small and big larvae ('the big animals were few times larger than the small') it would be useful to describe size ranges of these two groups. Firstly, someone might want to repeat your study. Secondly - how to use the indicator if we will not know individuals from which group we found in the stream? Information about concentration might be given clearer. 'Diesel fuel was added in concentration of: 3%, 9% and 15%' – I assume that you wanted to say '… was added in an appropriate amount to obtain the following concentrations:… '. The sentence 'to each box aprr. 4 mayflies were added (accurate numbers for each body size are shown in tab.1)' is also inaccurate, because in the table 1. There is only information about TOTAL number of mayflies of given size. After cited sentence I expected details about the number of larvae in each box. Also, you did not explain in methods why you conducted another experiment on small individuals. More information about resignation of some traits ('According to Warne and van Dam (2008) we skipped use of the LOEC (Lowest Observed Effect Concentration) and NOEK (No Observed Effect Concentration) approach.') would give the reader better understanding of what and why you did (or dis not). Graphs descriptions might be clearer. You intelligibly wrote what dashed line shows but nothing about what exactly solid lines means (even if it is obvious after looking on the graph).

As you noticed experimental conditions were significantly different from the natural environment. Moreover, what you did not consider in discussion – maybe providing smaller (therefore: young and probably intensively growing) larvae food might increase their survivability? Also, in natural conditions the water flows and in described experiment it was not. All of these inconveniences in mayflies life may influence the mortality even stronger than examined oil. Finally, the sample size (number of mayflies in experimental containers, about 4) was way too small. To examine mortality in population you should use much bigger sample.

Some rather colloquial expressions has been used in whole report. I recommend you to purify your language (for example 'serious issue might appear' and 'experiment was terminated/completed' instead of 'important problem can be met here' or 'experiment was stopped').

Have a magnificent day with improving your report,

<u>Terézia Horváthová</u>

This study documents how mayflies respond to fuel contamination of water and if this animal group is suitable for bioindicatior-based studies. Such studies are very needed as the contamination of the water sources is becoming a serious issue in the environmental conservation. It is generally known that Ephemeroptera are widely used as bioindicators for oxygen depletion. The information about the effect of another stressor can be used for assessing Ephemeroptera as general bioindicator.

This manuscript is very nicely written. The problem of fuel contamination and use of Ephemeroptera as biondicators is well described (both in introduction and discussion). I generally like the manuscript however I still have some minor concerns.

-I have a problem with lack of species determination in your study. In introduction, you claim that *the easiest way to measure contamination is to check out abundance of all mayfly larvae, without assigning them to particular families.* It might be the easiest way but not the most proper. You can state that assigning to species is not important but you have to have a data to prove it. I would rather turn it around and wrote that your study showed that all mayflies showed similar pattern, however it would be interesting to look at the response of specific species.

-you write about the fuel concentration in percentages (in your aims) but later in the text, you always refer to the volume. I would write volumes in methods but always use percentages (it is more intuitive for the reader).

-you write that you arbitrary divided your animals to big and small group, however this was based on '*big animals were few times larger than the small*'. It does not look good, I would take a subsample, measure them with ruler and state…'small animals were always smaller than xy mm'.

-tables: I don't see the point to include any of your tables. Table 1 says only about the sample size, this can be written as a text.

- What did you mean by this sentence? '*According to Warne and van Dam (2008) we skipped use of the LOEC (Lowest Observed Effect Concentration) and NOEK (No Observed Effect Concentration) approach.'* If you really skip it, you should write what was the reason (to skip it).

-results: Table 2 does not make much sense to me. You found out that your smallest concetration kills almost everything. Thus stating that $LS_{(10,25,50)}$ is always <3% is not proper as the value is not really informative (it can range between 0 to 3%). Also, please check if you wrote a right value for LS_{50} (adults). Is that really larger than 15%?

-graph 1 (correct is Figure 1) is ok but I would use the legend close to your survival lines (write near each line the concentrations, it is much easier to follow)

-I would include short paragraph in the discussion which concerns LS values for different water insect species. The comparison with fish is not very informative.

-you state that '*our result supports the hypothesis that mayflies can be used as good petrol contamination indicator, because they are vulnerable to even small amounts of contaminant'.* In the same paragraph, you write that your results can be strongly affected by the temperature and oxygen which you did not control for. Furthemore, in the conclusion part, you mention that your small sample size and shortage of time most likely biases your results. **So, if your results are biased, how can you wright that mayflies can be used as a good petrol indicator?**

-small comments:

-please consider that we all try to do World but not Polish (eventually Slovak) science. Commas in graphs and tables simply does not exist!

-if the number is at the beginning, always write is as a word (197 in your abstract)

-why is further investigation needed? I agree but I would like to read why.

5.2.4. Final version of report

Usefulness of mayflies (Ephemeroptera, Insecta) as bioindicators of fuel contamination of water

Andrzej Antoł, Aleksandra Piontek, Beniamin Wacławik

Abstract

Search for proper water pollution boindicators is important problem concerning environmental conservation. Mayfly (Ephemeroptera) larvae, which are common, easy to find and also vulnerable to pollution, are good candidates to be such indicators. In this study we tested sensibility of mayfly larvae from Jaszcze stream (Gorce Mountains, Poland) to different concentrations of diesel petrol. To detect size-dependent effect we divided larvae into two groups: large and small. We tested specimens for concentrations: 15 %, 9 %, 3 %, 1%, 0,5 %, 0,25 %. Study showed high vulnerability of these larvae and significant size-dependent difference (higher mortality of small ones). Mayfly larvae in general can be used as proper bioindicators of fuel water pollution and small onesare more sensitive indicators although further studies on particular families should be conducted.

Keywords: macroinvertebrates, oil pollution, streams, bioindication

Introduction

Anthropogenic water pollution is one of the biggest environmental problems (Fleeger et al. 2003). Pollution coming from fuel leaks – both results of big catastrophes, such as Exon Valdez in 1989 and also everyday human activity is significant part of this problem. Oil pollution has big influence on biota living in fresh and sea water, but sometimes because of lack of proper bioindicators it is hard to detect. Water macroinvertrebrates, for example many beetles (Coleoptera), crustaceans (Crustacea) or mayfly (Ephemeroptera), caddisfly (Trichoptera) and stonefly (Plecoptera) larvae are useful bioindicators of water pollution. However, to obtain proper information about contamination, level of their vulnerability to particular substances must be known.

To detect such destructive and common pollution as fuel pollution, the best way is to find bioindicators that might be used fast and easily. Mayflies larvae are good candidates to be used as petroleum contamination bioindicators, because of their abundance in environment, accessibility during long part of year (Landa 1968) and vulnerability to oil pollution (Russell et al. 1979).

In mayfly live cycle, dominant stadium is nymph, which may live even for some years, while the adult often lives for one day (Landa 1968). Moreover, these nymphs are usually very abundant and are one of dominant taxonomic groups in running water benthos, which makes them more useful than other groups, which are not available for such a long time in large numbers. Mayflies are vulnerable to natural and anthropogenic contaminations (Wesner et al., 2014), thus, they are widely used as bioindicators (Kłonowska-Olejnik et al. 2012, Kłonowska-Olejnik & Skalski, 2014). Abundance of mayfly larvae is part of EPT index (Ephmeroptera + Plecoptera + Trichoptera) which is considered as proper way to measure level of environment degradation (Hickey & Clements 1998). Being quite sensitive to industrial pollution, populations of mayfly nymphs has lower densities in part of streams below places in which this kind of leaks appeared (Schloesser et al. 1991).

The easiest way of preliminary measurements of water contamination is to check out abundance of all mayfly larvae, without assigning them to particular families (which should be done later). Thus, in our research we want to find if there is some similar pattern of all mayfly larvae reaction to the diesel petrol. But one important problem can be met there - because mayflies moult and swarm more than once a year and also different species and populations moult in different periods (Landa 1968), larvae of many ages and sizes appear in one habitat. Many experiments showed that small stream invertebrates are more sensitive to contamination than large ones (Kiffney and Clements 1996) and this is also connected with life cycle stadium (McCahon et al. 1989).

The aim of our study is to check the vulnerability of mayfly larvae to petroleum pollution in different concentrations in relation to their body size.

Materials and methods

We collected animals in Jaszcze mountain stream in Ochotnica Górna, Gorce mountain range, southern Poland. Animals were collected by hand, from stones. After the collection we separated the mayflies from other similar taxonomic groups using the binocular. Then we arbitrarily divided them into two body size categories: big (more than 6 mm of body length) and small (less than 6 mm of body length).

We put 40 ml of stream water into each of 32 plastic vessels of 100 ml volume (Fig. 1). Then we added diesel fuel to obtain concentrations of: 3%, 9% and 15%. In control group there was stream water only. We chose such concentrations basing on Rusell et al. (1979) study on vulnerability of caddisflies to oil. They used following concentrations: 0,27 %, 0,53 %, 1,06 %, 2,12 %. Their experiment lasted for few days and most of studied individuals survived. Thus, because our experiment was planned for only 5 hours, we decided to use set of higher concentrations, with the lowest being similar to the highest from Rusell's work. Relatively big intervals between values of concentrations were chosen to avoid situation when samples in all concentrations will give the same results.

In each treatment we prepared 4 replicates. To each vessel we added appr. 4 mayflies. After that we recorded the mortality of individuals every hour. To provide the oxygen we blew air into water each 30 minutes using short plastic pipe . After 5 hours we stopped the experiment.

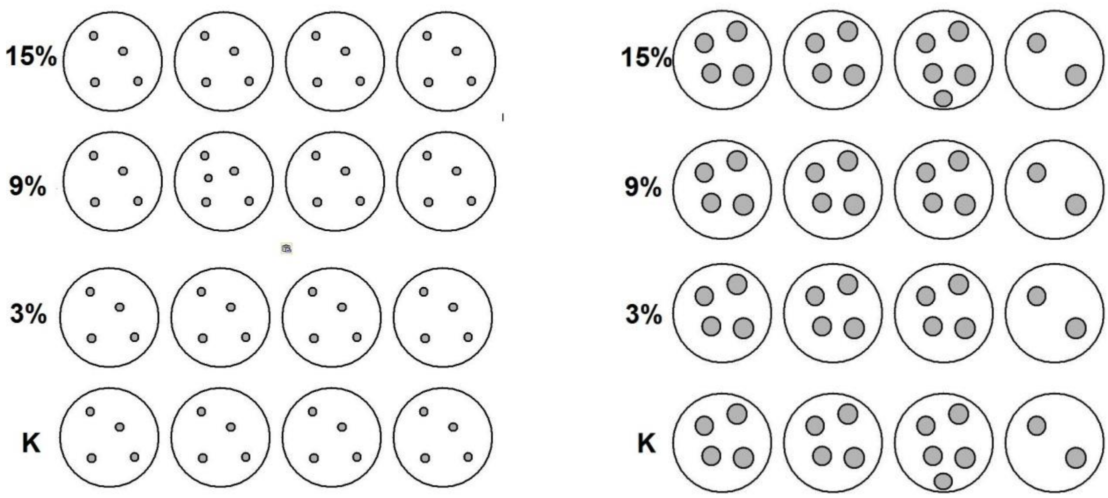


Fig 1. Design scheme of the first experiment. Total number of 32 vials containing 65 small larvae (left set) and 58 big larvae (right set). The % numbers show the percentage of oil in total volume of solution. K – control

After this experiment the mortality among small individuals was very high and mortality in their control group was over 60 %. In short exposure toxicological tests, results are not valid if control mortality exceeds 10 % (Rand et al., 1985). Thus, next day we conducted second experiment only on small individuals (n = 64) using lower fuel concentrations (0,25%, 0,5% and 1%) to obtain the values of LC_{10}, LC_{25}, LC_{50} (the lowest concentrations of toxicant in which 10, 25 and 50% of animals are dead after certain amount of time respectively (Robidoux et al. 1999)) for small animals more precisely (Fig. 2).

We counted these values for all animals in one concentration trail (appr. 16 individuals in first experiment and 16 in second).

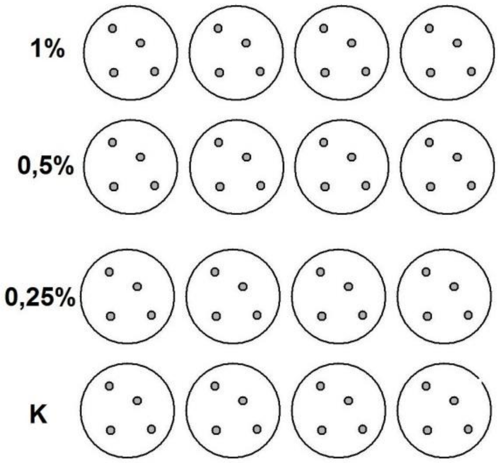


Fig 2. Design scheme of the second experiment. Total number of 16 vials containing 64 small larvae. The % numbers show the percentage of oil in total volume of solution. K – control

We analyzed time to death of every individual with Cox hazard model with size as qualitative and concentration as quantitative variable. We treated data from individuals which survived during the whole experiment as censored data. This method is used to compare the survivability curves and also other variables - such as the time which animals require to do some activity (Czarnoleski et al. 2011).It also allows to include censored data (as in our case) and do multifactorial analyses including interactions. Statistical analyzes were done using the STATISTICA 10 software (StatSoft, Inc. 2011).

Results

The high mortality caused by presence of diesel fuel was observed already after the first hour of experiment. The values of LC_{10}, LC_{25}, LC_{50} obtained in the first experiment after the first hour of exposition was for small larvae lower than 3%. For large larvae LC_{10}, LC_{25} were also lower than 3%, and only LC_{50} was higher than 15%.

The analysis of Cox model showed the significant influence of size (Waldχ^2 =27.56, $p<0.0001$) and concentration (Waldχ^2=11.51, $p=0.007$) on larvae survival. The survival decreased in the gradient of diesel fuel concentration and was higher among big animals (Fig. 3, Fig. 4). Interaction treatment x size was not significant (Waldχ^2=2.76, $p=0.097$).

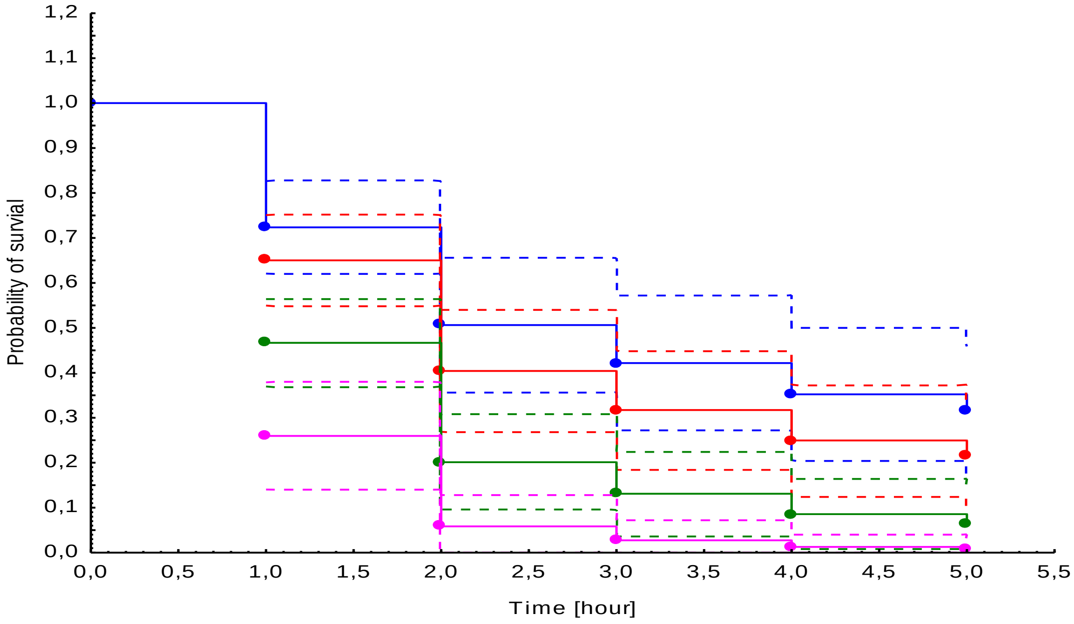


Fig 3. Function of survival in time for the small animals. Dashed line show 95% CI of the survival curve (solid line). Colour of line shows the concentration of diesel fuel in the treatment: blue line – 0% of diesel fuel, red line – 3% of diesel fuel, green line – 9% of diesel fuel, pink line – 15% of diesel fuel

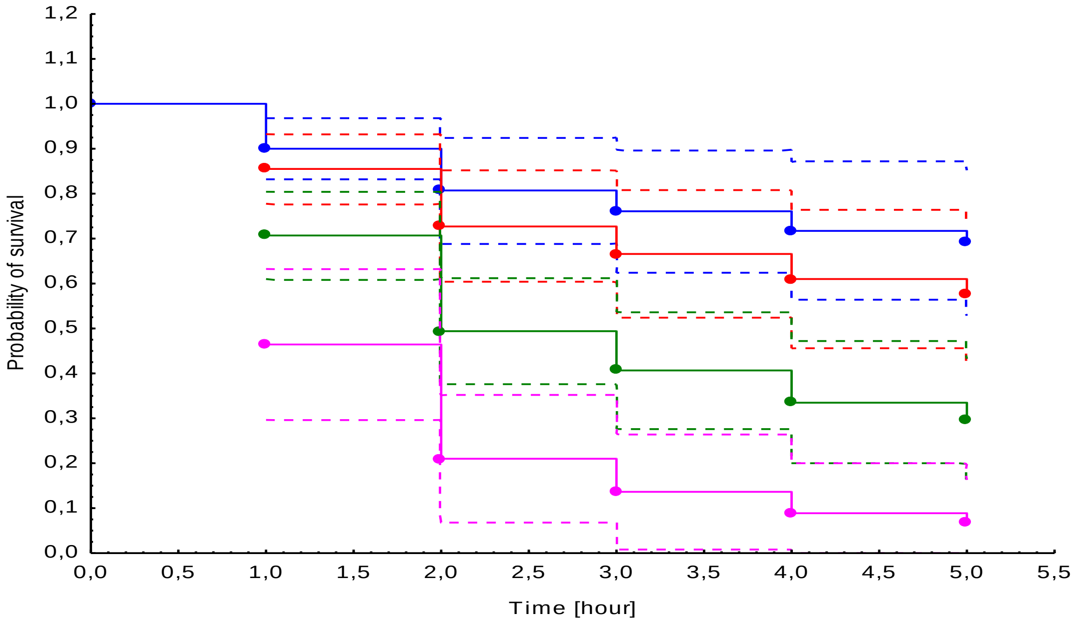


Fig 4 Function of survival in time for the big animals. Dashed lines show 95% CI of the survival curve (solid line). Colour of line shows the concentration of diesel fuel in the treatment: blue line – 0% of diesel fuel, red line – 3% of diesel fuel of, green line – 9% of diesel fuel, pink line – 15% of diesel fuel.

In the second experiment we also observed high mortality after one hour among small larvae and LC_{50} after one hour was below the lowest concentration for all trails. The Cox model analysis for second experiment showed significant influence of the treatment (Waldχ=8,59, p=0,003) (Fig. 5).

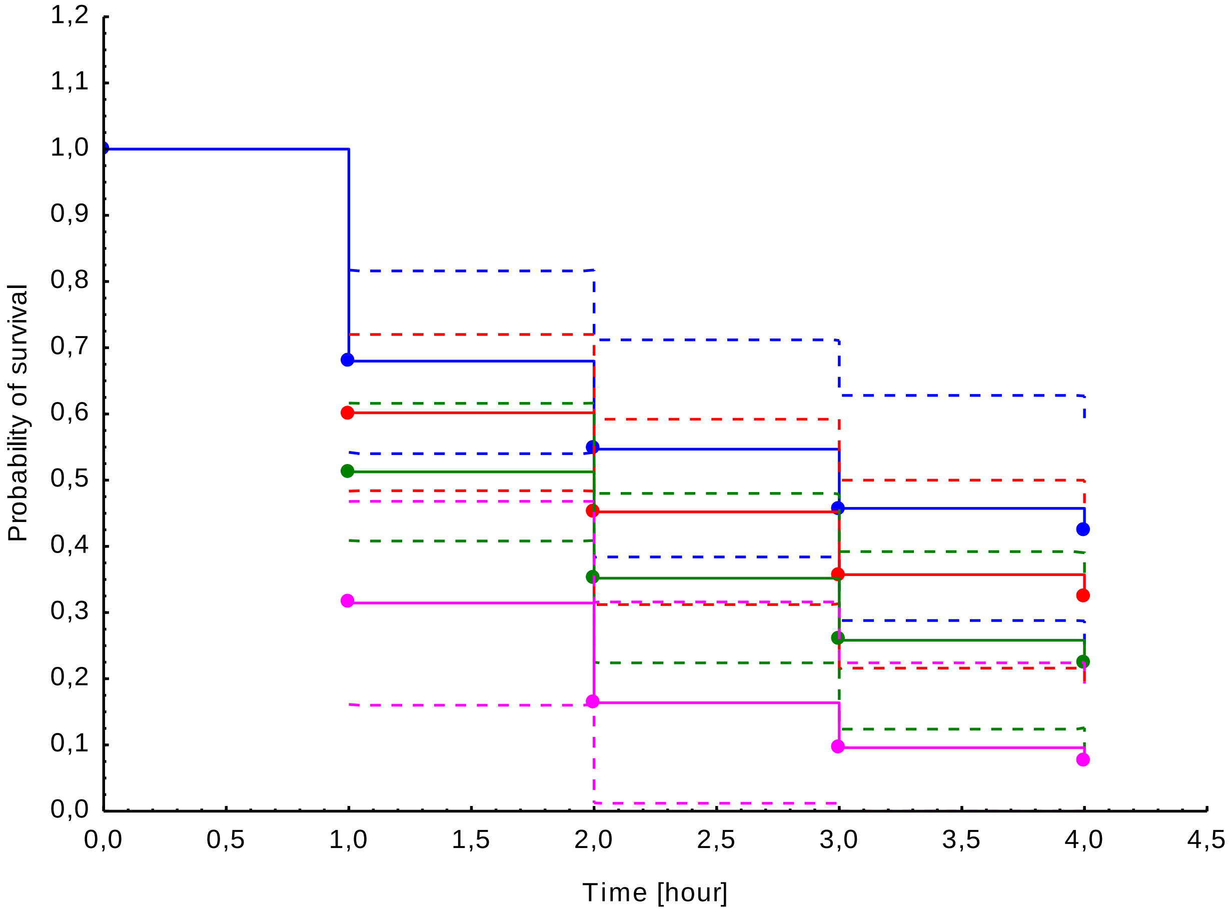


Fig 5. Function of survival in time for the small animals from the second experiment. Dashed lines show 95% CI of the survival curve (solid line). Colour of line shows the concentration of diesel fuel in the treatment: blue line – 0% of diesel fuel, red line – 0.25% of diesel fuel of, green line – 0.5% of diesel fuel, pink line – 1% of diesel fuel.

Discussion

All larvae in our study were vulnerable to oil pollution. For the highest contamination value (15%) almost no individual survived for more than 2 hours. The LC_{10} values for 1 hour exposure were lower than 3% for both small and big larvae. This confirms the value of mayflies as a bioindicators of petroleum pollution.

Studies on mayflies in natural conditions had shown the strong negative effect of oil spill on local populations of mayflies. Oil pollution of sediment was probably main reason of disappearing of *Hexagenia* mayflies from the Great Lakes (Schloesser et al. 1991). Also after spill of oil in Asher Creek, Missouri mayflies were not detected in the water for 9 months (Ort et al. 1995).

The long recovery time is connected to larvae mortality caused by direct exposure to oil but also lower rates of colonization of oil-contaminated substrates (Ort et al. 2005). Rosenberg et al. (1980) found lower numbers and diversity of species of mayfly occupying the stones contaminated with oil even after four months of colonization. We tested only the short-term influence of petroleum contamination on larvae survival but we assume that even small contamination may reduce the density and diversity of mayfly populations in long time after the leak spill.

The small larvae were much more vulnerable to oil contamination than big ones. The LC_{50} value for 1 hour exposure was below 3%. Both fish and invertebrates are known to exhibit size-dependent sensitivity to contaminants (Kiffney & Clements 1996). Kiffney and Clements (1994) found that small larvae of mayfly *Drunella grandis* were more sensitive to exposure on Cd, Cu and Zn than bigger larvae from the same population.

In our study we observed high mortality of larvae, also in the control groups (even over 60% in small larvae group after 5 hours in first experiment). The mayfly larvae are vulnerable not only to anthropogenic pollution but also naturally occurring factors such as temperature changes (which directly influence the water oxygen level) (Bridgeman et al. 2006). Thus, we think that higher mortality of small larvae in our study may be also the result of different temperature and oxygen level in comparison to their natural environment. Also other factors, such as lack of food or injury of individuals during moving to vessels could contribute to overall mortality in our experiments. Studies in better controlled conditions which imitate the natural conditions should be conducted to minimize the effect of abovementioned factors on experiment. However, problem of general vulnerability of mayflies to many different factors and substances must be considered also during bioindication, to avoid incorrect association of the absence of these insects with only single factor. Good way to supplement data obtained by this study on mayflies would be to take into consideration different groups such as Plecoptera and Trichoptera (Hickey et al 1998).

Our results support the hypothesis that mayflies can be used as good petrol contamination indicator, because they are vulnerable to even small amounts of contaminant. The size-specific response to contaminant would allow to indicate the fuel contamination just by estimating the amount of small individuals. Also without assigning individuals to the species we found similar response of larvae to contamination. Quite fast response to contaminant can be important especially when we observe the situation directly after petrol leak. This means that in some preliminary or general studies they can be treated as a group (or as a two groups:small and large larvae), but to obtain reliable and detailed results, further studies on particular families must be done. Due to lack of time it was impossible to study high number of individuals, thus we consider this report to be a pilot study and encouragement for more detailed research.

Acknowledgments

We would like to thank all participants of Evolutionary ecology – methodological workshop in Ochotnica Górna for the discussion and good advices and Mr Marian Krzyśko for donating the diesel fuel which we used in our research.

References

Bridgeman, T. B., Schloesser, D. W., & Krause, A. E. (2006). Recruitment of Hexagenia mayfly nymphs in western Lake Erie linked to environmental variability. *Ecological Applications 16*(2), 601-611.

Czarnoleski M., Muller T., Kierat J., Gryczkowski L., Chybowski Ł. (2011). Anchor down or hunker down: an experimental study on zebra mussels' response to predation risk from crayfish. *Animal Behaviour 82,* 543-548.

Fleeger J.W., Carman K.R., Nisbet R.M. (2003) Indirect effects of contaminants in aquatic ecosystems *The Science of the Total Environment 317* 207–233.

Hickey C.W., Clements W.H. 1998 Effects of heavy metals on benthic macroinvertebrate communities in New Zealand streams *Environmental Toxicology and Chemistry*, *Vol. 17, No. 11*, 2338–2346.

Kiffney, P. M., & Clements, W. H. (1994). Effects of heavy metals on a macroinvertebrate assemblage from a Rocky Mountain stream in experimental microcosms. *Journal of the North American Benthological Society*, 511-523.

Kiffney, P. M., & Clements, W. H. (1996). Size dependent response of macroinvertebrates to metals in experimental streams. *Environmental Toxicology and Chemistry*, *15*(8), 1352-1356.

Kłonowska-Olejnik M., Skalski T. (2014) The effect of environmental factors on the mayfly communities of headwater streams in the Pieniny Mountains (West Carpathians) *Biologia 69/4 Section Zoology*: 498—507,

Kłonowska-Olejnik, M., Stoch, T., & Skalski, T. (2012) Zgrupowania jętek (Ephemeroptera) w górnej części zlewni Koniny w Gorczańskim Parku Narodowym (Karpaty Zachodnie). *Ochrona Beskidów Zachodnich, 4*: 57–68.

Landa V. (1968) *Developmental cycles* of Central European *Ephemeropte*ra an their interrelations. *Acta Entomologica Bohemoslovaca* 65: 276-284

McCahon C.P., Whiles A.J., Pascoe D., 1989 The toxicity of cadmium to different larval instars of the trichopteran larvae *Agapetus fuscipes Curtis* and the importance of life cycle information to the design of toxicity tests. *Hydrobiologia Volume 185, Issue 2*, 153-162

Ort, M. P., Finger, S. E., & Jones, J. R. (1995). Toxicity of crude oil to the mayfly, *Hexagenia bilineata* (Ephemeroptera: Ephemeridae).*Environmental Pollution*, *90*(1), 105-110.

Rand, G.M.; Petrocelli, S.R., (1985). *Fundamentals of aquatic toxicology: Methods and applications*. Washington: Hemisphere Publishing.

Robidoux, P. Y., Hawari, J., Thiboutot, S., Ampleman, G., & Sunahara, G. I. (1999). Acute toxicity of 2, 4, 6-Trinitrotoluene in Earthworm (*Eisenia andrei*). *Ecotoxicology and environmental safety*, *44*(3), 311-321.

Russel, P. P., Resh, V. H., & Flynn T. S. (1979). A continuous flow bioassay technique for assessing the toxicity of oil-shale-related effluents: Preliminary results with two species of caddisfly larvae. *Oil Shale Symposium. Sampling, Analysis and Quality Assurance. March 197,* 416-431

Schloesser, D. W., Edsall, T. A., Manny, B. A., & Nichols, S. J. (1991). Distribution of Hexagenia nymphs and visible oil in sediments of the Upper Great Lakes Connecting Channels. *Hydrobiologia*, *219*(1), 345-352.

Warne, M. S. J., & van Dam, R. (2008). NOEC and LOEC data should no longer be generated or used. *Australasian Journal of Ecotoxicology*, *14*(1), 1.

5.3. Trade-off between stone size and number in caddisfy larvae cases

5.3.1 Project proposal

Summary

Larwy chruścików domkowych budują przenośne domki różnej konstrukcji i z różnego materiału. Szkielet jest utkany z wydzieliny gruczołu przędnego, a do niego dolepiony jest różny budulec: ziarna piasku, kamyki, fragmenty roślin wodnych. Domek chroni miękki odwłok larwy i w miarę wzrostu jest rozbudowywany. Wybiórczość materiału do budowy domków jest dobrze udowodniona w literaturze. Na przykład larwy Goera japonica wybierają kamyki o gładkiej powierzchni, co wiąże się z lepszym przepływem tlenu (Okano i Kikuchi 2009). Jedną z funkcji domków jest także ochrona przed drapieżnikami. Według Otto (2000), pomimo większych nakładów energetycznych, chruściki wybierają cięższe kamyki, które stanowią lepszą ochronę przed drapieżnikiem. Natomiast przyłączenie dodatkowych kamieni do zbudowanego już domku daje lepszą ochronę przed porwaniem przez prąd wody (Otto i Johansson 1995).

Wyprodukowanie nici do budowy domku wynosi 12% wydatków energetycznych larwy, co jest wysokim kosztem biorąc pod uwagę siedliska, w których żyją (Otto 1974). Inwestowanie większej energii w budowę domków młodszych stadiów larw może skutkować wytworzeniem lżejszego toraxu i skrzydeł u imago (Stevens i inni 1999). Na podstawie badań Otto (1982) larwy chruścików wybierając materiał jakim są kamyki zużywają więcej jedwabnej nici do budowy domków, co skutkuje tym, że ich formy dorosłe osiągają mniejsze rozmiary ciała niż te z rozwijające się w domkach z liści czy patyków. Mając dostęp tylko do kamyków chruściki mogą wybierać większe kamyki, aby produkować mniej jedwabnej nici, a co za tym idzie ponosić mniejsze koszty jej produkcji.

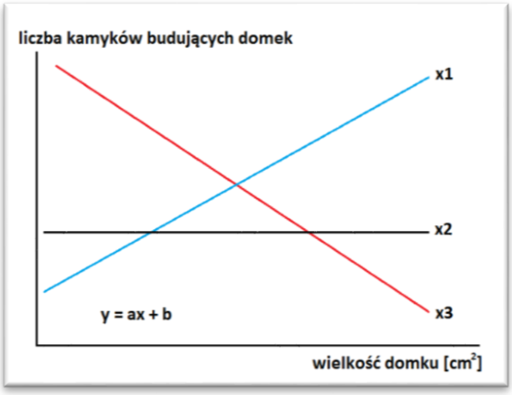
Aim / hypothesis

Zmiana wielkości domków zależy od zmiany wielkości kamieni, a nie od ich ilości.
Budowanie domków z większych kamyków jest mniej kosztowne energetycznie (wytwarzanie jedwabnej nici) niż budowanie z większej ilości drobnych kamyków.

Methods

Domki pozostałe po chruścikach zostaną zebrane spod kamieni w potoku Jaszcze. Wybierane będą domki jednego typu – zbudowane z kamieni. Wykonane zostaną zdjęcia domków na tle papieru milimetrowego. Na ich podstawie w programie ImageJ dokonane zostaną pomiary powierzchni domków. Następnie policzone zostaną kamyki budujące każdy z domków. Zastosowana zostanie metoda regresji liniowej w celu zbadania zależności pomiędzy powierzchnią domku, a liczbą kamyków, które go budowały.

Impact of results



Główny wynik proponowanego projektu zostanie przedstawiony w postaci regresji liniowej. W zależności od wartości współczynnika kierunkowego x w równaniu y=ax+b możliwe będą następujące interpretacje:

- x1 (dla x>0): wzrost wielkości domku spowodowana jest użyciem większej ilości kamyków

- x2 (dla x=0): wzrost wielkości domku spowodowana jest użyciem większych kamyków

- x3 (dla x<0): wzrost wielkości domku spowodowany jest użyciem mniejszej ilości większych kamyków

Otrzymane wyniki pozwolą na wnioskowanie na temat strategii dobierania materiału budulcowego do domków przez larwy chruścików oraz alokacji energii w wytwarzanie sieci jedwabnej.

References

Okano J. and Kikuci E. (2009) The effects of particle surface texture on silk secretion by the caddisfly *Goera japonica* during case construction. Animal Behaviour **77**: 595-602

Otto, C. 1974. Growth and energetics in a larval population of Potamophylax cingulatus (Trichoptera) in a South Swedish stream. *J. Anim. Ecol.* **43:** 339-361.

Otto, C. 1982 Habitat, size and distribution of Scandinavian limnephilid caddis £ies. Oikos **38**, 355-360.

Otto C. and Johansson A. (1995) Why do some caddis larvae in running waters construct heavy, bulky cases? Animal Behaviour **49**: 473-478

Otto, C. (2000). Cost and benefit from shield cases in caddis larvae. Hydrobiologia, **436**(1-3), 35-40.
Stevens, D. J., Hansell, M. H., Freel, J. A., & Monaghan, P. (1999). Developmental trade–offs in caddis flies: increased investment in larval defence alters adult resource allocation. Proceedings of the Royal Society of London. Series B: Biological Sciences, **266**(1423), 1049-1054.

5.3.2. First version of report

Summary

The building material selection strategy used by caddisfly larvae to build cases was examined. Cases of this larvae were collected from natural stream. 66 cases were analysed in the laboratory conditions. Surfaces of each case, total stones number and the biggest stones ratio were measured and counted. In this work we proved that caddisfly larvae use more stones and simultaneously more bigger stones to build bigger cases. Relationship between surface of cases and number of all stones was high statistically significant ($R=0.83$, $R^2= 0.69$, $p=0.000$), similarly proportion of the biggest stones and caddisfly cases was statistically significantly positive ($R=0.404$, $R^2=0.16$, $p<0.001$). Usage by the caddisfly larvae bigger stone is a strategy to reduce energetic cost which is reflected with lower production and secretion of the silk. Choice of resources often represents the best achievable balance of costs and benefits. Energetic costs for case construction are aff ected by case architecture, as the volume of silk required cementing organic or mineral particles together increases with decreasing particle size.

Keywords: caddisfly larvae, stone cases, stone size, selection strategy, optimization

Introduction

Caddisfly larvae build the cases with a variety design and from different materials such as small stones, grains of sand, parts of the water plants or leaves. The skeleton of the cases is made from the silk which is produced by silk gland. Secretion of silk is considered as essential for the evolutionary success of caddisfly larvae (Mackay and Wiggins, 1979). Caddisfly larvae secrete silk to line the inner walls of their portable cases to create smooth surfaces that enhance their respiratory efficiency (Okano and Kikuchi 2009). Functions of the cases are diverse. One of them is protection against predators or cannibalism. According to Otto (2000), despite of the higher energetic cost for production silk, caddisfly choose bigger stones which are the better protection against predations. The influence of larval case design on vulnerability to predators was evaluated in a series of laboratory experiments. Nislow and Molass (1993) conduct the experiment where caddisfly larvae still occupying their field-built cases despite of exposing to the two predators. Following 24 h exposure of caddisfly larvae to a specific predator, the pre-trial case length, case strength, case width, and case composition of victims and survivors were compared. Principal components analysis (PCA) indicated substantial independent variation for all four case parameters in the study population. The cases of larvae surviving exposure to either dragonfly naiads or fish were significantly stronger and wider than cases of victims of predation. Cases of survivors were also longer and had a greater mineral fraction than those of victims, but differences were either marginally significant or not significant. Moreover the addition of extra stones protect caddisfly larvae to current flow (Otto and Johansson 1995). Milne (1938) suggested the case may facilitate respiration in an aquatic environment, and Williams et al. (1987) presented empirical evidence indicating that this is indeed a function of cases in some species. Many aquatic predators forage using visual cues, and it has also been suggested that the cases function is to camouflage the larva inside.

Production the silk to build the case is 12% of the energetic cost caddisfly larvae, which is the high cost in environment where they live (Otto 1974). Investing more energy to building the cases with bigger stones by the younger stages of caddisfly larvae can result lighter thorax and the wings in adults (Stevens i inni 1999). On the basis of Otto (1982) research, larvae of caddis selects stones to building cases secrete more silk, what can result in imago forms achieve smaller body size than those which growth up in cases from leaves or sticks. If they have only stones in the habitat where they live**,** they can choose larger stones to produce less silk and bear less cost of energy to it production.

In this study we examined a trade-off occurring in enlarging case size. From one hand caddis-fly larvae can use more lighter (smaller) stones which demand less energy to transport them (but then it needs more costly silk to stick them one to another). From the other hand it may be beneficial to use bigger stones and produce less silk. To determine which from these two strategies is performed in order to enlarge case size we examined the relationship between the cases surface and the total number of stones which build the cases. We also check if the proportion of big stones is higher in bigger cases.

Material and Methods

We took 95 cases of the caddisfly larvae from the Jaszcze stream in Ochotnica Górna (National Park of Pieniny). Only cases build from stones were chosen. All cases were built with the same type of stones and most of them were taken from one big stone submerged in stream. This means that all cases might be belong to one species. 29 cases were incomplete, therefore were rejected from the experiment. Left 66 cases were separated individually on petri dishes and a piece of paper with the sample number was put into each dish (Fig.1). Each case was photographed with Sony DSC HX300 camera. Each case surface was measured ImageJ software (Fig.2 and Fig.3). Subsequently all separated stones from each case were counted. After that all stones from each case were sieved with kitchen sieve (net size 1.6 mm) in order to isolate and count the biggest stones. The big stones to all stones ratio was calculated to estimate the amount of big stones from each case.

To examine the relationship between case surface area and total amount of stones we used linear regression. The same analysis was used to show the relationship between case surface area and big stones to all stones ratio. For all statistical analyses the Statistica ver. 10 software was used.

Fig. 1. Caddis-flies cases used in our study

Fig. 2. An example of photograph used for measurements

Fig. 3. Green color – case outline

Results

Results of our experiments are showed on Fig 4. and Fig.5. Relationship between surface of cases and number of all stones is highly statistically significant (R=0.83, R^2= 0.69, p=0.000). Majority of caddisfly larvae had cases built with total stone number between 50 to 90 (visible on Fig.4).

Relationship between proportion of the biggest stones and caddisfly cases was statistically significant and was positive (R=0.404, R^2=0.16, p<0.001). The most frequent number of big stones was between 7 and 10 (visible on Fig.5).

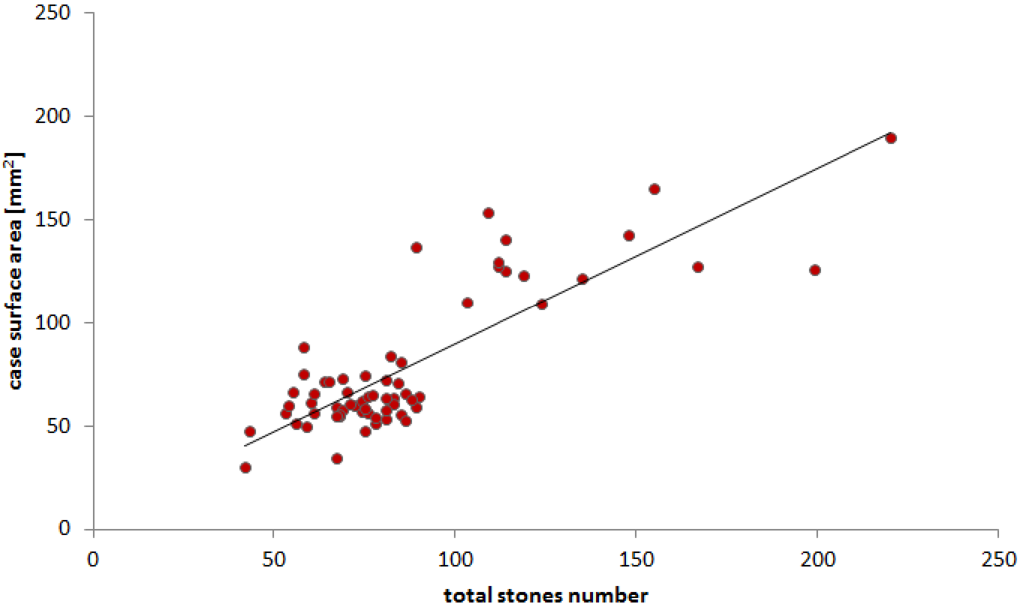


Fig. 4. The more stones, the bigger surface of case area is.

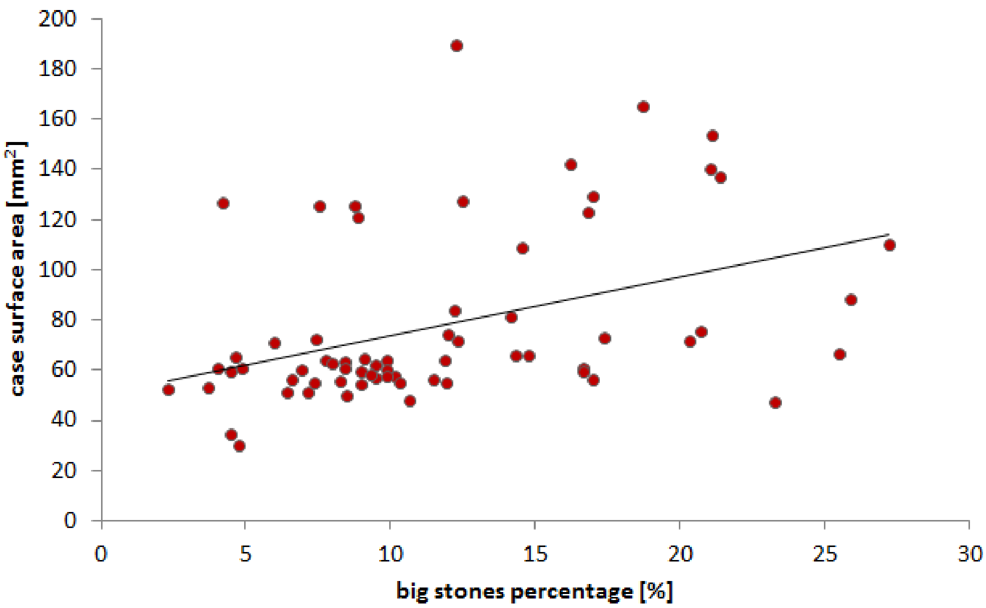


Fig. 5. The bigger the case, the higher big stones ratio is.

Discussion

Our experiment proves that caddisfly larvae use more stones to build bigger cases. Cases size strongly depends on the total number of stones. It might be linked with larvae maturation. Caddisflies have a several larvae stadium. As the larva became older and molt several times it periodically add new material to the anterior part of the case (Hansell 1972).

Caddisfly larvae also strongly selected the material which they use to build the cases. It usually depends on the season and different materials which are available in environment. Moretti and Loyola (2005) showed that *Barypenthus concolor* cases depends directly on the particle size composition present in the substrate. To build their cases *B. concolor* larvae use particles sizes in the same proportion as they are available in the habitat. Cases with homogeneous particle sizes could be less resistant to predators pressure. Also the insects could spend more time and require more adhesive substance to build their cases, which would represent a significant increase on energy costs, and cases architecture could differ from the one necessary for adequate breathing. Also the chasing material preference is influenced by local geology. They preferred smooth than rough particles, what enhance their respiratory efficiency (Okano and Kikuchi 2011).

According to Hansell (1968) after the removal of one anterior large particle and the anterior roof particle caddisfly larva repaired both roof and sides of its house with the same sized particles. It was concluded that this behavior was slightly modified in direction to small particle selection and that large particle selection behavior was blocked. It is suggested that large particles are selected according to the criterion of size, and probably not weight. Caddisflies shows a preference for using particles of certain grain size. Species building cases preferably with a certain grain size range of mineral particles switch to grain sizes near the range limits of the unavailable, normally preferred one (Hanna 1961, Tolkamp 1980). Concerning the control of the case architecture by the larvae, caddisflies use mouthparts and legs as tools to measure the particle size required at a given moment of case construction and to select the appropriate mineral size, or to shape organic material accordingly (Gorter, 1931; Hansell, 1974; Stuart and Currie, 2001). In addition, bristles and other sensors provide information about the required case length or diameter (Hansell 1973). As the size of the tools and the distance between the sensors increase during larval growth, the size of the building material and the case itself typically become larger with increasing larval size (e.g., Gorter, 1931; Hanna, 1961; Tolkamp, 1980), and many species change the overall case architecture in a certain development stage (Dudgeon 1990). Our research also provided that stone size is important in the selection of material which can be useful for case building. Caddisflies choose more bigger stones to build bigger cases. The proportion of big stones from cases in relation to whole cases was strongly statistically significant. There is connection with energetic cost which they must use to production silk which cemented organic particles. Optimality modeling suggests that the choice of resources often represents the best achievable balance of costs and benefits. These energetic costs for case construction are aff ected by case architecture, as the volume of silk required cementing organic or mineral particles together increases with decreasing particle size (Smart, 1976; Becker, 2001). Choosing larger stones caddisfly larvae can save the energy for the production and secretion silk.

This results provided that caddisfly larvae have strong preference for stones which are available in sediment. Using bigger stones allow them either minimize the energetic cost to produce the silk and increase the probability of surviving predators attacks.

Acknowledgements

We want to thank whole PhD student group which were participants in Ochotnica course for valuable comments and help in preparing this report.

References

Becker G., Larval size, case construction and crawling velocity at diff erent substratum roughness in three scraping caddis larvae, Archiv für Hydrobiologie 2001, 151:317–334

Gorter F.J., Köcherbauversuche an Trichopterenlarven, Z. Morph. Ökol. Tiere 1931, 20:443–532

Hansell M.H., The house building of caddis larvae: a source of projects for schools, Journal of Biological Education 1974, 8:88–98

Hanna H.H., Selection of materials for case-building by larvae of caddis flies (Trichoptera), Proceedings of Royal Entomological Society in London (A) 1961, 36:37–47

Hansell M.H. (1968) The house building behaviour of the caddis-fly larva *Silo pallipes* fabricius: III. The selection of large particles. Animal Behaviour **16**: 578–584

Hansell M.H. (1972) Case building behaviour of the caddis fly larva, *Lepidostoma hirtum.* Journal of Zoology 167: 179–192

Mackay R.J., Wiggins G.B., Ecological diversity in Trichoptera, Annual Review of Entomology 1979, 24:185–208

Milne M.J., Case-building in Trichoptera as an inherited response to oxygen deficiency, The Canadian Entomologist 1938, 70:177–180.

Moretti, M. S., & Loyola, R. D. (2005). Does Barypenthus concolor Burmeister (Trichoptera: Odontoceridae) select particles of different sizes for case building?. *Neotropical Entomology*, *34*(2), 337-340.

Nislow K. H., & Molles M. C. (1993). The influence of larval case design on vulnerability of Limnephilus frijole (Trichoptera) to predation. Freshwater Biology, 29(3), 411-417.

Okano J. and Kikuci E. (2009) The effects of particle surface texture on silk secretion by the caddisfly *Goera japonica* during case construction. Animal Behaviour **77**: 595-602

Okano J., Kikuchi E., Sasaki O., Ohi S., Geological variation in particle surface-roughness preference in the case-bearing caddisflies, Behavioral Ecology 2011, 22:1053–1063

Otto, C. 1974. Growth and energetics in a larval population of Potamophylax cingulatus (Trichoptera) in a South Swedish stream. *J. Anim. Ecol.* **43:** 339-361.

Otto, C. 1982 Habitat, size and distribution of Scandinavian limnephilid caddis £ies. Oikos **38**, 355-360.

Otto C. and Johansson A. (1995) Why do some caddis larvae in running waters construct heavy, bulky cases? Animal Behaviour **49**: 473-478

Otto, C. (2000). Cost and benefit from shield cases in caddis larvae. Hydrobiologia, **436**(1-3), 35-40.

Smart K., A progress report on the building motivation in the caddis larva, Lepidostoma hirtum, Proceedings of 1st International of Symposium on Trichoptera 1974, 185–186

Stevens, D. J., Hansell, M. H., Freel, J. A., & Monaghan, P. (1999). Developmental trade–offs in caddis flies: increased investment in larval defence alters adult resource allocation. Proceedings of the Royal Society of London. Series B: Biological Sciences, **266**(1423), 1049-1054.

Tolkamp H.H., Organism-substrate relationships in lowland streams, Agricultural Research Report Wageningen 1980, 907:1–211

Williams D.D., Tavares A.F., Bryant E.,Respiratory device or camouflage? - a case for the caddisfly, Oikos 1987, 50:42–52.

5.3.3. Reviews

Prof dr hab. Adam Łomnicki

Generally, I have not corrected the English of this report but if there were some obvious mistakes, I have introduces changes or if something was hard to understand I placed question marks on the msc.

The title of the report promises much more than it actually brings about. To determine trade-off, one should know the distribution of stone sizes in the habitat and then to find out what is the cost of finding or manipulating of larger stones as compared to saving of silk when using these larger stones. This was obviously not done. What we have really learned from this report is (1) that larger cases require more stones and (2) that in the larger cases the proportion of larger stones is statistically higher. The first statement is an obvious one and does not required any special explanation. Since the size of the case is related to the age of the larvae, when it increases, its size is increased by adding new stones. More interesting is the increase of the proportion of the larger stones in larger case. Unfortunately, this was not sufficiently discussed in this report. Since the stone of different size in the case make a better protection against predators and since larger stones require less silk, the problem is why they do not occur more often also in smaller cases. Are smaller larvae unable to manipulate with larger stones or are they not available in the habitat? This should be thoroughly discussed. In spite of these shortcomings, there are plenty of information from the literature on the biology of caddisflies which are not related to the size of stones that build the cases.

Two statement in the Material and Methods are not clear. What does it mean the caddisflies with their case where collected from the big stones submerged in water. Obviously not from the upper part of this stone but rather from the bottom of the stream after replacing this large stone. This should be described in more details together with the size of this large stone. What you have received after dividing number of large stones by the number of all stones is a proportion which can be also expressed as a percentage. The ratio would be if you divide number of large stones by the number of smaller stones. And finally, I do not understand what for you need 'the amount of large stones', the proportion or percentage is sufficient and you have only used it. The amount is not mentioned in the Results.

I do not know what for is Fig. 1 and I do not understand of the description of Fig. 3. The description of Fig. 5 is very naïve. This relation is not an obvious one but only statistically significant.

Dr Aleksandra Walczyńska

The report focuses on the issue anchored deeply in the theoretical evolutionary biology, a test for a trade-off. This is an interesting and ambitious idea, because looking for and testing trade-offs is very difficult empirically. It is therefore a valuable attempt of contributing in the field of the basic evolutionary theory. Below are my comments on what should be improved in the presented report.

Summary:

The whole summary section is somehow messy. It definitely lacks the traditional structure: background-method-results-conclusions-contribution.

Introduction:

1. It is too wordy. The considerable amount of information given is not necessary for this study.
2. The aim of the study is elusive. Testing whether a larger case is made of the bigger number of stones is quite trivial. Especially that, according to my intuition, enlarging a cylinder by using the smaller number of bigger particles is not necessarily effective.

3. What was traded off with what? I understand that the difference is in the costs of silk production vs. the costs of energy used for stones transport. The former is stated directly, while the latter is somehow treated as embarrassing and not enough space is devoted to it.
4. There is a lack of a clearly stated hypothesis, what has further consequences (see the comments to Methods and Discussion).

Methods:

1. There is no information on how was the accurate scale of photographed cases achieved. One needs to look at Fig. 1 to see that the millimeter paper was used for that.
2. In my opinion the “big stones” should be treated as the relative instead of the absolute measure – what is a large stone for a small caddisfly is still a small stone for a large caddisfly. The “big stone” criterion should be adjusted for this effect.
3. The well-defined and thorough description of statistics used is definitely lacking. This is a consequence of not posing a sound hypothesis, as I mentioned above. What is a dependent variable? What are the model effects?

Results:

1. As I mentioned above, the finding that larger case is made of the bigger number of stones is not very fascinating, nor is the increasing proportion of larger stones. I would suggest a different approach. First of all, instead of analyzing the ratios, I would do a covariance analysis, where the total number of stones covaries with the number of biggest ones. I am still not pretty sure whether case surface area should be a dependent variable in this analysis, but perhaps I was just not convinced enough by the authors.
2. Another possible thing to do is to divide all the cases into “small” and “large”, and to test whether the proportions of large stones differ between them.
3. The description of Fig. 5 means that the authors are also a bit confused on what affects what, because it designates the opposite relationship to what is actually illustrated.

Discussion

1. Discussion begins with one sentence on the authors’ own finding, followed by… 30 lines of text, which is perhaps a theoretical background for the study, but does not explain its results. The explanation goes next, but is not convincing. There is nothing about the energetic trade-off of silk production vs. stone transport, which was emphasized (though not confidently enough) in the Introduction.
2. The last two sentences (conclusions) are in my opinion unwarranted, because none of the effects mentioned was tested.
3. The “trade-off” attracting the reader’s attention from the title is used in the whole text only ONCE, in Introduction.

General conclusions:

Strongest point: the huge amount of work done and of the literature dug through.

Weakest point: too little time was devoted to pose a sound and clear question (hypothesis). It resulted in using of not the most proper methods of analyzing this interesting study material.

<u>Andrzej Antoł</u>

Firstly I'd like to look at all formally required parts of a paper. First issue is that we don't know where are the authors from, because they forgot to write an afliation.

In the summary I'd add one sentence of general introduction of the topic, not directly go to methods.

Introduction for me is generally good, but the paper of Otto and Johannson (1995) is described too detailed (why authors write about results of PCA used by Otto and Johannson?, it is unnecessary, enough if you write what they got as result in biological meaning).

I don't know the Jaszcze stream in Pieniny National Park, I know one in Gorce mountains, not whole stream is part of the Gorce National Park ;)

Methods are generally ok and precise, but I doubt if measuring digitally the surface of case is really justified. Stone can for example have different sizes, what can influence the total surface, but not mass. Maybe weighting the cases would be better?

In whole paper there are editorial and language error, but in result section the captions of graphs are not scientific but more popular. The caption should describe what is on the graph (like: Graph of relationship between number of stones and case area. p=..., r=..., y=ax+b), not be the first interpretation, like in reviewed paper. For interpretations the place is in discussion part.

In discussion I'd like to read more about the inconsistency between first regression and the second. There are for me to much description of other research and I cannot find the direct connection of it with the result obtained in presented research. Generally for me the discussion is the weakest and most unclear part of paper.

In general the topic is intersting, maybe not for Nature, but always something. In the discussion authors said about changing the case size during the season. Investigsating dynamic of this process also in the perspective of stone size could be interesting. Maybe some long term experiment in the future?

Advantages of this project is clarify of setup and results, simplicity of used statistics what facilitates following and undestanding the results. Also the sample size is really big and allows us to trust the results.

Disadvantages of this project are few measured variables, so that we loose some information, not really well thought measurements. Also the language and form of report is far from perfection.

<u>Aleksandra Piontek</u>

This study tries to answer the question which strategy of building cases is better for caddisfly. However, I’m not sure if the authors presented the good assumptions for each of the strategies given. First of all, in introduction you write “despite of the higher energetic cost for production silk, caddisfly choose bigger stones” but later on you claim that the caddisflies may be choosing bigger stones to reduce the costs of silk production – you need to fix this inconsistence, especially because it concerns the main question of your study.

If the bigger stones are better for protection against the predators and they need less silk to be connected than the benefits are pretty obvious. You suggest that the lighter stones require more energy to transport but it seems like a small problem compared to great energy loss on silk production. Moreover if the bigger larvae choose bigger stones it can mean that these bigger stones require proportionally the similar amount of energy to handle as the smaller stones for smaller larvae. I think you should clarify the potential benefits and disadvantages for caddisflies

You didn’t mention the Latin name of the studied taxonomic group anywhere in the report which may create difficulties for people searching for your paper in the future.

The part of the introduction about study of Nislow and Molass (1993) is too long and detailed for introduction – it suits discussion better.

In methods you wrote the wrong name of the national park. I think the methods of this study are not bad, although I think that measuring the diameter of the cases might have been the good idea as it provides more information about the actual size of the case (if differences in diameter of cases are marginal then it should be mentioned to justify the chosen method).

The titles of graphs are wrong – they should describe the graph instead of being a one sentence result. I don't really get the idea of final result "caddisfly larvae have strong preference for stones which are available in sediment" – do they have choice to prefer the stones that are not available for them?

From small mistakes – some Latin names in the references are not written in italic.

Mateusz Sobczyk

Jaszcze stream is in Gorce National Park not in Pieniny National Park.

In my opinion frequency of total stone number which compose cases should be presented on histogram not on regression slope like on figure 4.

Relationship between proportion of the biggest stones and caddisfly cases was statistically significant and was positive but explain only 16% of variance.

I don't understand figure 5 caption.

Figure 5 didn't show that the most frequent number of big stones was between 7 and 10.

I couldn't find in text clearly defined hypothesis and aim of study.

Authors incorrectly treat percent as substitute of proportion. In material and methods section was mentioned that big stones to all stones ratio was calculated but in figure 5 axis "x" contain percent as unit.

Researchers claim that caddisfly choose bigger stones to construct bigger cases because insects based on strategy which reduce energetic costs. Maybe explanation is more simple: bigger individuals choose bigger stone because are stronger.

Smaller stones not always have to be lighter than bigger, because all three dimension have influence on stone size. Material and methods section didn't have well defined expression "stone size".

Benjamin Wacławik

Not accepted at this stage, but a major revision may be resubmitted to Behavioral Ecology and reconsidered

Project „**Trade-off between stone size and number in caddisfy larvae cases"** presents interesting approach to study on behavior of caddisflies, more precisely, building cases. It concerns size of stones used by these insects in this activity. Problem is for sure novel, but that is pity that authors did not show that it is important for more people than for trichopterologists (for example that it could be a model study for different similar problems). In theoretical part, authors proved that this question is interesting, because caddisflies really exhibit some preference towards material they use. Question they asked is: do caddisflies prefer bigger stones? They demonstrated that choice of big stone is better for caddisflies, although they did not show that using smaller stones is the real alternative. They tell about lowering costs of energy during transport of small stones, but they immediately tell that insect will lose this saved energy during silk production. This makes all this paper less exciting, because I was not convinced, that this problem is real problem of trade-off. I rather had impression that it is something quite obvious.

Used methodology was proper and from the paper I can infer that it was well done. However, measurement of case surface should be described more detailed.

Authors obtained some interesting results, although I am not convinced to their interpretation. They found out that the bigger the case, the bigger the stones and on the other hand- the bigger the case, there are more stones. What are their conclusions? It is not clear formulated, but they try to convince reader that this means that caddisflies prefer big stones. For me it is totally not convincing. On the one hand- preference of bigger stones while building bigger cases can tell us something. But it is not mean that they just prefer bigger particles when building bigger aggregate? On the other hand they proved that caddisflies use more stones when building bigger cases. It really tells us something about the preference toward stone size. However, I am still not sure if it is tells the same thing as authors want to convince me. It is also connected with the tittle- this "trade off" made by using bigger stones does not seem to be problem of the experiment. Of course I allow the possibility that I just got confused and do not get idea. If it so- I am sorry ☺ But I think that means that report should be written more clearly.

Also I have some other remarks. Authors told in theoretical part, that it was proved that caddisflies often use material which is available in their habitat in proportions that are present in this habitat. Did they take into account that it might be the case? I am not specialist in geology and I do not know much about stones in Jaszcze, but I think that this problem should be somehow commented. Also there is a little mess with font in the references, which is totally not important, but should be corrected in last version.

To sum up, authors made a good work concerning interesting case and obtained significant results but they should think more about the interpretation.

<u>Wojciech Tokarz</u>

The report is generally well written and has only minor mistakes in its construction. There is no introduction into the subject in the summary, but just methods and results. The introduction is very thick and contain probably too many information which should be moved to the Discussion chapter, especially the literature review. The main aim of the project is not well visible in the content of this chapter. There is also no clearly stated hypothesis and predictions about the future results.

The methods are well described, but partially inadequate to the needs of the project, e.g. calculating side view area instead measuring the total surface of the cases, so they should be once more examined by the authors in order to find some possible modifications. A good idea would be also to compare the diameter of the case entrance and the diameter of stone walls to calculate the ratio representing the proportion of the size of stones to the size of individual inhabiting the case. Such improvements could help to adequately correspond with the aim of the experiment.

Results section is very clear and adequate to the methodology. Nice and informative photos, which increase the visual quality of the report (no other group used this element). Diverse references increase the quality of the discussion.

Broad literature review and clearly presented results are the best sides of the report and they directly show the big amount of work and dedication of the authors.

<u>Terézia Horváthová</u>

This study tries to understand how the relationship between stone size and number can explain the size variation of caddisfly larvae cases. It is an interesting question as the caddisfly larvae represent the example of the extended phenotype which is to my knowledge not very common research topic. I like the idea of using such a simple experimental design (collection of empty cases, counting the stone numbers, measuring the stone and case size) to explore a problem within an evolutionary context. Such an elegant approach can attract the attention of different researches and I believe that it could be even used for purposes such teaching biology in schools.

The introduction was written nicely, giving different explanations for the size-number relationship. I could follow the idea, the authors also gave me nice summary of what has been studied so far. This is the strongest part of the manuscript. However I have some major concerns about this manuscript:

-the language was in some parts weak (the mistake in the title, not *caddisfy* but *caddisfly*), I would check it again

-I miss formulation of your hypotheses (maybe avoid using 'from one hand…'and …'to other hand..'). You should describe more what you mean by measuring the proportion of big stone ratio (last sentence in introduction). This paragraph is the most important one, it should be explained clearly.

-I suggest to better explain your material and methods. How did you measure the surface area? The figure 3 is not informative at all (the title is not clear). You mention that all cases were built from the same material, however you do not provide any information about this material. This should be mentioned as it is important part in your discussion. More, big stones to all stones ratio can not be expressed in percentage. I would name it more simpler, just 'big stone ratio'.

-I was surprised not to see the graph with the relationship between stone number and your proxy for stone size. This is actually your title (trade-off between stone size and number...). I would run this analysis.

-I would rewrite the discussion. It is nicely written however it touches the topics which are not really connected with your experiment. Firstly, you mention the selection of the material in caddisfly, however you do not provide any data. Secondly, you wrote a long paragraph about the preference for the grain size and not for its mass. In your study, you did not measure neither the mass or the size. I would shorten the discussion and focus on parts which you can provide data with.

-the conclusion is very speculative, you should not be so strong. With your data, you cannot say anything about the minimization of energetic costs connected with silk production. However, you can still write it but use different words, for example...'our results may imply/ suggest that producing of cases with bigger stones may lower the cost connected with silk production..'And maybe add another sentence that you would like to run such an experiment in the future (silk production or energetics).

I believe that this study can attract the attention due to its interesting model system and simple experimental approach. I strongly advice the authors to work on the discussion and better explanation of the experimental set-up.

5.3.4. Final version of report

Optimazation of cases building strategy in caddisfly larvae (Trichoptera, Insecta)

Natalia Derus, Wioleta Kocerba-Soroka, Adam Krupski

Summary

Caddisfly larvae (Trichoptera) construct cases as protection against predators. Cases are built from mineral or organic particles like sand grains, stones or leaves. Particles in stone cases have different sizes during larvae life. Choosing stones of different size cause various energetic cost which can have an influence on further life.

The empty cases were collected from natural stream. One-side surfaces of cases were measured and the total stones number were counted. In addition the percentage of the biggest stones were calculated. Contribution of big stones to total case size was examined.

There is optimization between number and size within stone material, which are chosen by Trichoptera. We proved that caddisfly larvae use not only more stones to build bigger cases but also prefer bigger stones when they overbuild their cases. Usage of bigger stone by the caddisfly larvae might be a strategy to reduce the silk production and secretion for cementing stones, which is energetically costly.

Keywords: caddisfly larvae, stone cases, stone size, selection strategy, Trichoptera

Introduction

Caddisfly larvae build the cases with a variety design and from different materials such as small stones, grains of sand, parts of the water plants or leaves. Functions of the cases are diverse which can be protection against predators or cannibalism. The influence of larval case design on vulnerability to predators was evaluated in a series of laboratory experiments (Nislow and Molass, 1993). The skeleton of the cases is made from the silk, which is produced by silk gland. Production of the silk to build the case is 12% of the energetic cost of caddisfly larvae, which is relatively high cost in environment where they live (Otto 1974).

It is already proved that caddisflies do not choose the particles randomly (Hanna 1961, Tolkamp 1980). The selection of the bigger stones from the sediments give better protection against predators but it also more costly for larvae because they produce more silk for cementing stones (Otto 2000). Moreover, it is also known that the addition of ballast stones protect caddisfly larvae from current flow (Otto and Johansson 1995). Material composition of cases depends on the season and different materials which are available in environment. Also the material preference is influenced by local geology. It is already known that Trichoptera preferred smooth than rough particles, what enhance their respiratory efficiency (Okano et al. 2011).

Investing more energy to building the cases with bigger stones or adding extra stones by the younger stages of caddisfly larvae can result lighter thorax and the wings in imago (Stevens et al. 1999).

However it still remains unclear which strategy used by caddisfly larvae is predominate in natural habitat and which is more beneficial: selecting bigger stones to increase survival or building the case from small stones to save the energy by lower production of silk.

In this study we examined if big stones were more abundant in bigger cases. It would means that this larvae lose more energy for silk production and big stones transport but they had better protection against predations, therefore benefits of big stones possession outweighed the costs of silk secretion.

Materials and Methods

We took 95 cases of the caddisfly larvae from the Jaszcze stream in Ochotnica Górna (Gorce mountains). Only cases build from stones were chosen. All cases were taken from bottom side of big stones submerged in stream and were built with the same type of stones.. This means that all cases might belong to one species. All cases were empty because specimens reached imago stadium. 29 cases were incomplete, therefore were rejected from the experiment. 66 cases were placed individually on petri dishes and a piece of paper with the sample number was put into each dish (Fig.1). Each case one side surface was photographed with Sony DSC HX300 camera(Fig.2). All cases were outlined manually on the pictures with ImageJ software. Surface was calculated by the program from the outline with using 1mm scale. Subsequently all separated stones from each case were counted. After that all stones from each case were sieved (net size 1.6 mm) in order to isolate and count the biggest stones. The percentage of big stones to all stones was calculated.

To examine the relationship between natural logarithm case surface area, and total amount of stones, and relationship between natural logarithm case surface and big stones percentage. General Linear Regresion (GLR) was used. For all statistical analyses the Statistica ver. 10 software was used.

Fig. 1. Caddisfly larvae cases used in our study

Fig. 2. An example of photograph used for measurements

Results

Results of our experiments are shown on figures 3 and 4. Relationship between surface area of cases and total number of stones is highly statistically significant ($p < 0.00001$; $R = 0.79$, $R^2 = 0.63$). It indicates that the bigger case is, the more stones it consists of. The range of number of stones per case was from 42 to 220. Majority of caddisfly larvae had cases built with total stone number between 50 to 90.

Relationship between surface area of cases and percentage of big stones in given case is highly statistically significant too ($p < 0.001$, $R = 0.44$, $R^2 = 0.19$; Fig.4.). Therefore, also the size of the stones contributed into case size increase. The range of surface cases was between 29.9 and 189.9 mm^2. The most frequent number of big stones was between 7 and 10.

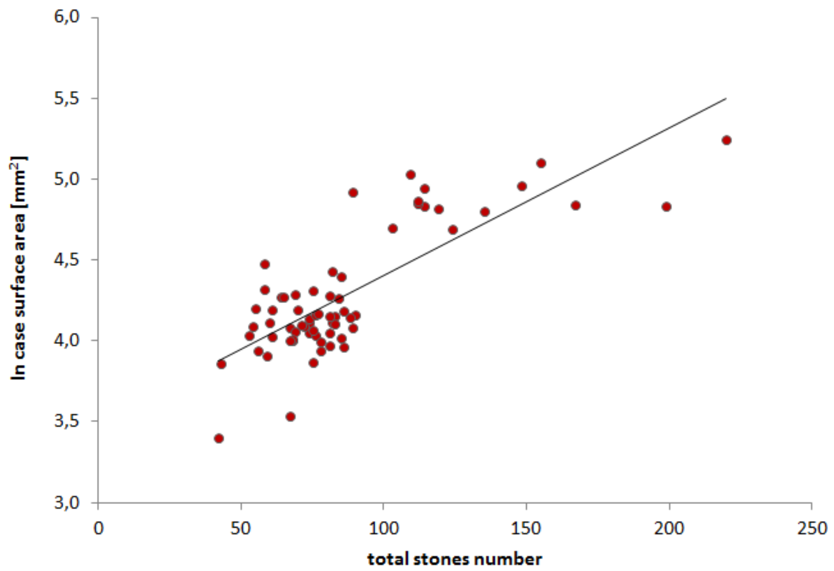


Fig. 3. Relationship between total number of stones and ln case surface area [mm^2]; $p<0.00001$, $R=0.79$, $R^2=0.63$, $y = 0,0091x + 3,4917$.

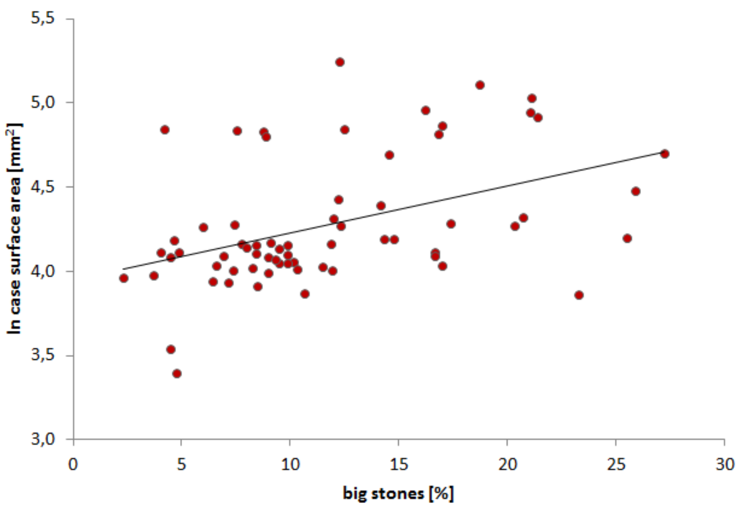


Fig. 4 Relationship between percentage of big stones and ln case surface area [mm^2]; $p < 0.001$, $R=0.44$, $R^2 = 0.19$, $y = 0,0281x + 3,9469$.

Discussion

Our experiment proved that caddisfly larvae use more stones to build bigger cases. It is obvious that cases size strongly depends on the total number of stones. The bigger case is, the more stones is needed. Increasing the cases surface is linked with larvae maturation. As the larva became older and molt several times it periodically add new material to the anterior part of the case (Hansell 1972).

More interesting result is the increase of the big stones percentage in larger cases. It suggest that caddisfly larvae might select the material which they use to build the cases. Depend on stadium of larvae growth they can choose bigger stones than before by using bigger mouthparts and legs as a measurements tool (Hansell 1974, Stuart and Currie 2001). Moretti and Loyola (2005) showed that *Barypenthus concolor* cases depends directly on the particle size composition present in the substrate. To build their cases *B. concolor* larvae use particles sizes in the same proportion as they are available in the habitat. It was proved that some species have clear preferences to choosing particles of certain size and even in experimental condition provided with particles with different sizes than they have in nature they choose particles with the most similar size (Hanna 1961, Tolkamp 1980). In relation to these results we suppose that caddisfly larvae whose cases we used to our experiment can be selective.

Our research provided that stone size is important factor during the selection of material which can be used for case building. The relationship between the percentage of big stones from cases and cases size was highly statistically significant. Bigger stones choice might be connected with high probability of meeting predators in the environment where they live. Therefore, better protection from being attacked is beneficial despite of bearing high energetic cost for silk production and transport heavy stones.

Acknowledgements

We want to thank whole PhD student group which were participants in Ochotnica course for valuable comments and help in preparing this report.

References

Hanna H.H. (1961) Selection of materials for case-building by larvae of caddis flies (Trichoptera). Proceedings of Royal Entomological Society in London (A), 36:37–47.

Hansell M.H. (1972) Case building behaviour of the caddis fly larva, *Lepidostoma hirtum.* Journal of Zoology , 167:179–192.

Hansell M.H. (1974) The house building of caddis larvae: a source of projects for schools. Journal of Biological Education, 8:88–98.

Moretti, M. S. and Loyola, R. D. (2005) Does Barypenthus concolor Burmeister (Trichoptera: Odontoceridae) select particles of different sizes for case building? Neotropical Entomology, 34(2):337-340.

Nislow K. H. and Molles M. C. (1993) The influence of larval case design on vulnerability of *Limnephilus frijole* (Trichoptera) to predation. Freshwater Biology, 29(3):411-417.

Okano J., Kikuchi E., Sasaki O., Ohi S. (2011) Geological variation in particle surface-roughness preference in the case-bearing caddis flies. Behavioral Ecology, 22:1053–1063.

Otto, C. (1974) Growth and energetics in a larval population of *Potamophylax cingulatus* (Trichoptera) in a South Swedish stream. Journal of Animal Ecology, 43:339-361.

Otto, C. (2000) Cost and benefit from shield cases in caddis larvae. Hydrobiologia, 436(1-3):35

Otto C. and Johansson A. (1995) Why do some caddis larvae in running waters construct heavy, bulky cases? Animal Behavior, 49:473-478.

Stevens, D. J., Hansell, M. H., Freel, J. A. and Monaghan, P. (1999) Developmental trade–offs in caddis flies: increased investment in larval defence alters adult resource allocation. Proceedings of the Royal Society of London. Series B: Biological Sciences, 266(1423):1049-1054.

Stuart A.E. and Currie D.C. (2001) Using caddisfly (Trichoptera) case-building behaviour in higher level phylogeny reconstruction. Canadian Journal of Zoology, 79:1842-1854.

Tolkamp H.H. (1980) Organism-substrate relationships in lowland streams. Agricultural Research Report Wageningen, 907:1-211.

6. KIKSY

TH: "Ja mam zacząć pisać to introduction, a kiedy to już mój problem!"

WT: „Idziemy na fajkę?"

TH: „NIE. Trzeba pisać, bo nie mamy nic, a jest 11. Discussion się najgorzej pisze."

MS: „To już Twój problem :D"

W KS: „Wiesz co, jak ja bym patrzyła na status społeczny mężczyzny… to teraz nie byłabym na doktoracie."

AA: (about obtaining fuel from the car tank) „I have no problems with sucking. I did it many time in my life."

TH: (about the real nature of AA) „Żylam sem w takim klamstwie"

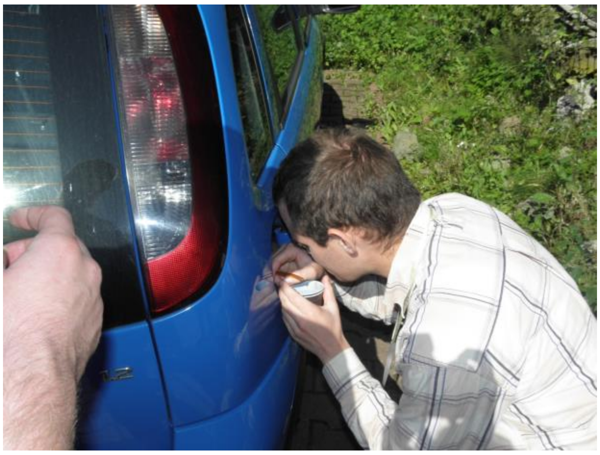
7. GALERIA

Wspólne gotowanie zbliża… (Wyszła pyszna zupa z dyni, którą jedliśmy 3 dni)

Nie mogliśmy przejść obojętnie koło takich okazów…

Co w potoku piszczy…?
(Szukamy inspiracji do badań)

Prawie jak zabawa w klasy…
(Teresa, Wojtek i Mateusz w trakcie pomiarów długości skoków koników)



„Spokojnie, robiłem to wiele razy…”
(Andrzej w trakcie pobierania benzyny do badań nad wrażliwością widelnic)