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RESEARCH TOPICS SUGGESTED BY PARTICIPANTS

chosed topics underlined

1) Do ants learn preference towards type of food? (JS)

2) How does cryptic environment influence speed of victim capture? (JS)

3) Does land usage affect mole activity? (JS)

4) The circadian activity of the forest ants (EC)

5) Are there temperature preferences of the mayflies larva? (EC)

6) Spatial differences in utilization of pastures (EC)

7) Differences in mass of above- and below-ground parts of plants growing in different stress conditions (JK)

8) The relationship between stem height and inflorescence mass (JK)

9) Do grasshoppers discriminate between native and alien food plants? (JK)

10) Stress influence on communication of ants (EP)

11) Influence of different kind of music on sound making of the grasshoppers (EP)

12) If and how the plant morphology depends on distance from river (EP)

13) How time of wilting depends on size of the plant (JG)

14) At which site of the tree appear colour leaves? (JG)

15) How does flower density depend on abiotic factors (JG)

16) Do bigger spiders occupy walls of particular exposure (DM)

17) Does the common sorrel limit the number of other plant species in its vicinity? (DM)

18) Does body mass of earthworms differ between meadow and forest? (DM)

19) Is there correlation between number and size of mole hills? (JW)

20) Do number of insects warming up on the wall depend on wall colour? (JW)

21) Structural body size in carabidae beetles in close and open areas (AG)

22) How different time spent in low temperature affect activity in ands (AG)

23) Abies alba perimeter in deep forest and ecotone, and the relationship between tree perimeter and needle length (AG)

24) Do environments differ in number of snails? (GC)

25) Do snails from different environments have different food preferences? (GC)

26) Do environments differ in biodiversity? (GC)

27) Ants motivation to obtain food after period of starvation (JW)

RESEARCH PROPOSAL

Acclimation time effect on ant survival in minus temperature and time of recovery

Giulia Casasole, Agnieszka Gozdek, Ewa Prawdzik

Aims

We aim to determine what is the effect of acclimation time on survival in minus temperature and on recovery time from chill-coma in 2 different ant species (*Lasius* niger and Mirmica *rubra*). We predict that cold-tolerance (by which we mean both survival and recovery time) will be higher if the acclimation time is longer and that will differ between species.

Existing knowledge

Temperature has indisputable impact on organism life history traits. Efficient adjusting to the thermal fluctuations is in particular important for the temperate organisms, which have to deal with seasonally, daily and even hourly changing temperature (Angilletta, 2009). Rapid cold-hardening process (RCH) protects insects against hoarfrosts and sudden temperature drop when they are not in overwintering phenotype. In RCH process short exposure to nonlethal low temperature (minutes to hours) efficiently increases the insect resistance to cold shock (Lee et al., 1987). The duration and temperature of acclimation affect chill-coma temperature (Renault et al., 2012) and cold-tolerance mechanism (Waagner et al., 2013). Significant differences between species in response to critical temperature after acclimation are observed, but the ultimate cause of that variation remains unclear (Chown et al., 2009).

Methods

Two species of ants are used for the investigation: *Lasius niger* and *Mirmica rubra*. The ants are caught in Ochotnica Jaszcze at the nest and are kept in room temperature overnight. Then they are placed in boxes (10 individuals per box) with standard amount of forest litter (0.5 g per box) containing leaves and humus. Small amount of litter in each box protects them against temperature shock during the experiment.

Pilot study

We performed a pilot study to find time essential for ants to acclimate to minus temperature. Boxes containing 10 individuals were placed in cooling chamber with a constant temperature above 0°C (fridge). Animals were kept in these conditions for different time periods (15, 30, 45, 60, 75, 90 minutes) and later transferred to minus temperature (freezer) for 4 min. Specifically at one time a group of 12 boxes was placed in the fridge. A group consisted of 6 boxes per species: one box for each species at each acclimation time. Then a second group of 12 boxes was placed in the fridge following the same procedure applied for the first. After this time survival was checked in room temperature and recovery time was measured for the first and last living individual.
GLM with recovery time as dependent variable, time of acclimation in the fridge as covariate, species and group as fixed factors was performed. An interaction time x species was found and also the group was significant (Table 2). The interaction can be seen in the Figure 1, while the significance of the group in the Figure 2. GLMM using the GLIMMIX macro in

SAS with logit link function and binomial error variance (Krackow & Tkadlec, 2001) was fitted to compare survival between species and to see if there was any effect of the group and of time of acclimation on survival. Survival was included as dependent variable, time of acclimation as covariate, species and group as fixed factors. Only a difference in survival between species was found (Table 3, Figure 3).

Table 2. GLM with recovery time as dependent variable, time of acclimation as covariate, species and group as fix factors. Statistically significant values are in bold (p<0.05)

Source	df	DEN df	F	P
Time	1	10	50.47	<.0001
Group	1	10	17.33	**0.0019**
Species	1	10	141.07	<.0001
time*species	1	10	24.78	**0.0006**

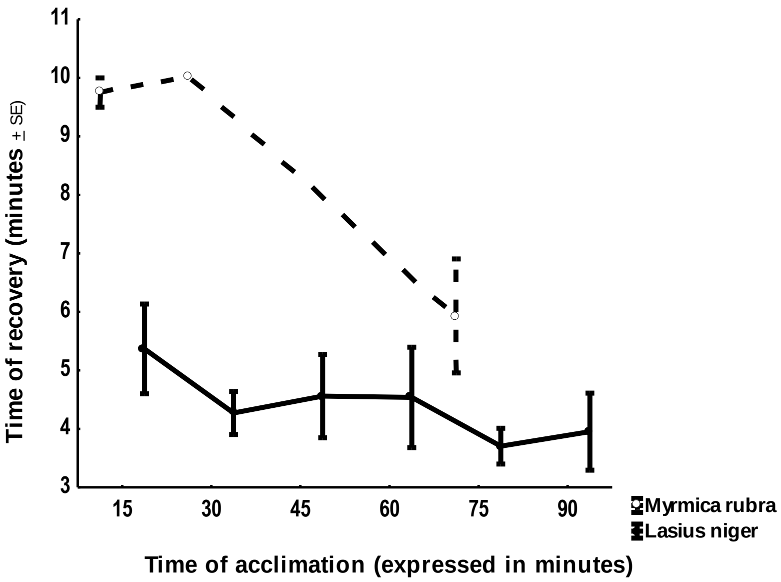

Figure 1. Average time of recovery (from 2 groups) in relation with time of acclimation in two ant species (*Lasius niger* –continuos line and closed circle-and *Myrmica rubra*-open lines and empty circle)

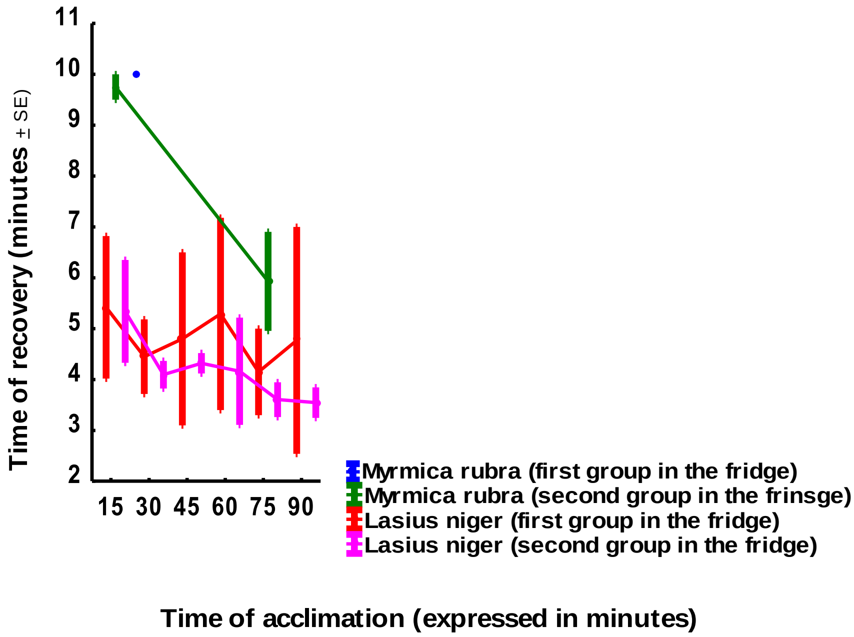

Figure 2. Time of recovery in relation with time of acclimation for two groups in two ant species (*Lasius niger* and *Myrmica rubra*)

Table 3 GLM done with GLIMMIX in SAS with survival as dependent variable, time of acclimation as covariate and group and species as fix factor ($p<0.05$)

Source	df	DEN df	F	P
Species	1	44	32.54	**<.0001**
time	1	44	2.33	0.1338
group	1	44	1.47	0.2325

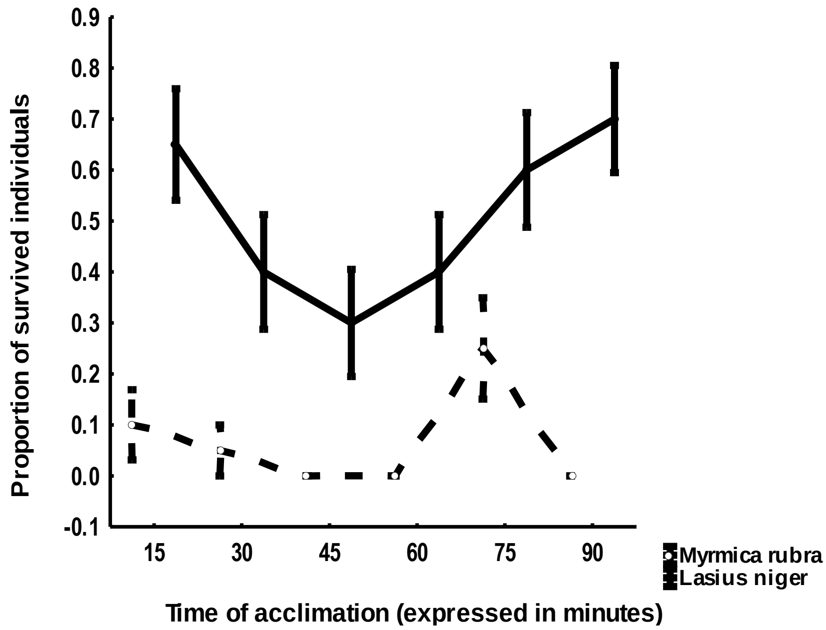

Figure 3. Average proportion of survived individuals (from 2 groups) in relation with time of acclimation for two ant species (*Lasius niger*- continuos line and closed circle-and *Myrmica rubra*-open lines and empty circle).

Experiment

Based on pilot study results we decided to change the experimental design. We shortened the time spent in the freezer to 3 min considered the high sensibility of Myrmica rura to minus temperatures. We also increased the acclimation time in the fridge to 2 hours with intervals of 30, 60, 90 and120 min because we observed the highest survival of *Lasius niger* after 1 hour of acclimation and we wanted to investigated it. In the experiment we plan to use 2 ant species (*L. niger* and *M. rubra*), the same species used in the pilot. Boxes containing 10 individuals each are placed in the fridge for acclimation (for 30, 60, 90 and 120min respectively). At one time a group of 16 boxes is placed in the fridge. A group consisted of 8 boxes per species: one repetition for each species at each acclimation time (Figure 1 and Figure 2). After acclimation boxes are transferred to the freezer for 3 min. After that time we start activity measurements (the starting point for the measurements is the moment when animals are transferred to room temperature). Then a second group of 16 boxes is placed in the fridge following the same procedure applied for the first. In this way we have 2 repetitions for each species at each acclimation time.
We plan to analyses the data with SAS version 9 performing GLM with time of recovery as dependent variable, species and repetition as fixed factors. The interactions (species x time, species x repetition, repetion x time and time x species x repetition) are sequentially removed from the models if not significant ($P>0.05$) starting from the higher order interaction. GLMM using the GLIMMIX macro in SAS with logit link function and binomial error variance (Krackow & Tkadlec, 2001) is fitted to compare survival between species and to see if there is any effect of the repetition and time of acclimation on survival. Survival is included as dependent variable, time of acclimation as covariate, species and repetition as fixed factors.

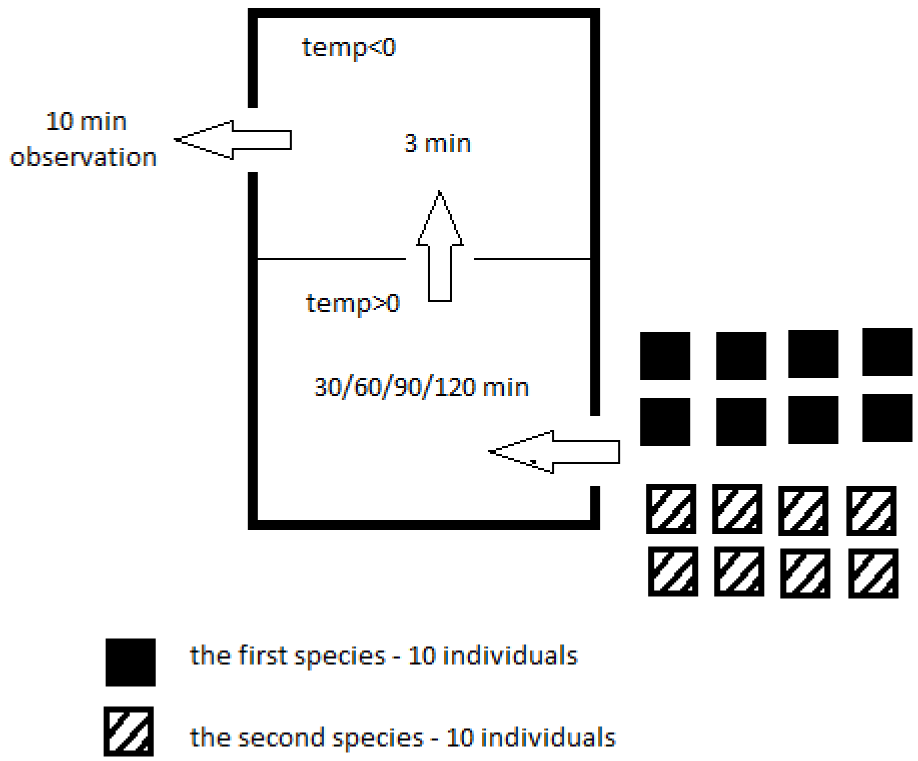

Figure 1 Experimental design. All colored squares illustrate one group of boxes (containing each 10 individuals). The boxes are placed at one time in the fridge for different acclimation times (30, 60, 90, 120 minutes), then in the freezer for 3 minutes and finally their time of recovery is checked during 10 minute observation

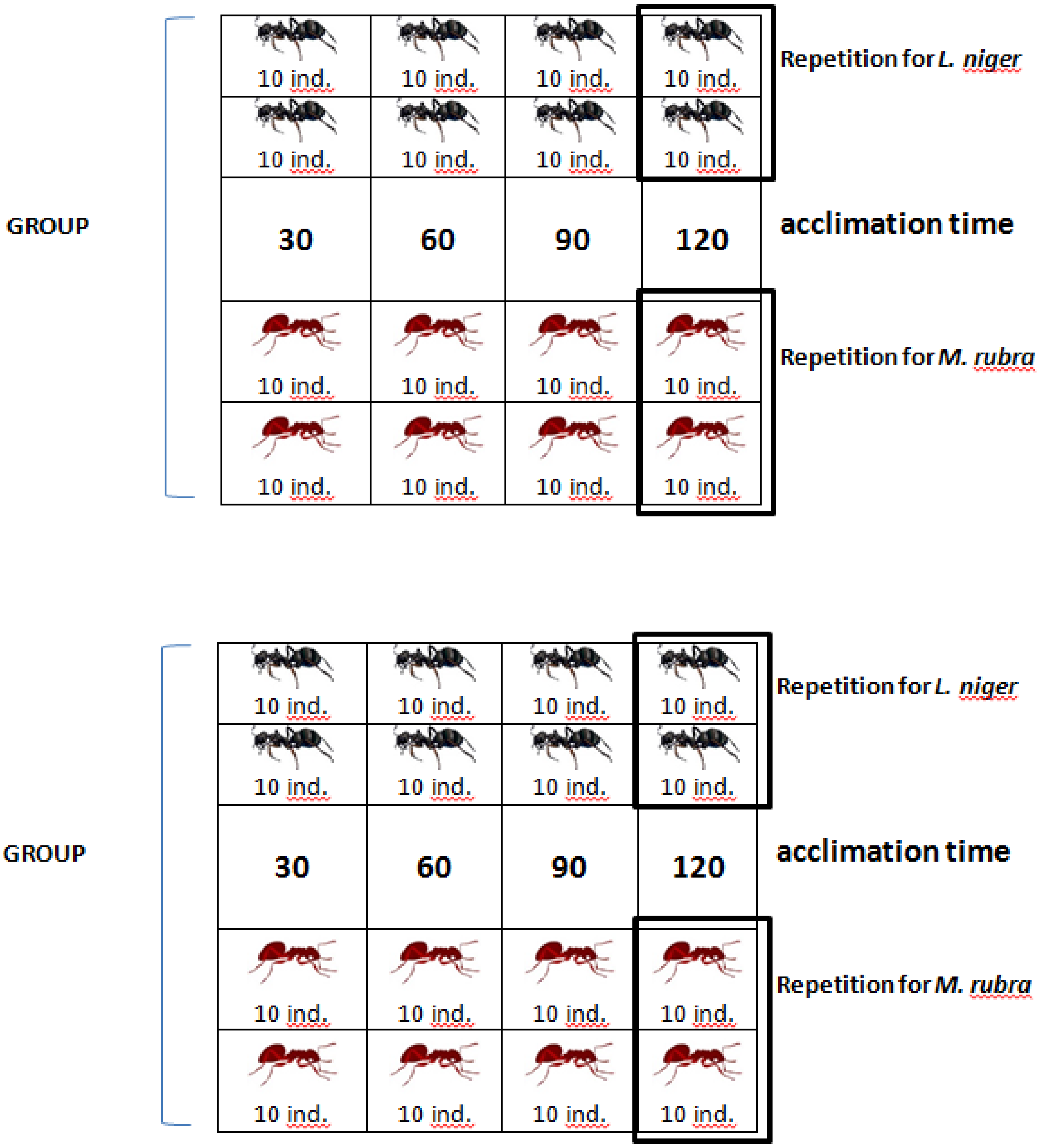

Figure 2 Experimental design. In the experiment we plan to have 2 groups. Each group consists of 8 boxes per species: one repetition for each species at each acclimation time. Each group is placed at one time in the fridge. So in total we'll have 2 repetitions for each species at each acclimation time.

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FIRST VERSION OF REPORT

Acclimation time effect on ant survival in minus temperature and time of recovery

Giulia Casasole, Agnieszka Gozdek, Ewa Prawdzik

Abstract
Rapid cold-hardening process ensures insect survival after short temperature drop and cold-tolerance mechanisms depend on the acclimation time. Three different ant species were used in experiments in order to compare the effect of acclimation duration on survival in minus temperature and the time to recover. The species differ significantly in survival and recovery time, but no significant effect of acclimation time on cold-tolerance was found. However we observed repeated similarities in the survival pattern, probably due to not precise cooling chamber.

1. Introduction

Temperature has indisputable impact on organism life history traits. Efficient adjusting to the thermal fluctuations is in particular important for the temperate organisms, which have to deal with seasonaly, daily and even hourly changing temperature (Angilletta 2009). Insects, as ectotherms having small body size, lose heat to the environment and therefore have evolved mechanisms that enable them to survive low temperature. They are rearly cold-hardy all the year. Instead they use environmental cues about incoming winter to switch on physiological processes that strengthen cold tolerance (Teets 2013). Interestingly insects are also able to cold-harden on shorter than seasonal time scale. Rapid cold-hardening process (RCH) protects them against hoarfrosts and sudden temperature drop when they are not in overwintering phenotype. In RCH process short exposure to nonlethal low temperature (minutes to hours) efficiently increase the insect resistance to cold shock (Lee 1987). The duration and temperature of acclimation affect chill-coma temperature (Renault 2012) and cold-tolerance mechanism (Waagner 2013).
We aim to define what is the effect of acclimation time on survival in minus temperature and recovery time from chill-coma in different ants species. Because surface area/volume ratio affects sensitivity to temperature, species that differ in body size were chosen. Increased ratio means higher exposure and more heat lost (Gunn 1942), so we predict that bigger animals will cope better with minus temperature even after relatively shorter time of acclimation.

2. Methods

Animal model

Three species of ants are used for the investigation – *Formica rufa, Lasius niger*, *Mirmica rubra*. They vary in size: *Formica rufa* is 9 mm long, *Lasius niger* - 5 mm and *Myrmica rubra* is the smallest ant in our experiment (3-4 mm). Total number of ants used in the experiment was 400 individuals from 3 species.

Pilot study

In pilot studies we aim to find time essential for temperate ants to acclimate to minus temperature. Ants are placed in boxes (10 individuals per box) with standard amount of litter (0,5 g gram per box) in cooling chamber with a constant temperature above $0\,^{0}$C. Each 15min (starting from 15min and ending at 90min) boxes were transferred to minus temperature for 4min. After this time survival was checked in room temperature and recovery time measured for the first and last living individual. Pilot studies are carried out separately for two species (Supplementary materials).

Main study

Based on pilot study we defined the amount of time spent in minus temperature as well as acclimation time. 4min in minus temperature was crucial time for *Myrmica rubra*, whose survival was very low, so we decided to shorten that time to 3 min. Because increased survival after longer acclimation time was observed, we extended twice time check point and test duration to two hours (30/60/90/120min). Four repetitions of each test were carried out as trial effect in pilot study was significant.

Experiment 1

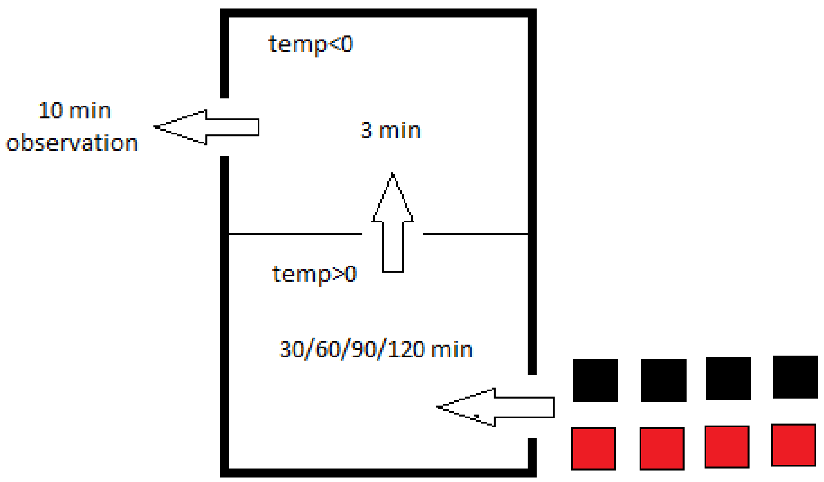
Ants (*L. niger* and *M. rubra*) were divided into 8 experimental groups (two species for each acclimation time) and placed in cooling chamber with a constant temperature (10 individual in box with litter). Small amount of litter in each box protects against temperature shock. Groups were treated with low temperature for different time periods – every 30min 4 groups were transferred to the freezer for 3 min. Only two repetitions were carried on and the last check point had no repetition because of scarcity of *M. rubra*, which had not been found in the previously discovered nest.

Experiment 2

Because of the scarcity of *M. rubra* we decided to carry on another experiment utilizing *Formica rufa*. Ants (*L. niger* and *F. rufa*) were divided according to above protocol. Number of repetition increased to four for each species.

Activity measurements

After 3 min spent in minus temperature animals were transferred to room temperature and survival was checked. Chill-coma recovery time was measured for each alive animal during 10min observation (the starting point for the measurements was the moment when first animal started moving). 10 min was enough time to count ants because half and one hours after treatment there were no more living ants then during 10min observation (Picture 1).



Picture 1 Experimental design. Coloured squares illustrate two species

Statistical analysis

All the analysis were done in SAS apart from non-parametric test for survival (performed in SPSS). GLM were performed in recovery time analysis with recovery time as dependent variable, time as covariate, group and repetition as fix factors .

3. Results

Experiment 1

We found significant differences in survival (test Kruskala-Wallisa, p=0.001, Figure 3) and recovery time after 3min in minus temperature between species (Table 1 and Figure 1). Repetition-group interaction is significant (Figure 2).

Table 1 GLM with recovery time as dependent variable, time as covariate, group and repetition as fix factors. Statistically significant values are in bold (p<0.05).

Source	df	DEN df	F	P
Time	1	7	2.62	0.1494
Trial	1	7	0.25	0.6336
Group	1	7	22.72	**0.002**
repetition*group	1	7	6.53	**0.0378**

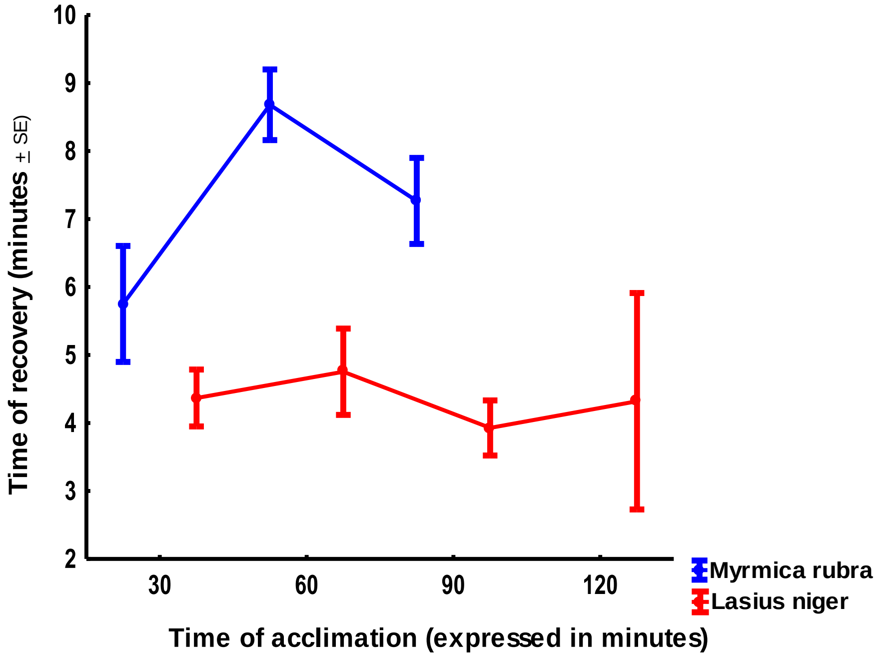


Fig.1 Average time (from two repetitions) of recovery for two species after 3min spent in minus temperature in relation with the acclimation time.

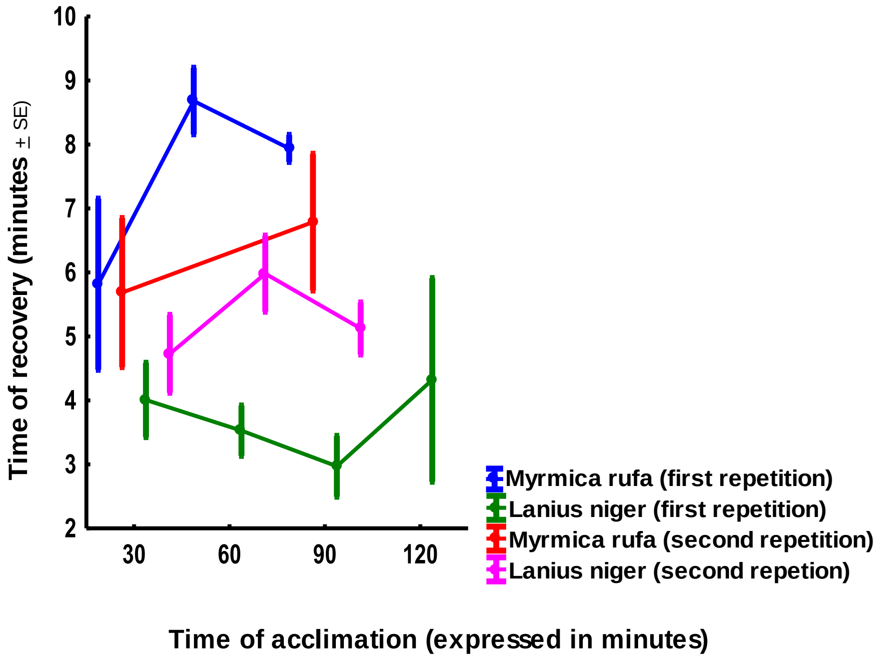


Fig.2 Recovery time in relation with acclimation time and repetitions

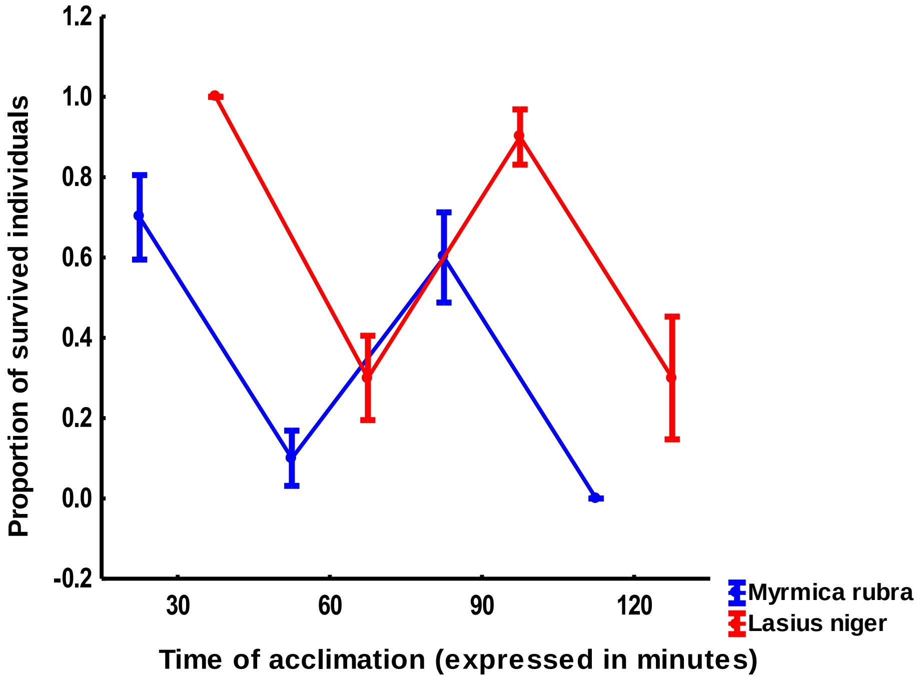


Fig.3 Average survival (from two repetitions) of animals in relation with acclimation time (averages±SE)

Experiment 2

Significant difference in survival (test Kruskala-Wallisa, p=0.04, Figure 9) and recovery time between species (Table 2, Figure 4) was observed. We did not observe significant effect of acclimation time. There was significant effect of repetition number (Table 2, Figure 4, 5, 6, 7, 8).

Table 2 GLM with recovery time as dependent variable, time as covariate, group and repetition as fix factors. Statistically significant values are in bold (p<0.05).

Source	df	DEN df	F	P
Time	1	7	2.62	0.1494
Trial	1	7	0.25	0.6336
Group	1	7	22.72	**0.002**
repetition*group	1	7	6.53	**0.0378**

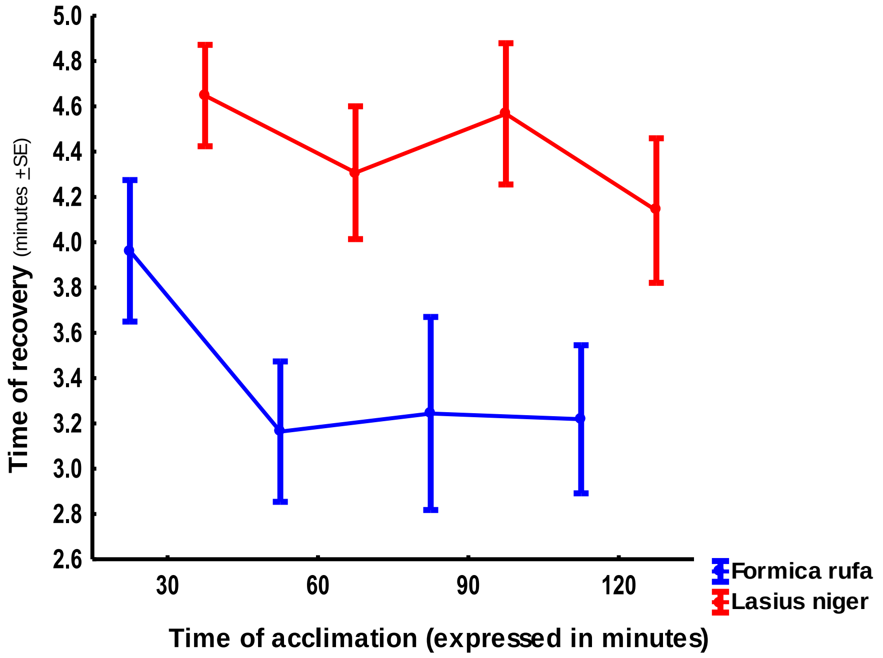


Fig.4 Average recovery time (from 4 repetitions) in relation with acclimation time

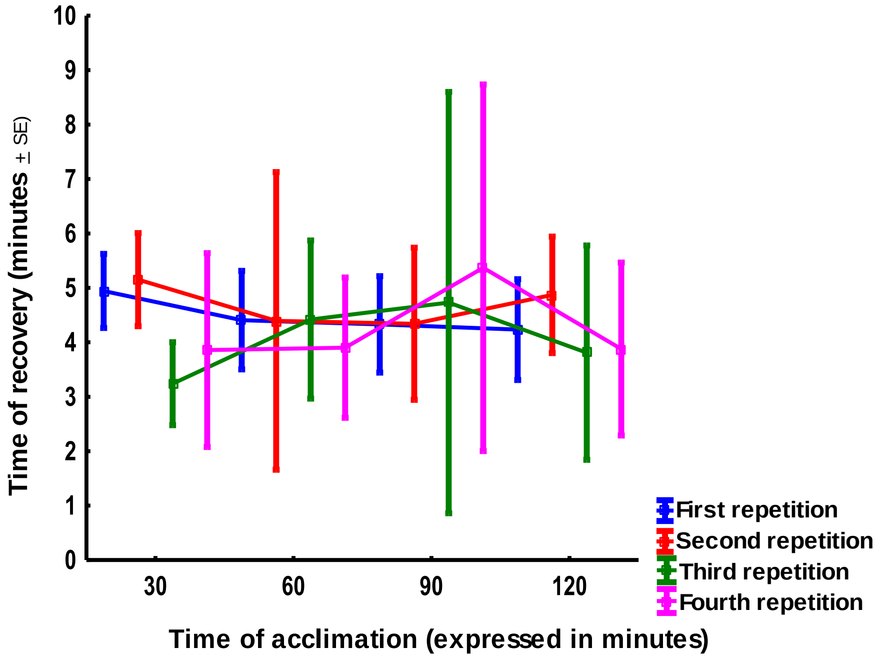


Fig.5 Recovery time in relation of time of acclimation in *Lasius niger* with all four repetitions

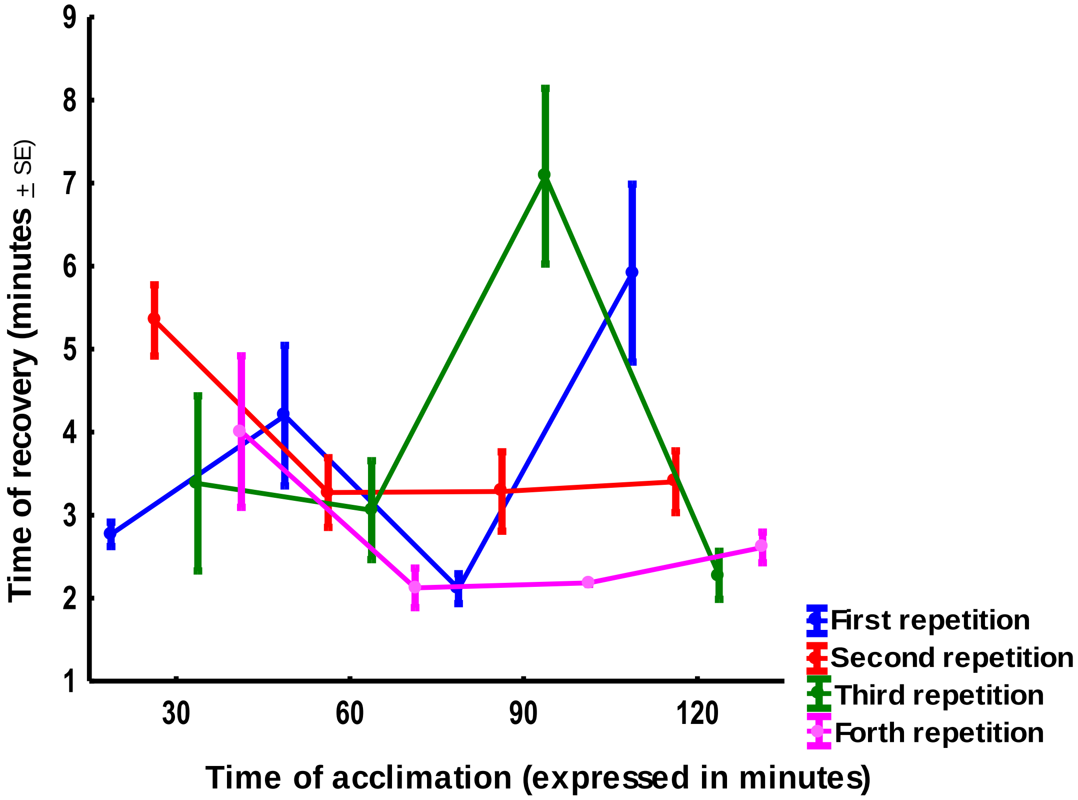


Fig.6 Recovery time in relation of time of acclimation in *Formica rufa* with all four repetitions

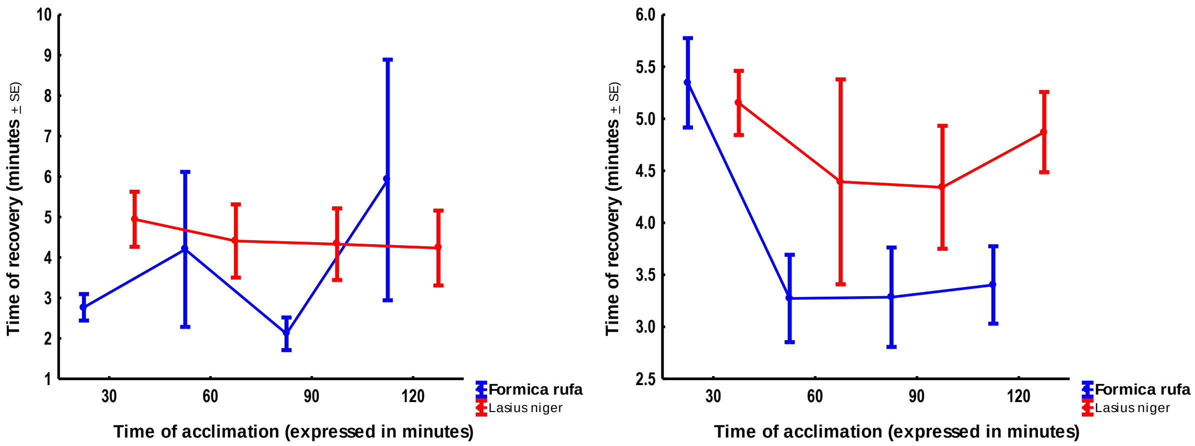


Fig.7 Recovery time in relation with acclimation time (Repetition 1 – graph on the left, Repetition 2 – graph on the right, were placed together in the cooling chamber)

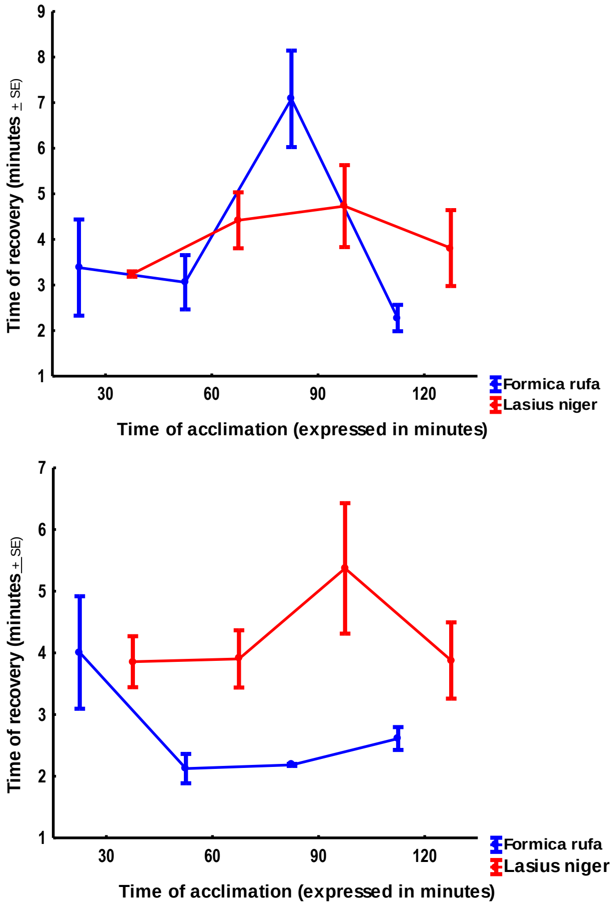


Fig.8 Recovery time in relation with acclimation time (Repetition 1 – graph on the left, Repetition 2 – graph on the right, were placed together in the cooling chamber)

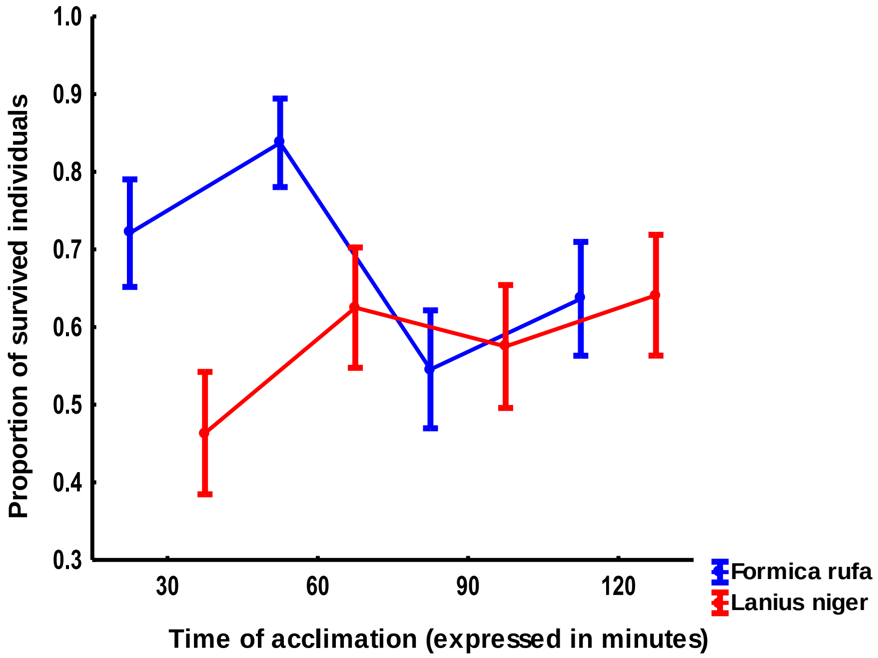


Fig.9 Average survival (from 4 repetitions) in relation with acclimation time (average±SE)

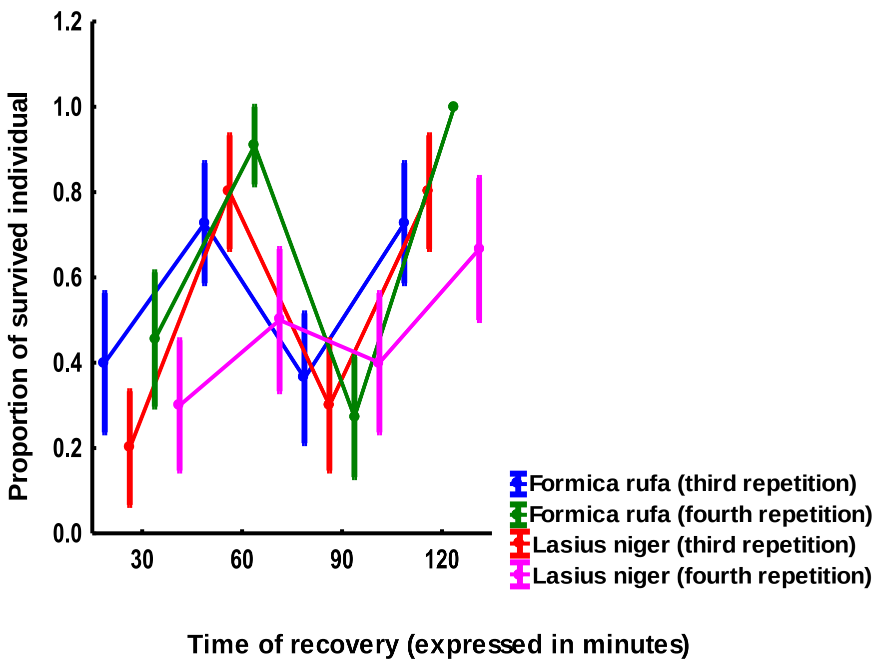


Fig. 10 Proportion of survival in relation with time recovery and group for the third and fourth repetition (they were placed at the same time in the cooling chamber)

4. Discussion

According to literature, acclimation time influences cold-tolerance mechanism (Waagner 2013). Investigated species have different tolerance to low temperature (significant diffrences in survival and recovery time between species), however we did not find significant effect of acclimation time on recovery, what is consistent with other studies (Clarke 2013). *Myrmica rubra* was more sensitive to low temperature than *Lasius niger* and *Formica rufa* was less vulnerable to low temperature than *Lasius niger*. Because species vary in size, the cold-tolerance differences can at least in part result from body size variation (Gunn 1942).

We also observed huge variability between samples. During one test two repetitions of each group were placed together in the cooling chamber (number of observers was a limiting factor). We cannot be sure about constant conditions in the chamber. In the first experiment one can notice similar pattern concerning survival in both species after the same acclimation time – for instance survival at 60 and 120 minutes decreased in both groups whereas increased at 90 minutes (Figure 3). Similar trend can be observed in the second experiment, especially for the repetition third and fourth (Figure 10). Survival is low at 30 and 90 min while it is increasing at 60 and 120min in both species. That can result from alternating switching on and off the automated cooling process in the refrigerator. Obviously more repetitions are required in future studies and more precise cooling chamber.

5. References

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Supplementary material

<u>Results of pilot study</u>

Table 1. Average length [mm] for two ant species *Myrmica rubra* and *Lasius niger*

Species of ant	**Average length**
Myrmica rubra	4.3
Lasius Niger	5.8

Table 2. GLM with recovery time as dependent variable, time as covariate, group and repetition as fix factors. Statistically significant values are in bold (p<0.05)

Source	df	DEN df	F	P
Time	1	10	50.47	<.0001
Trial	1	10	17.33	**0.0019**
Group	1	10	141.07	<.0001
time*group	1	10	24.78	**0.0006**

Table 3. GLM with recovery time as dependent variable, time as covariate and repetition as fix factors by group. Statistically significant values are in bold (p<0.05)

Group	Source	df	DEN df	F	P
Black ant	**Time**	1	9	5.35	**0.046**
	Trial	1	9	11.77	**0.0075**
Red ant	Time	1	0		
	Trial	1	0		

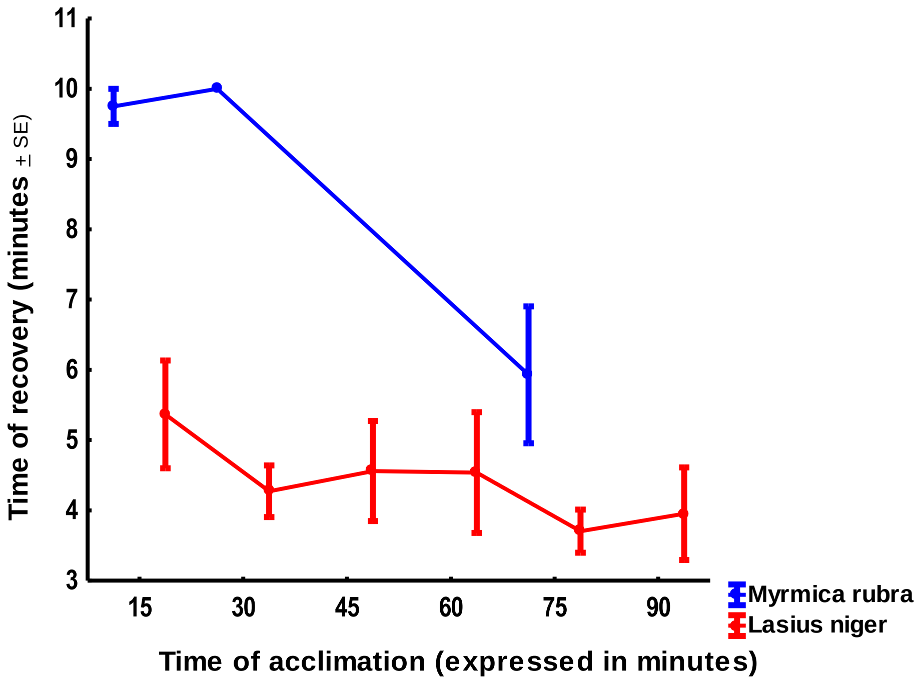


Figure 1. Average time of recovery after different time of acclimation in two ant species (*Lasius niger* and *Myrmica rubra*)

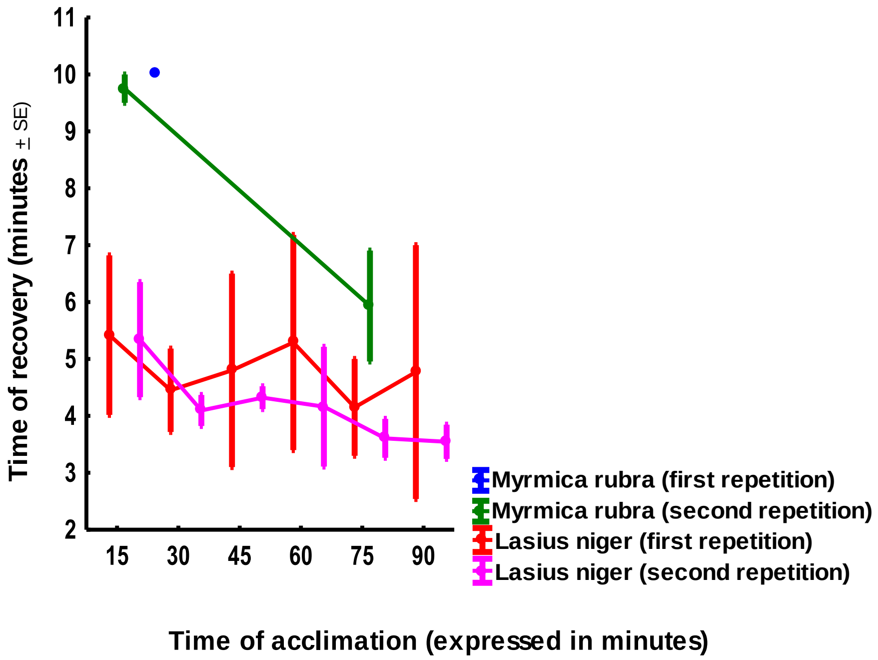


Figure 2. Average time of recovery after different time of acclimation for two repetitions in two ant species (*Lasius niger* and *Myrmica rubra*)

Table 4. GLM done with Glimmix in SAS (non-parametrical analysis) with survival as dependent variable, time as covariate and trial and group as fix factor ($p<0.05$)

Source	df	DEN df	F	P
group	1	44	32.54	**<.0001**
time	1	44	2.33	0.1338

trial	1	44	1.47	0.2325

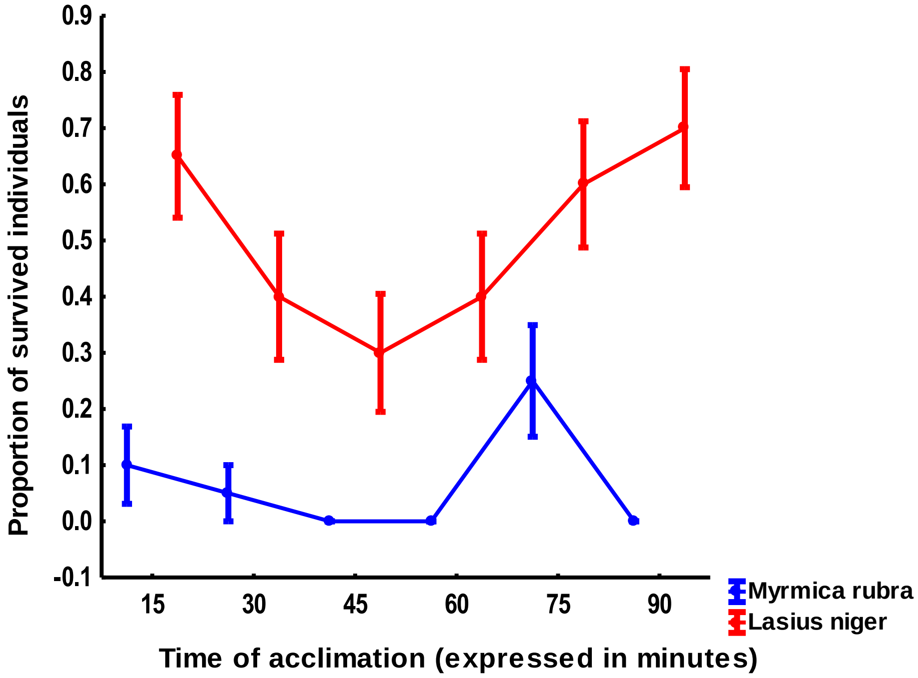


Figure 3. Average proportion of survived individuals after different time of acclimation for two ant species (*Lasius niger* and *Myrmica rubra*)

REVIEWS

Adam Łomnicki

The review of the report „Acclimation time on ant survival in minus temperaturę and time of recovery”

Formally, this report contains all the necessary elements but most of them are so badly prepared, that they do not seem to exist. The research problem is clearly stated in the Introduction and the hypothesis presented can be falsified but the reader can hardly follow how the entire investigations were carried on.

The Methods are written in such a way that the reader is unable figure out what was really done and for what reasons. For example, it is not clear what the word ‘repetitions’ mean. Are they replicates within each treatments or repetition in time? What is the relation between experimental groups and repetition? Eight experimental group doe not imply whether there are 8 group in each species or 8 groups for all species? Since I really was unable to learn from the manuscript what group and repetition mean, I am not sure whether it is proper application of statistics to consider them as a fix factor.

Since no name of species are given in Tables 1 and 2 with the results of GLM, I suppose that the name ‘group’ mean different species and this is even more obscure since in the Method 8 groups are mentioned but only two species. In the Results there are too many diagrams presented but similarly to the tables of statistical results without any comment which would explain why they were presented. Three lines of description for each experiment at the beginning of the parts with so many diagrams is not sufficient, a little bit more should be written. Some comments for each figure should be given or the figure without comments rejected. The report of Kruskal-Wallis test should also contain its statistics and degree of freedom.

This report can be quite good if the experimental design and the results are more precisely an clearly described. This is the mistake made by many young people. If they fully understand experimental design they do not realize that the report is the only way for the reader to learn what was really done. Science is a social activity and without ability to explain clearny to others was was done, it cannot be properly carried on.

Ulf Bauchinger

The authors present an interesting manuscript on the cold hardiness of ant species and how acclimation time may affect survival rate and recovery time. Despite the fact that some people claim ‘insects rule the world’ it appears that small experiments can diminish ant abundance significantly. This contribution is exciting and could be published after some fine-tuning and selection of the presented data.
The structure of the paper is mainly clear, the procedure quite nicely described. The importance of the research is built up in a stringent way. However, the ms seems in imbalance in terms of the amount of data presented and the rather limited discussion. One Picture and 10 (in words: ten) figures is even for this journal, ‘the Krakovian ant specialist’ beyond the scope that is acceptable. In the discussion the authors on the other hand refer to only two figures.

The methods are described in a reasonable way and especially the use of a pilot study is of interest. Unfortunately, this pilot stiudy is presented in another three figures and four tables.
Due to the extensive data set the authors seem to have overlooked the need to discuss data in a way that warrant its presentation.
The title does not seem to summarize the outcome of the paper. In the introduction the mass effect is predicted, but subsequently ignored in the approach, analysis and discussion.
One of the most troublesome issues for myself is however the last sentence in the abstract '… probably due to not precise cooling chamber.' I hope this study is not funded by a company that produces cooling chambers other than the one used in this study. This would raise a strong argument about a typical conflict of interest. Luckily the name of the cooling chamber is not mentioned, which is however necessary in research publications.
Please find some minor comments in the word file.

Justyna Gutowska

The subject of study is very interesting. Moreover, it seems the story of the experiment could be very good for belles-letters, however it doesn't make the research report easy and clear to read (and assess).
It is not clear if to look for the effect of acclimation time on cold-tolerance was the aim of the research, as e.g. it is not defined in an abstract, but showed later in the introduction and discussion. In an abstract it seems that the only aim was to compare the effect of acclimation duration on survival in three different species. It should be specified in the abstract, which species were chosen for the study.
Picture 1 is very helpful to understand the experimental design. However, it is not clear why the graphs look like different species had been treated by different acclimation times in given experiment. (It is possible to put them into the acclimation chamber starting in different time, but place each species to the minus temperature after acclimation of the same time).
The results section starts with clear presentation of the data from the experiment 1, but that the reader is bombarded with enormous number of graphs without text description. This makes the report very difficult to understand. Also discussion section seems to be very chaotic.

Justyna Kierat

The study raises an interesting topic related to global temperature changes and interspecific differences in temperature tolerance. I especially like the discussion of results, it is clear. Explaining differences in survival by different body masses of species is convincing for me. The differences between trials may have impaired discovering patterns which otherwise might exist. However, this huge variation was most probably because of equipment failure but not because of experimental design which was well planned.
I like the selection of literature and that newest publications are cited. Authors provide examples of studies both agreeing with their own results, and those where findings were opposite. Thanks to that they do not create false picture of perfect agreement of current knowledge with their study, and indicate research fields where results are unequivocal at present and where more research is needed.

In methods some details can be unclear if one did not know the design previously, and I mention them below.

Some detailed remarks:

- I like the visual aspect of the report. Pictures and graphs are clear and aesthetic.

- Some typos or small mistakes are in the text but they do not impair understanding.

- You write in large part in present time, I would suggest using past time because you already did the experiment.

- In “methods” section, when you mention about 400 specimens used, I would like to know how many specimens were taken from each species. You had 3 species so I suppose they were not equally represented. I have no objections against non-equal samples, I would only like to have the detailed information.

- Pilot study – you write that each 15min, boxes were moved from fridge to freezer. I miss an information if it was one box removed at each time interval, because from that fragment alone I would think that you sequentially moved all the boxes from fridge to freezer and back.

- Main study – you did four trials and it is OK even if the trial effect would not be significant in pilot, so I would not mention that “*Four repetitions of each test were carried out as trial effect in pilot study was significant*”. It is always better to have several trials (repeats) than one.

- Experiment 1 – the information about the role of litter in the experiment should appear at the beginning of methods (in the description of pilot where you first mention using litter) and then does not need to be mentioned here.

- Your “repetition” was one box with 10 ants, am I right? For me, it is not clearly stated in the text.

- Experiment 1,2 – I do not fully understand the thing with number of repetitions. At the beginning you write that you used 4 repetitions, but later you mention that in Exp. 1 there were only 2 of them and in Exp.2 – 4 repetitions. Maybe you could only write about repetitions number in description of Exp 1 and 2, and not in general description of main study?

- Activity of ants – “*the starting point for the measurements was the moment when first animal started moving*” does it mean that the first „awoken” animal had a time-to-recovery set as 0? Or only that from that time point you waited up to 10 minutes for awakening of remaining ants? If you set a first ant’s recovery time as 0 then you would lose information about time-to-first-recovery which probably differs between species studied.

- I'm not sure if all the plots are necessary. On figs 5,6 I see only a little chaos, and if they do not help to understand some patterns discovered by you but only variation, then I would prefer to have only the summary graphs (with averages from trials) in a paper. "Trial" was your random factor so we are not particularly interested how parameters varied between the trials (but only if they differed significantly or not).

Diana Maciąga

The authors pursue an interesting question of insects adjustments to thermal fluctuations by investigating an effect of acclimation time on survival in minus temperature and recovery time from chill-coma in different ants species of various body sizes.

Abstract and Introduction

The abstract seems too short to do report's justice, and lacks some words that could prove useful during indexing, such as the names of the chosen species. There are no key words listed, nor is any information about the authors provided.

The aims and predictions of the study are clearly stated and are supported by concrete biological foundations. The study design and the result section follow the assumptions described in the introduction.

Methods, Results and Discussion

At the beginning it is unclear how exactly the experiment was conducted. How many of the boxes were transferred into the cooling chamber each 15 minutes as it is reasonable that not all of them, and some were left inside for further 'acclimatizing'. Not until the 'experiment' section it becomes more clear. The picture attached is very good and greatly helps understanding the protocol. Perhaps it would be better to put it at the beginning of the methods section.

The sentence 'Because increased survival after longer acclimation time was observed, we extended twice time check point and test duration to two hours (30/60/90/120min' needs to be rewritten and corrected. Perhaps: we extended the time between time check points twofold, and extended test duration to two hours.

There should be a comma it this sentence (without it, it is very confusing at first): Myrmica rubra was more sensitive to low temperature than Lasius niger**,** and Formica rufa was less vulnerable to low temperature than Lasius niger.

The honest explanation of unexpected variability between samples reflects authors' fairness.

Language and layout

The report is generally clear and comprehensible, and forms a logical whole. The authors use register and vocabulary adequate for scientific papers.

The attached figures help in understanding the research design, though there are some errors in descriptions, for example the word '*lanius*' instead of '*lasius*' is used. Fig 10 might have been wider as the groups of points merge slightly.

The report should be read for typographical and punctuation errors (e.g. the descriptions of figures lack full stops), and minor corrections are needed. For example there is "test Kruskala-Wallisa" instead of its English translation, not all Latin names are in italics. Some corrections are also needed in terms of tenses used – in the methods section present tense is used instead of past.

The **Reference** section should be standardize.

Ewa Chmielowska

No information about Authors by their names☺
I would suggest to make sentences shorter, to make the text easier to read.
(…) Insects, as ectotherms having small body size, lose heat to the environment and therefore have evolved mechanisms that enable them to survive low temperature (…)-I hope to find more about other research about those mechanisms in your report, or at least some citation here
I am not sure, if the description of your pilot study should be put into your report. It is always conducted, I suppose, but It is rather not mentioned, unless it was incorporated into main study as its part.
I don't understand why you used a room temperature for recovery time counting, while the ants lived outdoor and were used to different conditions? It could produce some bias in your results.
About experiment design- maybe it would be useful in data analysis context to measure the speed of temperature dropping by placing the thermometers into cooling chamber and the freezer first, before the proper experimental part?
Can any social behavior in any of species be observed? If some ants cluster to avoid cold stress, and some not- it may be a moderating factor.
And unfortunately, I cannot see any suggestion about practical application of your experiment's results.

Joanna Sudyka

Study design appears to be clear and well thought considering given time limitations. However we do not know what are the implications of the fact that different ant species display different sensitivity and why was it studied at all? Is it advantageous in any way to cope better with minus temperatures for different ants? This should definitely be developed in discussion.
Abstract does not include any hypotheses. The Methods section lacks a few information as well. Were those ants actually measured by the researchers in the current project or size is taken from the literature (no citation)? If they were measured, some statistical tests should show that the differences (even if they appear to occur at first sight) are really significant, because it is fundamental for study design. There is no data on how many individuals were studied in each species. Definitely there are too much results shown in the paper- it creates confusion. It is not known which result is the most important. Tables with statistical analyses are also unnecessary. If the authors want to share them with others so much, they could do it in Supplementary materials.
Some technical problems need more attention. Not all graphs are properly signed there is no explanation what is represented by the bars (only Fig. 3 and 9 have it). I have noticed some minor spelling mistakes (line 14, 17, 86, 108, 141), grammatical inconsistencies (present and past tense in one paragraph- e.g. Pilot study,) and problems with sentence order and logic (line 50, 69-70). Citations of some works show one author, whereas in the literature it turns out that there are many authors of cited paper. Text should be edited more carefully (like adjusting text to the page size). I don't think it is necessary to put pictures and figures separately , all could be treated as figures, it would be more cohesive.

Summarizing, the project has some good points such as neat study design and large sample size allowing to expect robust results. However there are many weak points; the most important thing that lacks, is the justification of undertaking this study and some biological context for explaining the results. Discussion needs to be expanded.

Julia Wyszkowska

First of all I want to mention, I really like your research topic. It is not only interesting but seems also contain some novelty in this field.
Entire manuscript has got a clear and logic construction and all parts are separated what makes it easy to read. I like it also very much that you (performed and) described the pilot study. Moving the additional results into the supplement I think as a good idea as well.
What I would wish you to consider in final version of your paper:

- In the second paragraph of introduction I feel lack of explanation what the “surface area/ volume ratio” refers to.
- It is nowhere mentioned where are your ants from.
- You have a nice scheme of the design of experiment, but the reader has to read it from the end (the beginning is on the right side).
- It seems that you translated the name of Kruskal-Wallis test into Polish (results: ” test Kruskala-Wallisa”).
- It will be much more simple if you would unificate your graphs and stay with one colour for the same species all the time and so one.

FINAL VERSION OF REPORT

Acclimation time effect on ant survival in minus temperature and time of recovery

Giulia Casasole, Agnieszka Gozdek, Ewa Prawdzik

Abstract

Rapid cold-hardening process ensures insect survival after short temperature drop, while cold-tolerance mechanisms depend on the acclimation time. We hypothesize that there are differences in the effect of acclimation time on survival and time recovery between species. Three different ant species: *Formica rufa, Lasius niger, Mirmica rubra* were used in experiments. Ants were acclimated in temperature above zero and then transferred to minus temperature in order to check their cold-tolerance. The species differ significantly in survival and recovery time, but significant effect of acclimation time on cold-tolerance was found only in the first experiment. However we observed similarities in the different ant species survival pattern, what was probably due to not precise cooling chamber.
Key words: rapid cold-hardening, acclimation time, cold-tolerance, *Formica rufa, Lasius niger, Mirmica rubra*

Introduction

Temperature has indisputable impact on organism life history traits. Efficient adjusting to the thermal fluctuations is in particular important for the temperate organisms, which have to deal with seasonally, daily and even hourly changing temperature (Angilletta 2009). Insects, as ectotherms having small body size, lose heat to the environment and therefore have evolved mechanisms that enable them to survive low temperature. They are rarely cold-hardy all the year. Instead they use environmental cues about incoming winter to switch on physiological processes that strengthen cold tolerance (Teets et al. 2013). Interestingly insects are also able to cold-harden on shorter than seasonal time scale. Rapid cold-hardening process (RCH) protects them against hoarfrosts and sudden temperature drop when they are not in overwintering phenotype. In RCH process short exposure to nonlethal low temperature (minutes to hours) efficiently increase the insect resistance to cold shock (Lee et al. 1987). The duration and temperature of acclimation affect chill-coma temperature (Renault et al. 2012) and cold-tolerance mechanism (Waagner et al. 2013). Significant differences between species in response to critical temperature after acclimation are observed, but the ultimate cause of that variation remains unclear (Chown et al., 2009). We aim to determine what is the effect of acclimation time on survival in minus temperature and recovery time from chill-coma in different ants species (Formica rufa, Lasius niger, Mirmica rubra). We predict that cold-tolerance (by which we mean both survival and recovery time) will be higher if the acclimation time is longer and it will differ between species.

Methods

Animal model

Three species of ants were used for the investigation: *Formica rufa*, *Lasius niger, Mirmica rubra*. They varied in size: *Formica rufa* was 9 mm long, *Lasius niger* - 5 mm and *Myrmica rubra* was the smallest ant in our experiment (3.5 mm). The measurements of body length were conducted on sample size of 10 individuals per species and the average was presented with ruler (to the nearest 1mm). Total number of ants used in the experiment was 400 individuals from 3 species (*F. rufa* – 160, *L. niger* - 230, *M. rubra* - 70 individuals). The ants were caught in Ochotnica Jaszcze at the nest and were kept in room temperature overnight. Before the experiment they were placed in boxes (10 individuals per box) with standard amount of forest litter containing leaves and humus (0.5 g per box). Small amount of litter in each box protected against temperature shock.

Experimental design

Before starting the experiment a pilot study was performed to find time essential for ants to acclimate to minus temperature. We run out two different experiments using each time two species of ants. The general experimental design was as follows and the differences between the two experiments are provided in the next paragraphs.

Boxes containing 10 individuals each were placed in the fridge for acclimation (for 30, 60, 90 and 120min respectively). At one time a group of 16 boxes was placed in the fridge. A group consisted of 8 boxes per species: one repetition for each species at each acclimation time. Each repetition consisted of two boxes of one species. After acclimation boxes were transferred to the freezer for 3 min (Figure 1). After 3 min spent in freezer animals were transferred to room temperature and survival was checked. Chill-coma recovery time was measured for each alive animal during 10min observation (the starting point for the measurements was the moment when animals were transferred to room temperature). 10 min was enough time to measure recovery time because half and one hour after treatment there were no more living ants than during 10min observation.

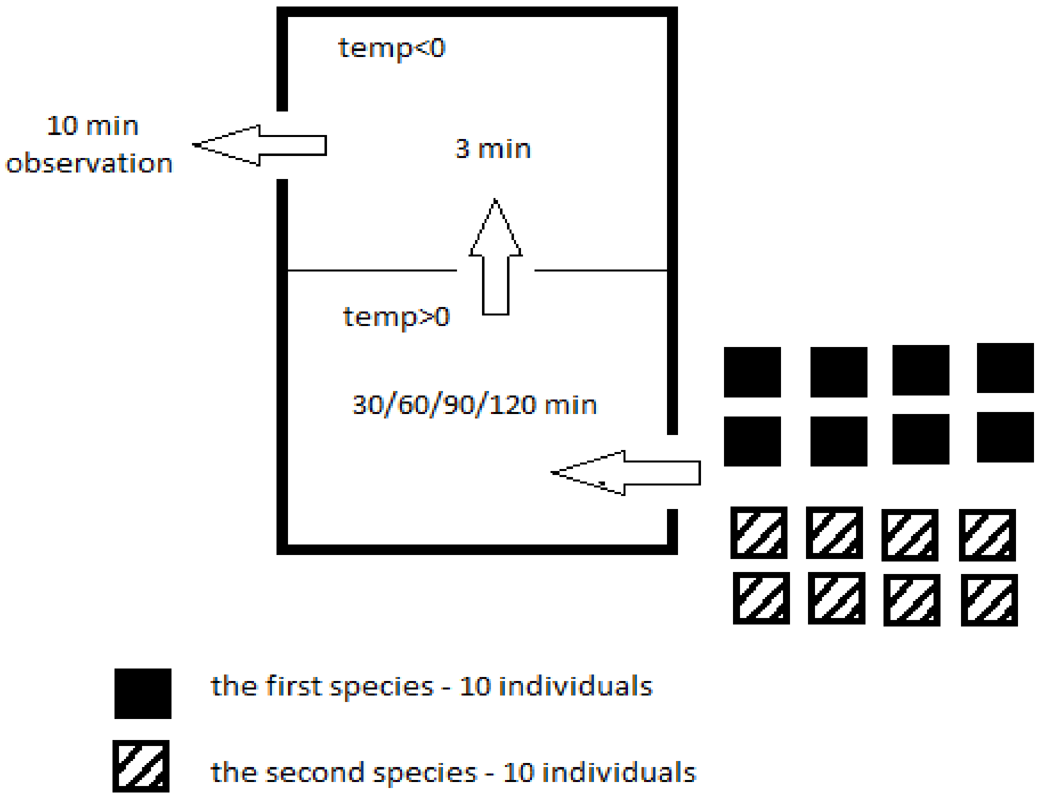


Figure 1 Experimental design. All colored squares illustrate one group of boxes (containing each 10 individuals). The boxes were placed at one time in the fridge for different acclimation times (30, 60, 90, 120 minutes), then in the freezer for 3 minutes and finally their survival and time of recovery was checked during 10 minute observation

Experiment 1

In the first experiment we used 2 ant species: *L.niger* and *M.rubra*. Because of the small amount of *M.rubra* that we could collect in the field we performed only one repetition per species per time (Figure 2). The experiment was carried out according the above protocol (Figure 1).

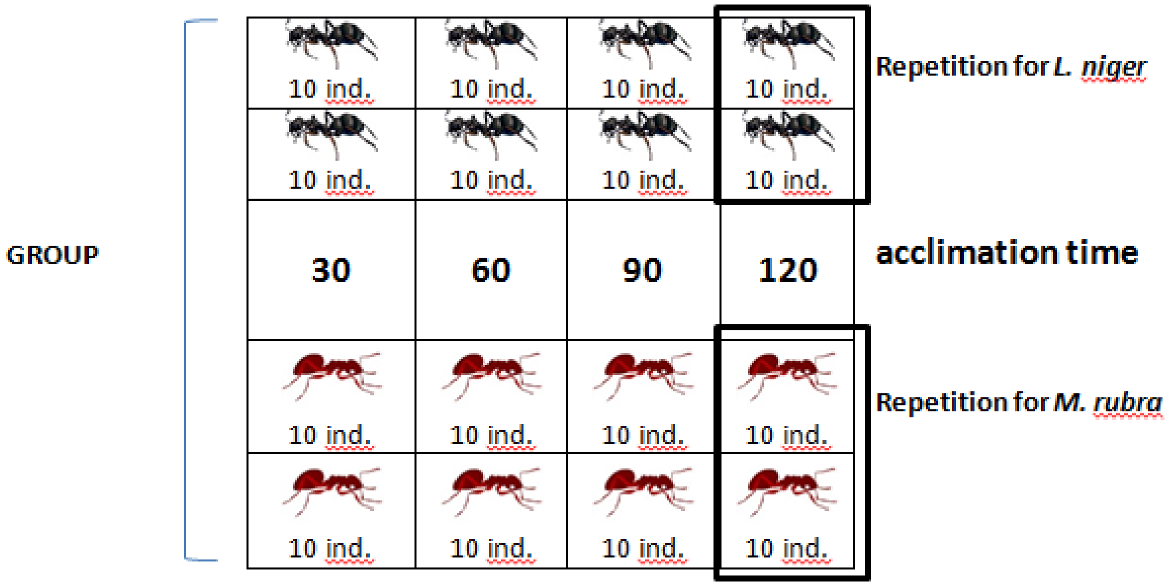
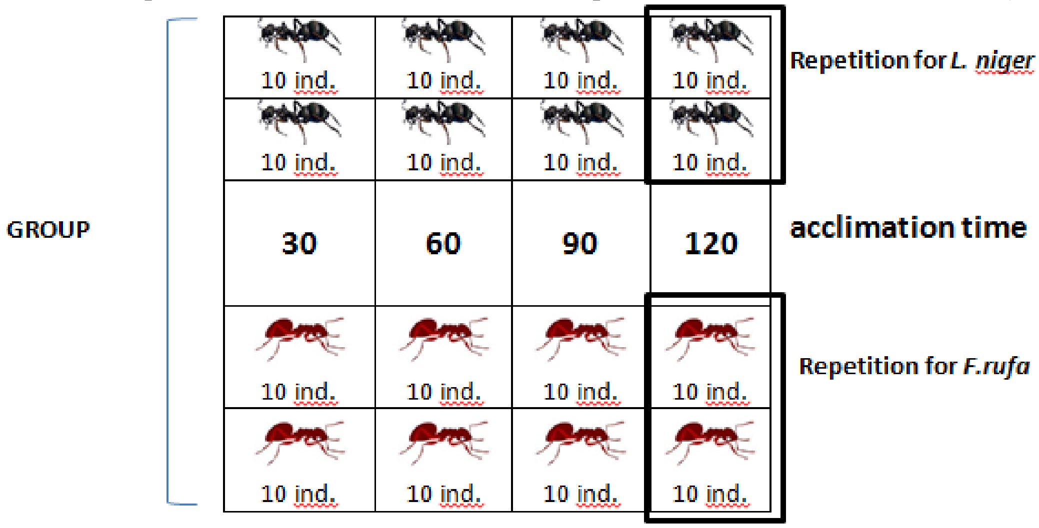


Figure 2 Experimental design – experiment 1. A group consisted of 8 boxes per species: one repetition for each species at each acclimation time. The group was placed at one time in the fridge.

Experiment 2

Because of the lack of *M. rubra* we decided to carry out another experiment utilizing *Formica rufa* and *L. niger*. Ants were divided and treated following the above protocol (Figure 1). The number of groups increased (compared to the first experiment) to two and the number of repetition increased to two for each species at each acclimation time (Figure 3).



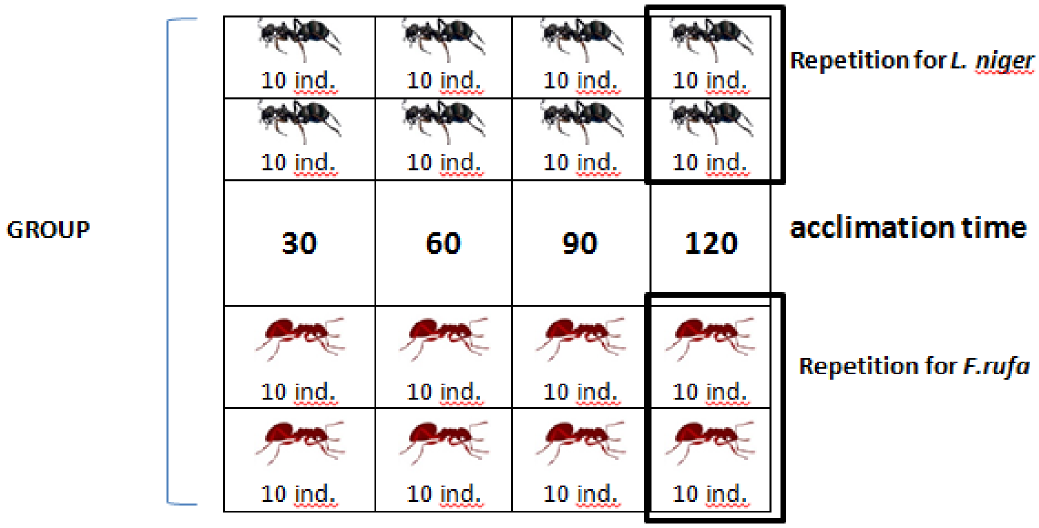


Figure 3 Experimental design – experiment 2. Each group consisted of 8 boxes per species: one repetition for each species at each acclimation time. Each group was placed at one time in the fridge. So in total we had 2 repetitions for each species at each acclimation time.

Statistical analysis

All the analyses were done in SAS version 9.

For the first experiment a GLMM was performed with recovery time as dependent variable, time of acclimation, species as fixed and box as random effect. Interaction between time of acclimation and species was considered. A GLMM using the GLIMMIX macro in SAS with logit link function and binomial error variance (Krackow & Tkadlec, 2001) was fitted to compare survival between species and to see if there was any effect of time of acclimation on survival. Survival was included as dependent variable, time of acclimation and species as fixed factors and box as random effect. Interaction between time of acclimation and species was considered.

For the second experiment a GLMM was performed with recovery time as dependent variable, time of acclimation, species and group as fixed factors and box nested in group as random effect. Interaction between time of acclimation and species was considered. A GLMM using the GLIMMIX macro in SAS with logit link function and binomial error variance was fitted to compare survival between species and to see if there was any effect of time of acclimation on survival. Survival was included as dependent variable, time of acclimation, group and species as fixed factors and box nested in group as random effect. Interaction between time of acclimation and species was considered.

Results

Experiment 1

An effect of acclimation time on survival was found and also a significant difference between species was observed (Table 1, Figure 4).

Table 1 GLMM with GLIMMIX macro in SAS: survival was included as dependent variable, species and time of acclimation as fixed factors and box as random effect .The interaction species x acclimation time was deleted because not significant.

Source	DF	Den DF	F Value	Pr > F

species	1	22.1	13.95	**0.0011**
Acclimation time	3	22.2	23.14	**<.0001**

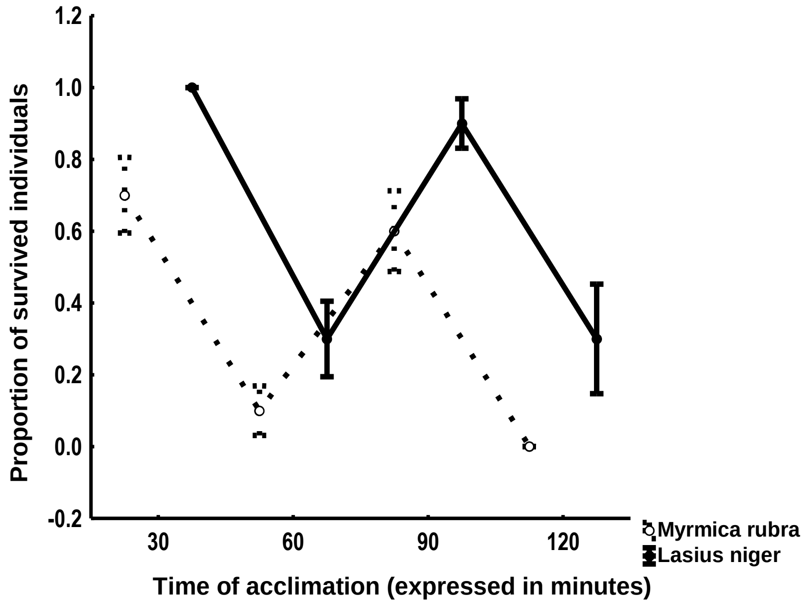


Fig.4 Average survival of animals (from one repetition containing 2 boxes) in relation with acclimation time (averages±SE)

There was no effect of acclimation time on recovery time, but we found a significant difference between species in recovery time (Table 2, Figure 5).

Table 2 GLMM with recovery time as dependent variable, acclimation time and species as fixed factor and box as random effect. The interaction species x acclimation time was deleted because not significant. Statistically significant effects (P<0.05) are reported in bold

Source	DF	Den DF	F Value	Pr > F
acclimation time	3	6	1.83	0.2414
species	1	6	22.5	**0.0032**

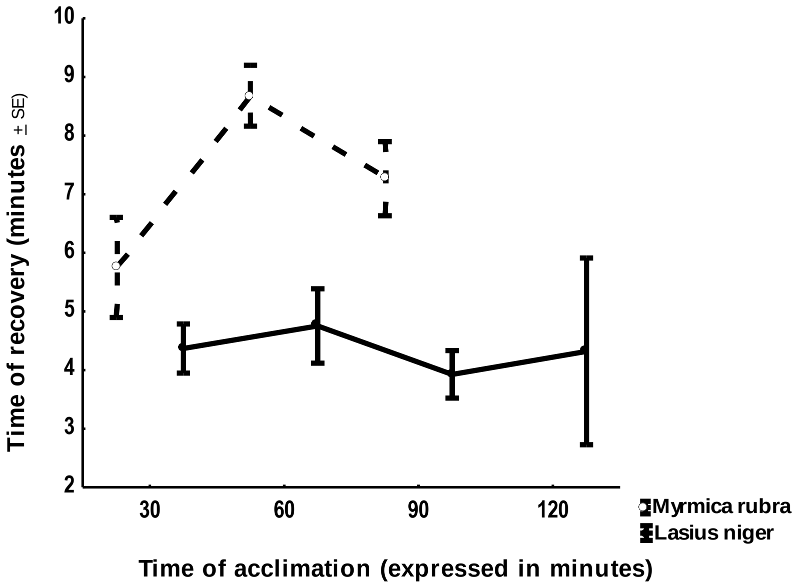


Fig.5 Average time (from one repetition containing 2 boxes) of recovery for two species after 3min spent in minus temperature in relation with the acclimation time

Experiment 2

No effect of time of acclimation on survival was found. There was a significant difference in survival between species and a trend in species-time interaction was possible to observe (time x species $F_{3, 52.8}$=2.70 P=0.0547) (Table 3, Figure 6).

Table 3 GLMM with GLIMMIX macro in SAS: survival was included as dependent variable, species, group and time of acclimation as fixed factors and box nested in group as random effect .The interaction species x acclimation time was deleted because not significant. Statistically significant effects (P<0.05) are reported in bold

Source	DF	Den DF	F Value	Pr > F
Species	1	55.8	5.82	**0.0191**
Acclimation time	3	55.8	2.29	0.0885
Group	1	1.82	4.87	0.1703

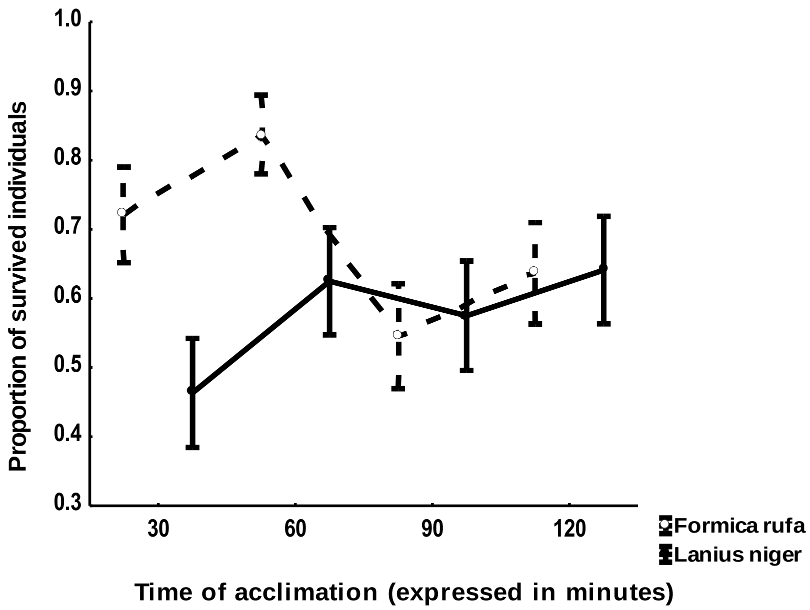


Figure 6 Average survival (from 2 repetitions, each one containing 2 boxes) in relation with acclimation time (average±SE)

There was no significant effect of acclimation time on time of recovery and no differences between species. Also the experimental design with two different groups did not affect the results (Table 4, Figure 11).

Table 4 GLMM with GLIMMIX macro in SAS: survival was included as dependent variable, species, group and time of acclimation as fixed factors and box nested in group as random effect .The interaction species x acclimation time was deleted because not significant.

Source	Num DF	Den DF	F Value	Pr > F
Acclimation time	3	24	0.3	0.8255
Species	1	24	3.45	0.0757
Group	1	2	1.66	0.3268

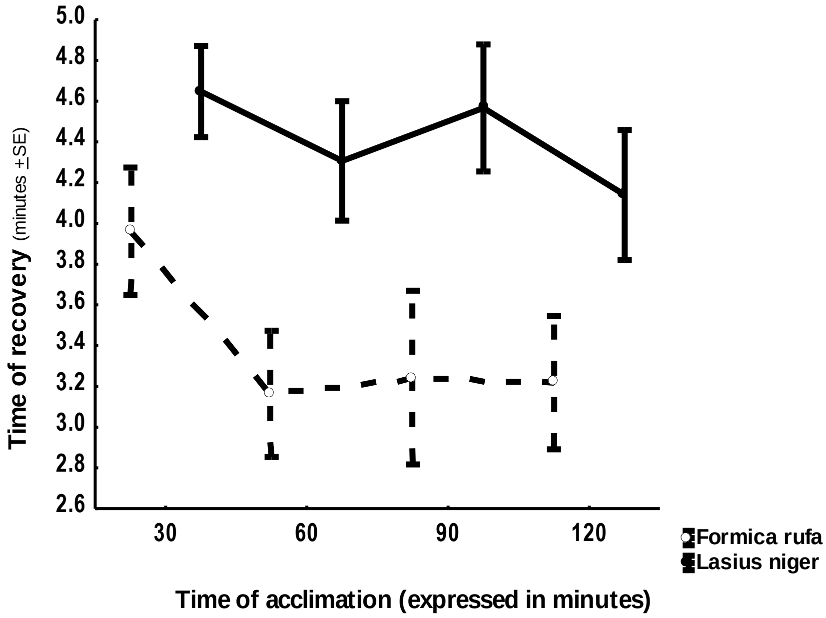


Figure 11 Average recovery time (from 2 repetitions each one containing 2 boxes) in relation with acclimation time

Discussion

According to literature, acclimation time influences cold-tolerance mechanism in insects (Waagner et al. 2013). We found significant effect of acclimation time on survival in the first experiment, however lack of sample repetition (due to problems with collecting *M.rubra*) is a major constraint of that experiment. We did not observe this significant effect in the second experiment and we did not find significant effect of acclimation time on recovery, what is consistent with other studies (Clarke et al. 2013). It is probable that investigated ant species are not particularly cold-tolerant, they rely on burrowing in the soil in order to avoid temperature extremes (Clarke et al. 2013,) and are lacking rapid cold-hardening (Lee et al. 1987, Chown et al. 2009).

Investigated species had different tolerance to low temperature (significant differences between species in survival and recovery time in first experiment and in survival only in the second experiment). *Myrmica rubra* was more sensitive to low temperature than *Lasius niger* and *Formica rufa* was less vulnerable to low temperature than *Lasius niger*. Because species vary in size, the cold-tolerance differences can at least in part result from body size variation (Gunn 1942).

We observed similar patterns concerning survival in both species after the same acclimation time - for instance in the first experiment survival at 60 and 120 minutes decreased for both species whereas increased at 90 minutes (Figure 4). We cannot be sure about constant temperature conditions in the chamber and observed survival patterns can result from alternating switching on and off the automated cooling process in the refrigerator. Obviously more repetitions are required in future studies and more precise cooling chamber.

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RESEARCH PROPOSAL

Differences in mass of above- and belowground parts of plants growing in different stress conditions

Diana Maciąga, Julia Wyszkowska, Justyna Kierat

Background:
The ratio of plant aboveground (shoots, leaves) and underground (root) biomass has been shown to depend on mechanical stress level. Different kinds of mechanical disturbance do not necessarily have the same qualitative influence on this plant characteristics. Plants exposed to intensive grazing tend to invest more in biomass of aboveground parts than in roots (Deng et al. 2013, Wen-tao et al. 2011, Bagchi and Ritchie 2010). *Plantago major* exposed to leaf brushing in laboratory conditions allocates lower proportion of biomass in roots (Anten et al. 2010). However, artificial windy conditions did not affect the proportion of biomass allocated into root in this species (Anten et al. 2010). Trampling is yet another form of mechanical stress and in laboratory it has been shown to change leaf morphology (Suonhara and Ikeda 2003). When simulated in pot cultures, trampling decreased overall biomass but in some species root biomass decreased more than aboveground biomass while other species responded conversely (Sun 1993).
As studies in trampled areas have indicated, plants that have a rosette form are frequently identified as having trampling resistance (Sun et al 1993). *Plantago major* L. is a perennial plant that belongs to the Plantaginaceae family. It reaches 15 cm height, but the size varies a lot depending on the growth habitat. The leaves are ovate to eliptical and grow in rosettes (Samuelsen 2000). *Plantago major* is a typical species occurring on paths, although it is not exclusive to them (Bates 1995) and can be found on grasslands.

Aims:
The aim of this study is to assess differences in root to shoot biomass ratio (R/S) in plants growing in conditions of different environmental stress. The study will be conducted in the field. In the research high stress conditions correspond to high degree of trampling down.

We hypothesize that plants growing in stressful (highly trampled) conditions will react similarly to plants exposed to grazing, and they will have lower R/S ratio than plants growing in less trampled area.

Methods:
Plantago major was chosen as the object of the research.
Three research plots will be studied. Each of them consists of two habitats: a dirt road used by farm animals and vehicles (trampled stand) and adjacent bands of grassland (non-trampled stand).
On each plot, samples from both trampled and non-trampled stands are taken randomly. The observer stands with their back to the research plot and throws a stone onto a plot. The nearest plant to which the stone lands will be chosen. However, all visibly damaged specimens will be rejected. Predicted sample size is 6 specimens from each stand (36 plants in total). The plants will be dug out using a shovel. The roots will be cleaned off the soil by placing the plant in the stream so that the soil get soft and can be detached from the roots without damaging them. The plants will be kept in fresh water until measurements to avoid withering.

In laboratory the plants will be dried with paper towels. The above- and belowground parts of each specimen will be separated and fresh mass will be weighted on a scale (to the nearest 0,01 g). The root/shoot ratio (R/S) will be calculated for each specimen.

R/S ratio will be analyzed using two-way ANOVA with trampling/no trampling as a fixed factor and plot as random factor.

Literature

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FIRST VERSION OF REPORT

Root to shoot ratio does not correspond to the level of mechanical stress in *Plantago major* L.

Justyna Kierat, Diana Maciąga, Julia Wyszkowska

Abstract

A balance between roots and above-ground biomass is necessary to ensure proper functioning of a plant. In response to mechanical disturbance, plants can alter the strategy of investment in their above- and below-ground parts. We hypothesize that specimens of *Plantago major* growing in highly stressful conditions (trampled on dirt roads) will invest more into shoot recovery, thus having lower root/shoot ratio than plants growing in less trampled area.
Root/shoot mass ratio between trampled and non-trampled areas was compared on four plots. No relation between R/S ratio and the degree of trampling was found. Plot x root mass interaction was significant, which indicates that differences in environmental conditions had stronger impact on *P. major* allocation decisions than the level of mechanical stress. The level of trampling, although too high for other species to survive, might have not been sufficient to affect *P. major*'s biomass partitioning between above- and below-ground parts. Also, the level of trampling might not vary enough between areas to reveal significant relation. Differences in environmental conditions (e.g. water availability, soil structure, fertility) may be responsible for the observed variability of R/S ratios between plots. Further studies on effects of additional environmental conditions on *P.major*'s R/S ratio is required.

Key words: *Plantago major*, root/shoot ratio, resources allocation, trampling

Introduction

The relationship between mass of above-ground and below-ground parts of plant has been of interest in agriculture (Harris 1992) and ecology (Monk 1966, Bagchi and Ritchie 2010). The main role of roots is to supply water and nutrients to a whole plant, and the above-ground parts produce carbohydrates from photosynthesis. A balance between roots and above-ground biomass is necessary to ensure proper functioning of a plant as a whole. Root/shoot (R/S) ratio depends on a species and can differ even in magnitude of 10 (Monk 1966). Deviation from root/shoot ratio characteristic for a given species is thought as harmful for a plant (Harris 1992). However, plants can modify relative investment in above- and below-ground parts accordingly to environmental conditions. R/S ratio of one species can vary depending on nutrient supply (Harris 1992), water content (Monk 1966) or disturbance level (Sun 1993, Anten 2010).

The grazing by herbivores has been a commonly studied type of mechanical stress plants are exposed to. It is known that grazing affects plants' allocation into above- and below-ground parts. R/S ratio is lower in grazed than non-grazed plants (Archer i Tieszen1983). Resources are moved from root to leaves in order to rebuild damaged and eaten tissues and maintain an efficient photosyntesis.

In anthropogenic areas plants have to cope with another type of mechanical disturbance, which can be called trampling. Plants growing on roadsides or paths are exposed to treading by passers, being drived over by cars and bikes etc. Similarly to grazing, this type of stress affects primarily above-ground plant parts. However, trampling causes squeezing of leaves and stems rather than tearing them off, thus the response of plant in the means of investment in above- and below-ground parts may be different for these two types of disturbance. The existing evidence is insufficient to draw strong conclusions because previous study gave different results depending on species (Sun 1993). Laboratory studies on mechanical stress without tearing shoots apart (leaves brushing) suggest that R/S ratio response to it may be similar as to grazing (Anten et al. 2010). The question if plants in natural conditions will respond in the same way as the ones in pot cultures remains open.

We examined R/S ratios in a common perennial plant *Plantago major* (L.). We hypothesize that plants growing in stressful conditions (highly trampled) will react similarly to plants exposed to grazing, and they will have lower R/S ratio then plants growing in the less trampled area.

Methods

Plantago major (Fig.1.) is a perennial plant which can be typically found in heavily-trampled places (Bates 1995). It has got a relatively thick roots that grow shallow under the ground what makes *P. major* a good object for root studies.

The study site was located in Ochotnica Górna in Western Carpathian Mountains. Four experimental plots were chosen. Half of each plot was highly trampled (sparse vegetation with patches of bare soil, traces of treading or driving over (Fig.2.), 'trampled' hereafter) and half of it was clearly less-trampled (vegetation with no traces of intensive treading (Fig.2.), 'non-trampled'). From each part of a plot 6 specimens of *P.major* were randomly chosen. A person standing on a border of a plot, with their back to it, threw a stone onto it. The plant nearest to the stone was chosen. Plants with damaged (torn) leaves or blooming were not included into the study. Lack of leaf part would result in a lower above-ground mass which would not reflect plant allocation but loss of mass caused by external factors. Because torn leaves were found on some plants on both trampled and non-trampled parts, we assume that it is not typical damage for trampled patches. Blooming plants were excluded as reproduction itself can alter plant's investments into its organs.

A chosen plant with some surrounding soil was carefully dug out using a shovel. The soil was wetted and *P.major*'s roots were carefully cleaned off it. Plants' roots were submerged in water in order to avoid withering, and they were transported to the laboratory. Each plant was carefully dried off with a paper towel and divided into below-ground (roots) and above-ground (shoots) part. Each part was weighed to the nearest 0,01 g.

Total fresh biomass per plant was compared between plots and trampling degrees using GLM. In order to compare root to shoot ratios between trampled and non-trampled plot parts, and between plots, analysis of covariance was performed. Shoot biomass was a dependent variable and root biomass a covariate. Trampling degree was a fixed variable and plot was a random one.

Figure 1. *Plantago major* L.

Figure 2. An example of area classified as trampled. No direct surrounding of other plants, bare soil visible.

Figure 3. An example of area classified as clearly less trampled ('non-trampled'). Prosperous vegetation visible.

Results

Plant's biomass was dependent neither on trampling degree (F=7,0555, p = 0,0766) nor on plot (F=2,9524, p=0,1988), interaction of these variables was non-significant (F=0,3725, p=0,7733).

Analysis of covariance showed a non-significant interaction of plot×trampling degree (F=0,5747, p=0,6355) and root mass×trampling degree (F=0,0525, p=0,8201), and these interactions were removed from further analysis. Root mass' effect was significant which indicated a relationship between root and shoot biomass. Plot×root mass interaction was significant, indicating that R/S ratios differed between plots. Detailed results of analysis are shown in table 1.

Table 1. Results of analysis of covariance with shoot mass as a dependent variable. Non-significant interactions were excluded from analysis.

	Effect	df	MS	F	p
plot*root mass	Random	3	0,0447	0,5881	0,6265
degree of trampling	Fixed	1	0,2909	3,8306	0,0575
root mass	Fixed	1	8,6103	47,6485	0,0020
plot*root mass	Random	3	0,2415	3,1806	0,0345
error		39	0,0759		

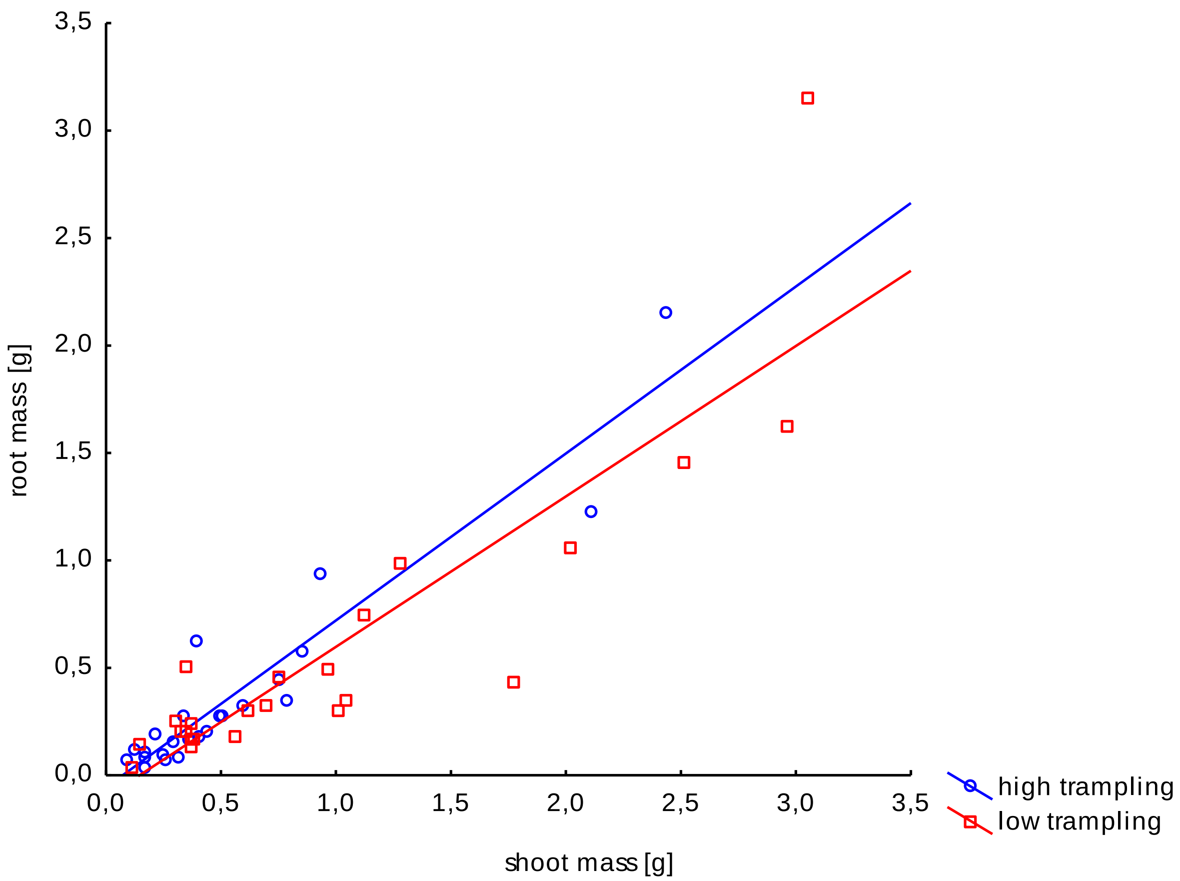

Figure 1. Relationship between root and shoot biomass of *P. major* in habitats differing in the degree of trampling. Difference in root/shoot ratio is not significant as indicated by the slopes.

Discussion

Our analysis revealed no effect of interaction between root biomass and trampling degree which indicates no dependence of R/S ratio between different levels of trampling stress. However, we found that R/S ratio differed between plots as revealed by significant root×plot interaction. This result suggests that for *P. major* differences in environmental conditions had stronger impact on their allocation decisions than the level of mechanical stress. It can be reasonable, taking into consideration that *P. major* belongs to species resistant to mechanical stress. The level of trampling experienced by the examined specimens, although too high to suffer by other species, might not have been sufficient to substantially affect *P. major*'s biomass partitioning between above- and below-ground parts. Also the level of trampling itself could not vary enough between areas called 'trampled' and 'non-trampled', despite the visible differences in vegetation density. On the other hand, in the field exist many factors which can potentially affect R/S ratio of a plant. Nutrient content and water availability in soil are some of them. In conditions of high soil fertility R/S ratio is commonly reduced in trees (Harris 1992). In xeric conditions R/S ratio increases what facilitates getting sufficient amount of moisture from soil (Hertel et al. 2013). Even such factors as CO_2 content in the air influence relative allocation in parts of plants: elevated CO_2 concentrations result in proportionately higher root biomass (Nie et al. 2013). During the study we found several differences between plots, e.g. in soil structure and density, the amount of stones in the soil, relief and slope of the plots. Some or all of these differences could contribute to observed variability in R/S ratio, although we cannot determine which were important and which ones were not.

Our results suggest that future studies on R/S ratio in *Plantago major* should focus on influence of multiple environmental variables and their possible interactions. Because of multitude potential factors existing in the field, which may affect allocation decisions of plants, laboratory cultures and long-term experiments should be incorporated.

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REVIEWS

Adam Łomnicki

The report contain all the necessary elements of the standard manuscript, Research problem is clear and the hypothesis tested can be falsified. In the title I would prefer a stronger statement not correspondence of R/S to mechanical stress but it changes to the mechanical stress. In the methods description one point is very enigmatic. It is not clear whether in each treatment (trampled and not trampled) there were four experimental plots or 4 plots placed close to a path, so that part of the plot was trampled and path not trampled. If so it implies paired plots. This should be clarified.

When reporting the results statistical tests using *F* statistics (page 4 and 5), the value of *F* and *p* are not sufficient, the name of the test and the degree of freedom should be also given.

In the Discussion where effects of trampling on R/S ratio were not found but only the effects due to different environmental conditions in the plots, it would be nice to look again on these plots to find out the features of the plot in which the R/S ratio is the highest and that in which it is the lowest. It can support or not support some information from the literature given in the Discussion.

Joanna Rutkowska

“To root or to shoot” seems to be the third(!) title of the study. The report has lots of good aspects, but I will focus mainly on weaker parts.

Abstract and Introduction clearly mention the single prediction of the study, namely the positive effect of trampling on root/shoot ratio. Such result would indicate that more extensive trampling should lead to higher investment into shoots. Surprisingly, the report puts disproportionally significant emphasis on some other aspects of the study, i.e. total biomass of the plant (and of its parts) and interactions involving study plots.

The Introduction has a perfect balance between general review of the field, ecological significance of the topic and aims of the study. Methods is sufficiently detailed, except for the fact that drawing of *Plantago major* shows generative parts, while not showing roots of that plant. In the case of the current study it is misleading.

Result section of the report is really trampled. Most importantly, a reader expects analyses concerning root/shoot ratio. Instead, there is analysis to total biomass followed by complex GLM model in which plot × root mass is on top of the table while “trampling” and any potentially interesting interactions are hidden below. Analyses do not include degrees of freedom. The second “Figure 1” reveals that the distribution of masses of plant parts was not normal.

In the revised version of the report, I would consider doing more replicates of the study, to check whether a significant trend of differences between trampled and non-trampled areas would emerge as significant. I would also avoid using synonyms for the main factor of the study. Currently, authors use expressions such as “degree of”, “level of trampling” “highly and less-trampled”, which is confusing. There are some linguistic faults, such as “too high to suffer” and “in the field exist” that need fixing.

The last line of the report is a killer. Is it really a “take home message” that the study should have been done in the lab?

Giulia Casasole

First of all I would like to congratulate with the authors of this report for the big work that they made in such a short time. I found your idea interesting and quite original, at least to me that I am not a botanist and so I don't have much knowledge in this field. I could read the report easily without problems connected with the way it was written in English. I could find only minor mistakes in the language due to constrains of time the authors had to deal with and to the fact that for no one English is the mother tongue. Coming to the structure the report contains all the necessary parts of a manuscript. The abstract summarizes the project, but reading it I started having doubts on the meaning of "some expressions" used in all report. First of all you mentioned the "degree of trampling". This expression made me think of a scale with assessed different levels of trampling that I expected to find explained in details in the method. Then in the method section I found out that you meant with "degree of trampling" trampled areas and not trampled areas (or less trampled), so that this expression meant the 2 groups of lands that you used for your study. There was also not any scientific and "objective" way to distinguish them and that could help me if I would like to reproduce the study. You put the pictures, but still for me going to field and choosing them would be not so easy and I'm not sure that my choice would correspond to yours. The size of each plot is not mentioned and your definition of plot confused me a little at the beginning. So actually you had 8 study plots, 4 for each group (trampled and not trampled). You told that each trampled area is combined with a no-trampled one, but I don't know which is the distance between each "combined plots" and the others. Considering that a trampled area was always attached to a no-trampled one I suggest you to take it into account into the statistical analyses. In the result section you did not specify the degrees of freedom in brackets and there were some mistakes in the table where the interaction plot x root mass was repeated twice and each time with a different P value. In the discussion you referred again to a "level of trampling" but this time not indicating a group (trampled or not trampled), but a gradient, a scale that you did not explain. I think that the discussion is quite consistent with the hypothesis and predictions explained in the introduction and the literature presented quite good (they mentioned 8 papers related to the main topic of the research project, some of them published in 2013). As final comment I only add that you should have considered other factors that could have characterize your study plots (exposition to the sun, slope, etc.): you minimized your study to only one factor (trampling or not trampling) that cannot explain alone a trade-off in plants between the investments in roots and shoots.

I hope that my review will be taken in a positive way as a help to improve your project.

Justyna Gutowska

The report is generally well structured, the research problem and hypotheses are clearly stated and all the text parts have a logical flow. However, an abstract could be shorter, e.g. contain less of explanation of the results. The title already reveals the result, so it doesn't encourage to read the whole report.

Definitely, if the literature suggests that the differences in root/shoot mass ratio can be caused by environmental conditions, they should be taken into account in such experiment. The report concludes that the laboratory studies could be more adequate for studying this problem.

Way of presenting the research in the report could be significantly improved. Exemplary, sentence about torn leaves does not bring anything to the article. Some used arguments lack the references, e.g. “reproduction itself can alter plant’s investments into its organs”. Language check is definitely needed and comments deleted. It could be useful for someone to repeat the study if he/she knew how big were the experimental plots. The ‘results’ section is a little bit difficult for the reader to understand, as e.g. it is only stated in the table description that the shoot mass was a dependent variable in the covariance analysis, and by the way, it the dependent variable should be on a Y axes on the graph. Describing the graph you write that Difference in root/shoot ratio is not significant as indicated by the slopes, whereas you indeed indicated the non-significance of difference by the covariance analysis.

Agnieszka Gozdek

The structure of the paper is logic and clear. The new paragraph begins from new thought. All the elements required in the manuscript are given here. The abstract is good and contains crucial information about results and suggests directions for further research.
Sentences in introduction are logically connected but last sentence concerns hypothesis can lead a readers in mistake. It may suggest that you also examine how plants react to grazing.
Methods are described in details but you missing two important aspects. In your study above-ground parts are very important so why you mention in this part only about roots morphology? Thick, flat and spreading leaves may protect plant from trampled. In methods where study site are described table with easy plot characteristic could be helpful (distance from houses, river, type of litter, presence of stones). It would be easier to consider another environmental factors what will enable more precise analysis of results. The most part of the references are relate to abiotic elements. Think about that.
The results are presented clearly. In the table you can bold significant effect and make it more visible.
The style in which the text is written is simple but precision, and make the whole text quite clear and easy to read

Ewa Chmielowska

The structure of your report includes all necessary parts, however I am curious what could be the practical application of your research results. I would also suggest to distinguish these parts with bold type.
The text is written in clear, simple style, which makes the report easy to understand
Plantago major is a common ruderal plant. It may survive near passages with hard traffic, however we do not know what is responsible for their morphological variations at those chosen plots. Is it for sure the trampling or maybe a disease? Or maybe different availability of nutrients or different history of succession caused the differences in plots and plants appearance? Therefore, the method of sampling (actually- what method??) seems to me not convincing enough. We cannot, for example rule out the possibility, that the plants were trampled to some degree in all chosen plots. However, as there is an obvious time limitation, I understand that there was no time to observe the trampling process at the plots.

And concerning the names: there is an inconsistency in calling samples: why the “less-trampled part” is called “non-trampled”? Was it trampled or not?
It would be much more informative, if the Researchers could provide a fuller description of analyzed habitat.
What is a “Root mass’ effect”?
Second figure 1 actually should be figure 4
In your discussion I didn’t found a finding, that the results may differ in different plots because of other plant species visible on the second photo.
And where is the root part of Plantago from the picture?:)

Ewa Prawdzik

At first I would like to congratulate on the style. In general the report is written in clear and straightforward way, what make it pleasant to read. The study problem is clearly stated and introduction explains why the problem is noteworthy. At least I am convinced. However the literature could be a bit more up to date (I mean the literature considering trampling, not the basic knowledge – the one cited is from 1993), but probably the authors looked for it days and nights and I believe that there was nothing newer.
Methods are presented clearly and I guess one could repeat the measurements relying on that description. I have just one suggestion: maybe it will be useful if the plots were briefly characterized (there are only 4 of them to describe). I think that short info about if one plot was just near the stream or sheltered from the sun while the other was in a very sunny place, etc. (or at least a note that they were chosen as similar as possible), could help to understand other factors, that could affect the results.
Results and Discussion parts do not detract from very good, in my opinion, structure of the text. For sure the plants from patches are exposed to bigger stress, and did you think over possible adaptation those plants developed? Like thicker leaves, more “procumbent phenotype”, etc. Maybe it is also worth to include into discussion.

Joanna Sudyka

The project appears as well thought and neat, research problem is accurately outlined. It may also be important as there seem to be no study on this kind of stress in natural environment. It is comprehensible and easy to read.
However it wasn’t exactly explained in the abstract how the ratio was calculated. Next, I get an impression that there are many irrelevant information in introduction and discussion. I guess it is because of the fact that authors tried hard to explain the inconclusive result. Tables with statistical analyses are also unnecessary, it was enough to show it in the text. On the other hand degrees of freedom for F in the first covariance analysis are missing.
There is no information on how four experimental plots were chosen, was it random, or belonged to one track. It is particularly important given that analysis demonstrated plot factor as significant for root mass. What was the size of the plot, were they equal? Were they distinct and remote enough to speculate that the conditions such as nutrient content, water availability and especially CO_2 content were so different to make *Plantago* alter its investment?

The report is well edited and technically coherent. I have noticed only some minor spelling mistakes (line 48, 50, 53), grammatical inconsistencies (line 28, 86) and logical flaws (line 77). There are two Figures 1 (drawing and graph) and the graph is not referred in the text.

Summing up: The project has many strong points, however the explanation of the differences between plots is not entirely convincing. The problems with analysis may result from small sample size taken by the authors.

FINAL VERSION OF REPORT

Root to shoot ratio does not change with the intensity of mechanical stress in *Plantago major* L.

Justyna Kierat, Diana Maciąga, Julia Wyszkowska

Abstract

A balance between roots and above-ground biomass is necessary to ensure proper functioning of a plant. In response to mechanical disturbance, plants can alter the strategy of investment in their above- and below-ground parts. We hypothesize that specimens of *Plantago major* growing in highly stressful conditions (trampled on dirt roads) will invest more into shoot recovery, thus having lower root/shoot ratio than plants growing in less trampled area.

Root/shoot mass ratio between trampled and less-trampled areas was compared on four plots. No relation between R/S ratio and between trampled and less trampled areas was found. Plot effect on R/S ratio was significant which indicates that differences in environmental conditions had stronger impact on *P. major* allocation decisions than the level of mechanical stress. Differences in environmental conditions (e.g. water availability, soil structure, fertility) may be responsible for the observed variability of R/S ratios between plots. Further studies on effects of additional environmental conditions on *P. major*'s R/S ratio are required.

Key words: *Plantago major*, root/shoot ratio, resources allocation, trampling

Introduction

The relationship between mass of above-ground and below-ground parts of plant has been of interest in agriculture (Harris 1992) and ecology (Monk 1966, Bagchi and Ritchie 2010). The main role of roots is to supply water and nutrients to a whole plant, and the above-ground parts produce carbohydrates from photosynthesis. A balance between roots and above-ground biomass is necessary to ensure proper functioning of a plant as a whole. Root/shoot (R/S) ratio depends on a species and can differ even in magnitude of 10 (Monk 1966). Deviation from root/shoot ratio characteristic for a given species is thought as harmful for a plant (Harris 1992). However, plants can modify relative investment in above- and below-ground parts accordingly to environmental conditions. R/S ratio of one species can vary depending on nutrient supply (Harris 1992), water content (Monk 1966) or disturbance level (Sun 1993, Anten 2010).

The grazing by herbivores has been a commonly studied type of mechanical stress plants are exposed to. It is known that grazing affects plants' allocation into above- and below-ground parts. R/S ratio is lower in grazed than non-grazed plants (Archer i Tieszen1983). Resources are moved from root to leaves in order to rebuild damaged and eaten tissues and maintain an efficient photosynthesis.

In anthropogenic areas plants have to cope with another type of mechanical disturbance, which can be called trampling. Plants growing on roadsides or paths are exposed to treading by passers, being driven over by cars and bikes etc. Similarly to grazing, this type of stress affects primarily above-ground plant parts. However, trampling causes squeezing of leaves and stems rather than tearing them off, thus the response of plant in the means of investment in above- and below-ground parts may be different for these two types of disturbance. The existing evidence is insufficient to draw strong conclusions because previous study gave different results depending on species (Sun 1993). Laboratory studies on mechanical stress without tearing shoots apart (leaves brushing) suggest that the R/S ratio response to it may be similar as to grazing (Anten et al. 2010). The question if plants in natural conditions will respond in the same way as the ones in pot cultures remains open.

We examined R/S ratios in a common perennial plant *Plantago major* (L.). We hypothesize that reaction of plants growing in stressful conditions (highly trampled) will be similar to that described in literature for grazed ones, namely they will have lower R/S ratio then plants growing in the less trampled area.

Methods

Plantago major (Fig.1.) is a perennial plant which can be typically found in heavily-trampled places (Bates 1995). It has got a relatively thick roots that grow shallow under the ground what makes *P. major* a good object for root studies.

The study site was located in Ochotnica Górna in Western Carpathian Mountains. Four experimental plots were chosen where a path with severely trodden ground was surrounded by visibly less trampled shoulder. The plot was divided into two areas on the basis of subjectively assessed vegetation condition. One area was highly trampled (bare soil with patches of sparse vegetation, traces of treading or driving over (Fig.2.), 'trampled' hereafter) and the second one was clearly less-trampled (vegetation with no traces of intensive treading (Fig.2.), 'less-trampled'). The plots differed in size but both areas of each plot had equal surfaces. Detailed differences between all study plots are shown in table 1. From each area (trampled and less-trampled) of a plot 6 specimens of *P. major* were randomly chosen. A person standing on a border of a plot, with their back to it, threw a stone onto it. The plant nearest to the stone was chosen. Plants with damaged (torn) leaves or blooming were not included into the study. Lack of leaf part would result in a lower above-ground mass which would not reflect plant allocation but loss of mass caused by external factors. Because torn leaves were found on some plants on both trampled and less-trampled parts, we assume that it is not typical damage for trampled patches. Blooming plants were excluded as reproduction itself can alter plant’s investments into its organs.

A chosen plant with some surrounding soil was carefully dug out using a shovel. The soil was wetted and *P. major*’s roots were carefully cleaned off it. Plants’ roots were submerged in water in order to avoid withering, and they were transported to the laboratory. Each plant was carefully dried off with a paper towel and divided into below-ground (roots) and above-ground (shoots) part. Each part was weighed to the nearest 0,01 g.

Total fresh biomass per plant and R/S ratios were compared using GLM. In both analysis trampling was a fixed variable, and plot was a random one nested in trampling.

Table 1. Individual characteristic of different plots

	stony soil	distance to a stream	exposition	insolation	distance to a road
Plot 1	+	< 30 m	flat	+++	< 10 m
Plot 2	+	>100 m	SW	+++	> 100 m
Plot 3	+++	< 20 m	flat	++	adjacent
Plot 4	+++	< 10 m	flat	++	adjacent

Figure 1. *Plantago major* L.

Figure 2. An example of area classified as trampled. No direct surrounding of other plants, bare soil visible.

Figure 3. An example of area classified as clearly less trampled ('less-trampled'). Prosperous vegetation visible.

Results

Plant's biomass was dependent neither on trampling ($F_{1,40}$=3,5703, p = 0,1077) nor on plot ($F_{6,40}$=0,7362, p=0,6235). Only plot had a significant effect on R/S ratio (tab.2). Distribution of residuals was visually assessed in all analysis performed.

Table 2. Effect of trampling and plot on R/S ratio (GLM)

Effect	df	MS	F	p

trampling	Fixed	1	0,0229	0,0943	0,7692
plot(trampling)	Random	6	0,2429	4,2223	**0,0022**
error		40	0,0575		

Discussion

Our analysis revealed no effect of trampling on R/S ratio. However, we found that R/S ratio differed between plots. This result suggests that for *P. major* differences in environmental conditions had stronger impact on their allocation decisions than the level of mechanical stress. It can be reasonable, taking into consideration that *P. major* belongs to species resistant to mechanical stress. The level of trampling experienced by the examined specimens, although too high to suffer by other species, might not have been sufficient to substantially affect *P. major*'s biomass partitioning between above- and below-ground parts. Also the intensity of trampling itself could not vary enough between areas called 'trampled' and 'less-trampled', despite the visible differences in vegetation density. It is also possible that *P. major* did react to trampling by changing shoots morphology without different allocation in above- and below-ground parts. Trampling has been shown to change leaf morphology (leaf blade length to width ratio, participation of petiole in total leaf length) in laboratory (Suonhara and Ikeda 2003). On the other hand, in the field exist many factors which can potentially affect R/S ratio of a plant. Nutrient content and water availability in soil are some of them. In conditions of high soil fertility R/S ratio is commonly reduced in trees (Harris 1992). In xeric conditions R/S ratio increases what facilitates getting sufficient amount of moisture from soil (Hertel et al. 2013). Even such factors as CO_2 content in the air influence relative allocation in parts of plants: elevated CO_2 concentrations result in proportionately higher root biomass (Nie et al. 2013). During the study we found several differences between plots, e.g. in soil structure and density, the amount of stones in the soil, relief and slope of the plots. Some or all of these differences could contribute to observed variability in R/S ratio, although we cannot determine which were important and which ones were not.

Our results suggest that future studies on R/S ratio in *P. major* should focus on influence of multiple environmental variables and their possible interactions. Because of multitude potential factors existing in the field, which may affect allocation decisions of plants, long-term experiments should be incorporated.

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RESEARCH PROPOSAL

Does land use type affect European mole (*Talpa europaea*) activity?

Ewa Chmielowska, Justyna Gutowska, Joanna Sudyka

1. Aims

European mole (*Talpa europaea*) is a common, yet protected mammal that feeds on soil invertebrates (especially earthworms). It is perceived to be a pest species as it forms multiple molehills on its territory causing visible, significant alterations in its habitat. However, it has also a very important function for the meadow ecosystem, mainly improves soil structure through aerating it. In this study we assess how land management alters mole activity, to explore the possibility of protecting moles by adequate land management. Our aim is to examine activity of European mole in relation to the land use type. We will study three land use types, namely mowed and grazed meadow, grazed meadow, and abandoned meadow. We can expect that activity of moles expressed by the number of molehills will differ according to land use type.

2. Existing knowledge

The European mole (*Talpa europaea* Linnaeus) is a solitary, territorial mammal (MacDonald et al. 1997). The principal food of moles are earthworms (Edwards et al. 1999). Molehills are exits from underground tunnels, which provide shelter, breeding and feeding place for the mole (Edwards et al. 1999). Edwards and co-authors (1999) found that molehill production in grazed areas was one-third that of hay meadows. Also, half as many molehills are formed in unlimed as limed plots (Edwards et al. 1999). Such effects can be explained e.g. by the fact that molehill production is lower with less food availability (Edwards et al. 1999). In another paper (Schaefer & Sadleir 1981), it was demonstrated that molehill densities are significantly positively correlated with earthworm weights (perhaps because moles dig more when they have more available energy), and with soil pH, probably because of its effect on earthworms (Satchell 1955). The significant correlation between soil moisture and molehill numbers may indicate that moles dig more when it is easier for them to dig. The ground would be easier to dig in if the soil moisture content was high, and more difficult when it was dry and hard (Scheafer 1981). Alternatively, soil moisture may influence earthworm weights or earthworm activity in some way.

Peak molehill production occurred in spring and autumn, with few molehills formed at other times of the year in UK (Edwards et al. 1999), so September in Carpathians is supposed to be also a time of high molehill production and this is a suitable time of year for such study.

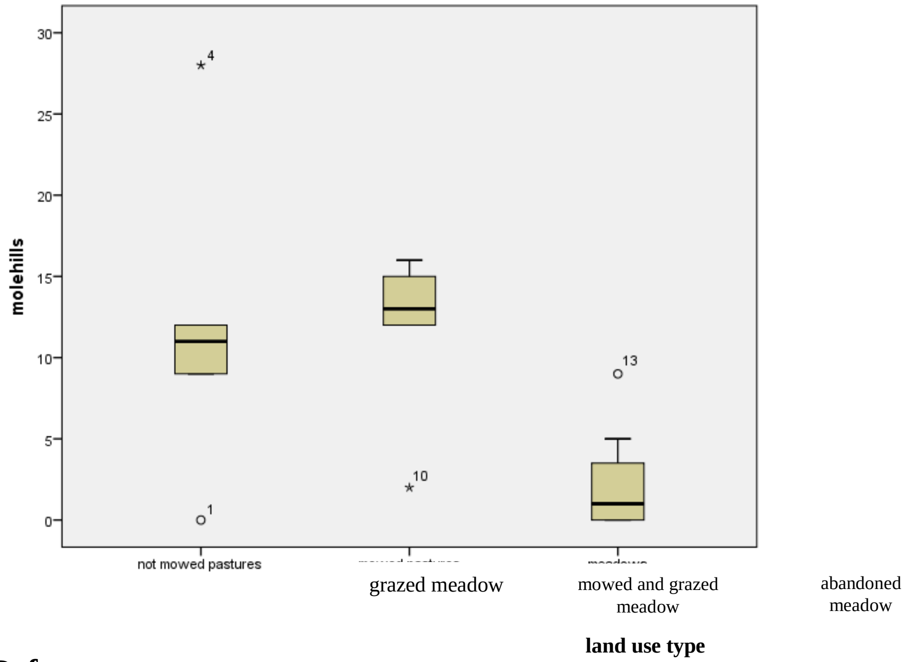
3. Research methods

Three types of habitat were chosen for this research: abandoned meadow, mowed and grazed meadow and a grazed meadow in Jaszcze hamlet in Ochotnica Górna (Nowotarski district, Małopolskie province, Southern Poland, N: 49º31’20.23; E: 20º13’19.55). From each type of habitat, we chose four replicates. Within each site, we randomly pick 7 different patches (altogether 84 samples) by throwing a stone. Then, from the point it was dropped, the circle of 3m radius is marked out with a piece of plastic rope (each circle area: 28,26m^2).

For each circle we count the number of molehills, and measure elevation, slope and exposure. The measurements are made with a Samsung Galaxy smartphone equipped with a GPS module able to obtain readings for slope, elevation and exposure, with a free software, namely “GPS status”, downloaded from Google Play service.

4. Preliminary results.

Our preliminary study showed that there are significant differences between three land use types ($F_{2,14}$=4,189, P=0,038). This gives promising perspective for the results of the full study on mole activity in different meadows.



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Weiner J., 1999, Życie i ewolucja biosfery, PWN Warszawa

FIRST VERSION OF REPORT

Does land use type affect European mole (*Talpa europaea*) activity?

Ewa Chmielowska, **Justyna Gutowska**, **Joanna Sudyka**

Abstract

Mole is considered to be a pest species as it forms multiple molehills on its territory causing visible, significant alterations in its habitat. This study explores the possibility of limiting mole-caused damage by adequate land use. Activity of moles, expressed by the number of molehills, was studied on three land use types: mowed and grazed meadow, grazed meadow, and abandoned meadow. Additionally, slope, elevation and exposure were considered as factors influencing mole activity. We expected that activity of moles expressed by the number of molehills will differ according to food availability and density of vegetation cover in each land use type. Mole activity was lower at abandoned meadows in comparison to other land types. This results offers promising perspectives for avoiding moles by the choice of specific land use type.

Introduction

The European mole (*Talpa europaea*) is a common, yet protected mammal that feeds on soil invertebrates (especially earthworms). It is considered to be a pest species as it forms multiple molehills on its territory causing visible, significant alterations in its habitat. Molehills are exits from underground tunnels providing shelter, breeding and feeding place for moles (Edwards et al. 1999). Different land use types are characterized by different biodiversity (Weiner 1999). We expected that those types will offer different food availability and soil conditions for digging tunnels for the mole. Our aim was to examine activity of European mole in relation to the land use type. We studied three land use types, namely mowed and grazed meadow, grazed meadow, and abandoned meadow.

The European mole is a solitary, territorial mammal (MacDonald et al. 1997). The principal food of moles are earthworms (Edwards et al. 1999). Edwards and co-authors (1999) found that molehill production in grazed areas was one-third that of hay meadows. Additionally, half as many molehills were formed in unlimed in comparison to limed plots (Edwards et al. 1999). Such effects can be explained by the fact that molehill production is lower when there is less food available (Edwards et al. 1999). It was also demonstrated (Schaefer & Sadleir 1981) that molehill densities are significantly positively correlated with earthworm weights (perhaps because moles dig more when they have more available energy), and with soil pH, probably because of its effect on earthworms (Satchell 1955). The significant correlation between soil moisture and molehill numbers (Edwards et al. 1999) may indicate that moles dig more when soil structure displays less mechanical resistance. The ground would be easier to dig in if the soil moisture content was high, and more difficult when it was dry and hard. Alternatively, soil moisture may influence earthworm weights or earthworm activity.

Peak molehill production occurs in spring and autumn (Edwards et al. 1999), so September in Carpathians is eligible for studying mole activity.

Methods

Three land use types were chosen for this research: mowed and grazed meadow, mowed meadow and abandoned meadow in Jaszcze hamlet in Ochotnica Górna (Nowotarski district, Małopolskie province, South Poland). Four plots with each type of habitat were chosen (Fig. 1). Within each site, 7 different patches were randomly picked (altogether 84 samples) by throwing a stone. Then, from the point it was dropped, the circle of 3m radius was marked out with a piece of plastic rope (each circle area: 28,3m^2).

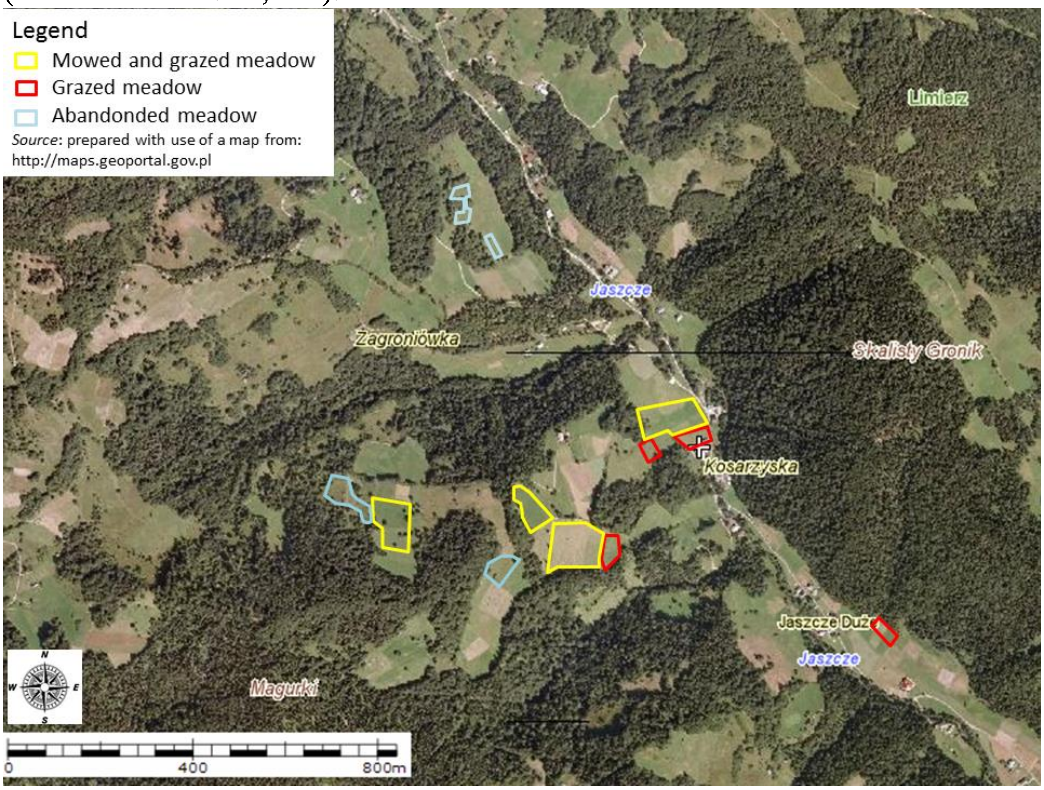

Fig. 1 Plots of each habitat type chosen for the research

For each circle the number of molehills, elevation, slope and exposure were measured. The measurements were made with a Samsung Galaxy smartphone, with a free software, namely "GPS status", downloaded from Google Play service. To test for possible differences in molehill abundance on each land type ANOVA was employed.

Results

The only correlation between recorded parameters was found for molehill number and slope (r=-0,409, P=0,031) in abandoned meadow. In general molehill distribution is not random but clustered across the field. As a result our molehill numbers do not display normal distribution. Therefore we calculated mean values for each plot within land use types (12 results for our three groups). We tested the differences with one-way ANOVA with type of land use as a fixed factor and found significant differences (Fig. 2, $F_{2,11}$=7,478,P=0,012).

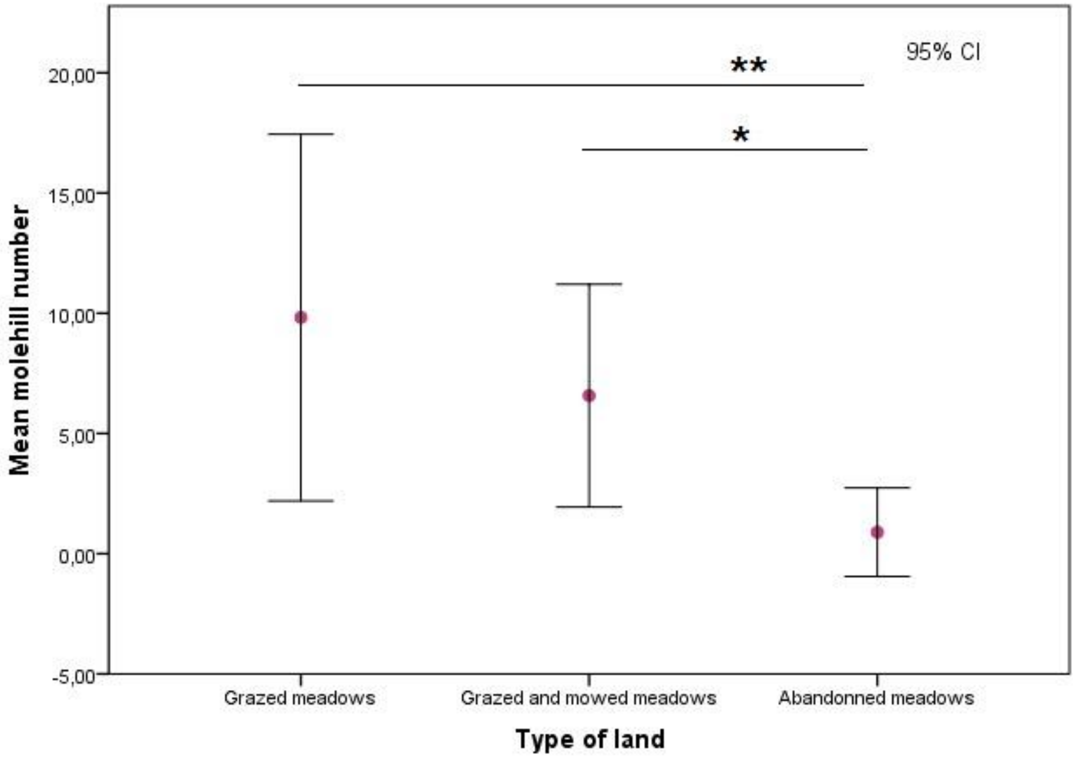

Fig.2 Mean molehill number found on different land types.

Discussion

Our study showed that mole activity differs across various land type use. Different land types offer distinctive habitats for the mole. Moles are significantly associated with drained mineral soils of pH greater than about 4.0. They cannot be found in soils with a very stony matrix which would inhibit the construction of an effective tunnel system (Milner & Ball 1970). The significant correlation between soil moisture and molehill numbers may indicate that moles dig more when soil structure displays less mechanical resistance. In our study plant cover on abandoned meadows was dense in comparison to other land types so we can expect that underground parts of those plants could prevent moles from digging their tunnels and creating molehills. Another function of molehill, namely aerating underground tunnels can also be reduced in densely grown area owing to aerating function of root structures resulting in less molehills. Additionally it appears that moles do not dig tunnels on steep slopes (probably more energetically costly) as it was shown by us for abandoned meadows.

Another important factor for moles is earthworm abundance (Funmilayo 1970). Each land type provides various limiting factors for earthworms (pH, soil type, heavy metal contents, presence of organic matter) (Eijsackers 2011). We may expect that studied land types display various earthworms abundances. Knowing that there is strong correlation between earthworm and mole number we may conclude that abandoned meadows are comparatively poor in earthworms. This could be an interesting perspective for further study.

Mole is considered to be a pest species as it forms multiple molehills on its territory causing visible, significant alterations in its habitat. Our study may contribute to possibility of limiting mole-caused damage by adequate land use. However, dense plant cover on abandoned meadows could be limiting factor for our study. It might have cause difficulties in spotting old and/or small molehills resulting in underestimation of molehills on abandoned meadows.

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Weiner J., 1999, Życie i ewolucja biosfery, PWN Warszawa

REVIEWS

Joanna Rutkowska

In is not clear what the main scope of the study is. One the one hand we get to know building habits of the mole, on the other hand authors seem to give advice on use of land by the farmers. In the latter case, the advice for the farmers is to abandon the meadows so that the land will not be damaged by molehills. How useful such recommendation is?
In the Introduction part I lacked the broader perspectives of the study and clearly defined predictions. The aim of the study was mentioned relatively early in the text and was followed by the literature review, which is quite a unique strategy. Disappointedly, after getting to know results of the previous studies, the reader starts wondering where the novelty in the current one is.
Methods section was too brief to allow repetition of the study. Specifically, the procedure of throwing the stone was not described. How heavy it was? Was the thrower doing it blindly? Was it thrown uphill or downhill? Etc. How were elevation and slope assessed? Finally, it seems that "Anova" is not a sufficient description of statistical methods used in the report.
Both Results and Discussion section seem to hide what was the main finding of the study. It should be clearly written at the beginning of each section. Unexpected, result section reveals problems with lack of normality in the data distribution. Even more unexpectedly, instead of transforming the data, authors reduced the power of their test by calculating means. Result section should have complete statistical analyses, including any post-hoc tests and explanation of the asterisks on the figure. I also suggest recalculating molehill number into density to make it more universal.
After reading the report I have more questions than answers. Some off those questions should be answered in the revised form. i) Sites of three types of land use differed in area. Was it anyhow included in the reasoning? ii) Was there a significant difference in slope between the three types of land?

Ulf Bauchinger

The manuscript investigates the abundance of molehills in relation to different land use types and additionally aims to address how elevation, slope and exposure. Because molehills are a huge disturbance for our perception of undisturbed landscape the topic is of super ordinate interest for mankind and I would want to see such research published in 'Nature'. However before publication some issues need to be addressed to warrant publication in 'Nature'.
The authors should use a '.' Instead of a ',' for decimal separation.
The general structure of the manuscript is clear, all main parts are in order, but I was wondering if adding acknowledgments has just been overlooked?
The abstract is concise and well written, but some of the statements may not be tackled with the outlined methods and data. For example the statement on 'food availability' to influence molehill presence seems not be testable and thus justified.
The general approach is outlined in the introduction, but some of the paragraphs (here and throughout the ms) could profit from a topic sentence to start with.

Method and Result sections are both to short and important methods and data that are interpreted subsequently are simply missing. Especially if such pressing questions have to be answered like 'what influences molehill abundance' and how can we avoid molehills important data have to be made available to the reader.

For example: How was elevation measured? What is 'exposure' and how is it measured? Both data are missing in the result section. Please give location of the study area in coordinates.
The description of the statistical analysis could require some more attention (please see respective comments in the ms).
Finally, I strongly recommend considering deletion of the last sentence in the discussion. If the molehill experts can not assure the readers of 'Nature' that it is possible to count all molehills within a circle of the diameter of 6 m to a certain degree of scientific certainty, who else would be able to generate data that will help to answer such important questions.

Please see also my comments made directly into the word document.

Agnieszka Gozdek

Project is logical planned and carried out. All the elements required in the manuscript are given here. The title well reflect content of work. The aims and predictions are clearly presented. In general project is interesting but results have not practical application as you mention. If we would like avoid moles we should not use land (which is contrary to the last sentence in abstract). The analysis shows that moles are more active in mowed and grazed meadow, grazed meadow then in abandoned meadow.
The methodology is clear, but you should consider whether the plots were choosen correctly (some plots have common border, others have not).
Sentence "We expected that activity of moles expressed by the number of molehills will differ according to food availability and density of vegetation cover in each land use type." Did you check food availability on the plot? This is only a guess.
In my opinion data like elevation, slope and exposure very well complete the experiment although not statistically significant.
The language is a bit heavy because some sentences are to long (four lines).

Ewa Prawdzik

In general the report is written in clear, understandable way. Introduction contains a vast amount of useful, carefully gathered information and that is for sure a big advantage (maybe it could be a little better organized but it is a petty issue). However I found hardly any information about significance of the research problem in the introduction. The possible practical application of the findings is mentioned in abstract, but according to the results the only significant difference in molehills number is between abandoned meadow and other types of investigated land (and not between grazed or grazed and mowed meadow). So Land abandonment is the way to avoid moles?

Methods are described in detail (classy map) and I guess it is possible to repeat measurements basing on the description. The smart idea with utilizing smartphone app enabled to collect additional, useful data.
Some up-to-date publications could be used in Discussion and Introduction parts, but that is of course limited by the time we had for writing the reports.

Giulia Casasole

First of all I would like to congratulate with the authors of this report for the big work that they made in such a short time. I found your idea interesting and reading your introduction and discussion made me discover the function of mole hills that I didn't know before. I read your report quickly and easily without the need to come back to unclear parts. It is well written in English and it follows the structure of a manuscript. In the abstract you summarize your study with methods, predictions and result, but the expression "this results offers promising perspectives for avoiding moles by the choice of specific land use type" does not convince me. You cannot change a land use to avoid moles. According to your result you should keep all lands as abandoned meadows to avoid the "mole pest" and I guess that you agree with me that it is not possible. Your distinction in 3 types of land use is not so clear and I do not know if I would be able to reproduce the study finding the same categories of lands in the field. From the photo I noticed that your plots are not at the same distance between each other. For example there are three grazed meadows attached to mowed and grazed meadows and one distant from all of them. There are also two abandoned meadows close to each other, but far from the other plots of the same land use and also of different land use. You should take it into account in your statistical analyses. In your conclusion you try to explain the fact that moles hills are less abundant in abandoned meadows with the scarcity of earthworms and/or the dense vegetation, but you do not consider the bigger difficulty of spotting them in this type of land use compare to the others. I hope that my review will be taken in a positive way as a help to improve your project.

Diana Maciąga

The authors make an attempt to investigate the relation between land use and activity of the European mole, indicated by the number of molehills.

Abstract and introduction

The following sentence: 'We expected that activity of moles expressed by the number of molehills will differ according to food availability and density of vegetation cover in each land use type' suggests that food availability and density of vegetation will be examined, while no such studies are performed. The question of food and density of vegetation is only raised in discussion of the relation between land use and moles activity, which in turn, is the main subject of the report and should be described as such in the abstract. Similar confusion is created by the first paragraph of the introduction.
In the abstract the authors also state that the report 'explores the possibility of limiting mole-caused damage by adequate land use', though the studies concentrate on the relation between land

use and mole activity. The authors do not pursue the question of how the results of the study may contribute to the possibility of limiting mole-caused damage by adequate land use.
The abstract lacks the list of key words.

Methods and results
The description of methods is clear and sufficient to understand the research design.
The attached figure help in understanding it.

Discussion.
The sentence about aerating function of root structure and how it affects mole activity is a bit unclear and requires further explanation.
The authors also suppose that abandoned meadows are poor in earthworms; perhaps it would be reasonable to add a few words as to why they state so? Without this, the conclusion seems unjustified.

Language and layout
Though the report should be read for typographical and punctuation errors and minor corrections are needed in the word order of some sentences (see below), the text is generally clear and comprehensible. Some amendments to the layout are needed (new paragraph should be indented). The authors use register and vocabulary adequate for a scientific paper.

Three land use types were chosen for this research: mowed and grazed meadow, mowed meadow and abandoned meadow(,) in Jaszcze hamlet in Ochotnica Górna - here the 'in Jaszcze...' part refers only to abandoned meadows, the word order needs to be changed, a comma is enough.

Molehills are exits from underground tunnels providing shelter, breeding and feeding place for moles – a change in word order needed, the sentence suggests that exits provide shelter etc.

Justyna Kierat

The project about molehills is interesting because it not only contributes to our knowledge about moles but indicates possibility of practical use of results of the study. The results agree with those from other studies, and are well discussed. I would like to read a bit more detailed description of statistical tests used.

Some detailed comments:

- The style of writing is very clear. I do not see any mistakes except a few misspellings.
- Introduction: at the beginning of second paragraph are some information repeated from the first one (about moles' food).

- Methods: You did not mention what statistics did you do for slope, elevation and exposure. Was it regression in all those cases? And was correlation performed on raw data, or on means like ANOVA? Was each meadow analyzed separately or all of them in one analysis?

- Results: I would be happy to see a graph with correlation of molehill number and slope.

- On the description to fig. 2 I do not see information of what are whiskers and asterisks.

- Discussion: The aspect of applicability in your study is interesting. It would be nice if you could write a bit more how land use can reduce negative impact of moles. I suppose, the most simple way would be to leave more meadows abandoned because they are less prone to "molehilling". However, farmers would be probably more interested in having less molehills on land which they exploit. Do you see any possibility to create some form of land mosaic, so that used (mowed/grazed) land would be protected from mole activity? E.g. surrounding mowed land by a border of unused meadow in order to deter moles from digging in that place?

Julia Wyszkowska

The entire manuscript is written in an understandable and simply way, what is it's value. The hypothesis and aims of the study are also directly given. In general I like your report, however I have following comments, which I think worthy to consider in final version of the report:

- You mention already in the abstract and then in the discussion that results could somehow help to avoid moles by the choice of specific land use type. My objection is, that you chose abandoned meadows as one of the habitat type. On those places not many moles occur, but they are also treat as barrens. So it works other way round: when farmers abandon grassland, moles are gone. It has very weak practical application.
- Your introduction, second paragraph, contains the sentence:

"The ground would be easier to dig in if the soil moisture content was high, and more difficult when it was dry and hard. Alternatively, soil moisture may influence earthworm weights or earthworm activity."

There is no reference given, so I understand it like your own prediction. It would be better to move this part to the discussion.

- In methods you do not mention if chosen plots (circles) cover each other.
- In first paragraph of your of discussion you are mentioning about pH and soil moisture – you did not study those factors and you do not even have any suspicions about their changing in research plots. I think it is unnecessary to write about those two factors.

FINAL VERSION OF REPORT

Does land use type affect European mole (*Talpa europaea*) activity?

Ewa Chmielowska, **Justyna Gutowska**, **Joanna Sudyka**

Abstract
Mole is perceived to be a pest species as it forms multiple molehills on its territory causing visible, significant alterations in its habitat. However, it has also a very important function for the meadow ecosystem, mainly improves soil structure through aerating it. In this study we assess how land management alters mole activity, to explore the possibility of protecting moles by adequate land use. Activity of moles, expressed by the number of molehills, was studied on three land use types: mowed and grazed meadow, grazed meadow, and abandoned meadow. Additionally, slope, elevation and exposure were considered as factors influencing mole activity. We expected that activity of moles expressed by the number of molehills will differ according to land use type. Mole activity was lower at abandoned meadows in comparison to other land use types. This results offers promising perspectives for protecting moles by land management planning.
Keywords: mole, molehills, land use type, meadows

Introduction
The European mole (*Talpa europaea*) is a solitary, territorial mammal (MacDonald et al. 1997). The principal food of moles are earthworms (Edwards et al. 1999). The mole is a common, yet protected species, that is perceived as a pest, because it forms multiple molehills on its territory causing visible, significant alterations in its habitat. Negative attitude towards this species creates the threat that it may become endangered, due to fast mole-repelling methods development (Edwards et al. 1999). However, it has also a very important function in the meadow ecosystem, mainly improving soil structure through aerating it.
Molehills are exits from underground tunnels, which provide shelter, breeding and feeding place for moles. Edwards and co-authors (1999) found that molehill production in grazed areas was one-third that of hay meadows. Additionally, half as many molehills were formed in unlimed in comparison to limed plots (Edwards et al. 1999). Such effects can be explained by the fact that molehill production is lower when there is less food available (Edwards et al. 1999). It was also demonstrated (Schaefer & Sadleir 1981) that molehill densities are significantly positively correlated with average individual earthworm body mass (perhaps because moles dig more when they have more available energy), and with soil pH, probably because of its effect on earthworms (Satchell 1955). The significant correlation between soil moisture and molehill numbers (Edwards et al. 1999) may indicate that moles dig more when soil structure displays less mechanical resistance. The ground would be easier to dig in if the soil moisture content was high, and more difficult when it was dry and hard. Alternatively, soil moisture may influence individual earthworm body mass or earthworm activity (Schaefer 1981).
Different land use types are characterized by different biodiversity (Weiner 1999). We expected that those types will offer different physical and soil conditions for digging mole tunnels. Our aim was to examine activity of European mole in relation to the land use type. We studied three

land use types, namely mowed and grazed meadow, grazed meadow, and abandoned meadow. Peak molehill production occurs in spring and autumn (Edwards et al. 1999), so September is eligible for studying mole activity.

Methods

Three land use types were chosen for this research: mowed and grazed meadow, grazed meadow and abandoned meadow. All these types are present in Jaszcze hamlet in Ochotnica Górna (Nowotarski district, Małopolskie province, South Poland, N: 49º31’20.23; E: 20º13’19.55), where the research was conducted. The differences between land types are expressed mainly by a different vegetation profile. The mowed and grazed meadow (Fig. 1) is characterized by very low vegetation, the large participation of *Poaceae*, and the low occurence of perennial species, as well as the presence of cattle dung. The vegetation on grazed meadows (Fig. 2) is comparatively higher, but the absence of perennial plants may still be observed. Abandoned meadows (Fig. 3) present more advanced stage of succession, with high vegetation, the abundance of perennial species (e.g. *Vaccinum*, *Rosa* and the plovers of *Fagus silvaticus*). Four plots of each land use type were chosen (Fig. 4). Within each site, 7 different patches were randomly picked (altogether 84 samples) by blindly throwing the same stone. Then, from the point it was dropped (if it rolled down the slope the point of hitting the ground was considered), the circle of 3m radius was marked out with a piece of plastic rope (each circle area: 28,3m^2). The total area of the plot was not important, because we picked the same number of identical patches at each of them.

Fig. 1 Mowed and grazed meadow

Fig 2. Grazed meadow

Fig. 3 Abandoned meadow

For each circle the number of molehills were counted, elevation, slope and exposure were measured. The measurements were made with a Samsung Galaxy smartphone equipped with a

GPS module able to obtain readings for slope, elevation and exposure, with a free software, namely "GPS status", downloaded from Google Play service.
To test for differences in molehill abundance on each land type one-way ANOVA with land use type as a fixed factor was employed in SPSS Statistics software. In general molehill distribution is not random but clustered across the area. As a result our molehill numbers do not display normal distribution. It is impossible to transform our data because in many cases we had 0 molehills present within the patch. Therefore we calculated mean values for each plot within land use types (12 results for our three groups). This enabled to receive normal distribution for our dependent variable. To examine influence of other factors we used raw data without any transformation, because the tests used in that part of the analysis do not require the normal distribution of data.

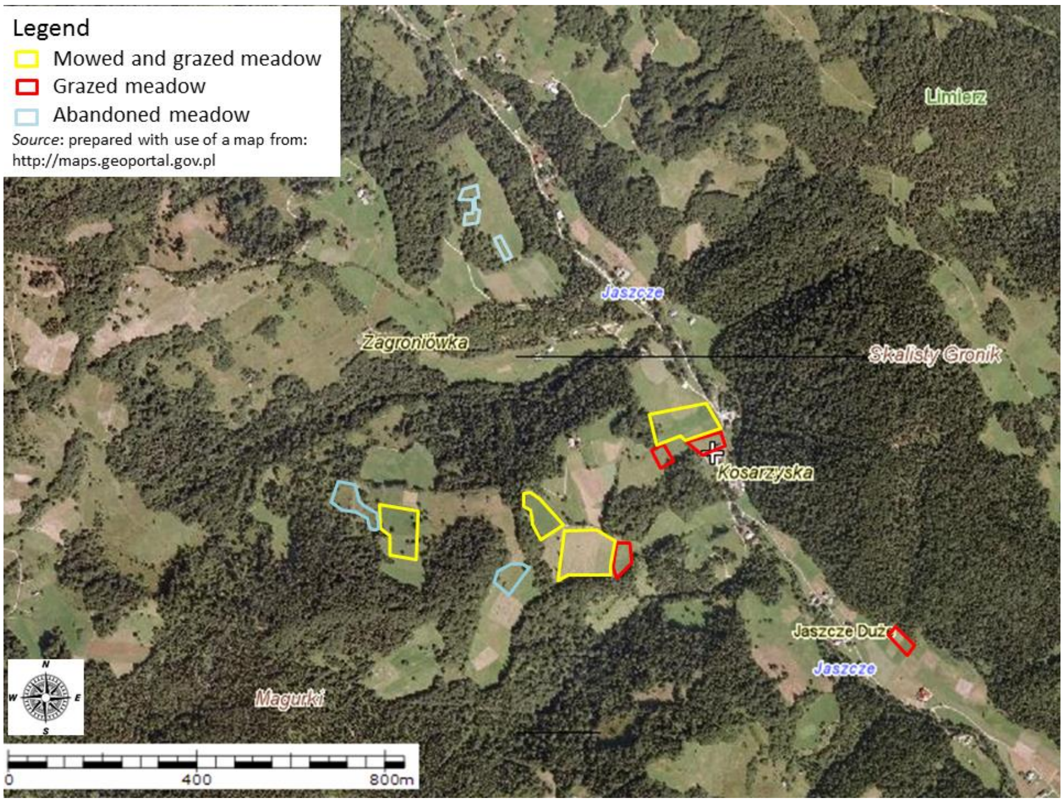

Fig. 4 Plots of each land use type chosen for the research

Results

We found the significant differences in molehill number between land use type (Fig. 5, $F_{2,11}$=7,478,P=0,012). Post-hoc analysis showed that significant differences occurred between grazed meadows and abandoned meadows (P=0,004) as well as between grazed and mowed meadows and abandoned meadows (P=0,038).
Another step was to detect influence of other studied factors on our result (raw data). We examined correlations of measured parameters (Supplementary material Tab. 1). Because elevation and exposure were correlated with land use type we excluded them from our model. We left type, slope and interaction of those two factors (Supplementary material Tab. 2) in Generalized Linear Model. We found that interaction was significant (P<0,000). Therefore we examined correlations of slope and molehill number within each type separately. The only

correlation was found for molehill number and slope (Supplementary material, Fig. 3, r=-0,409, P=0,031) in abandoned meadow.

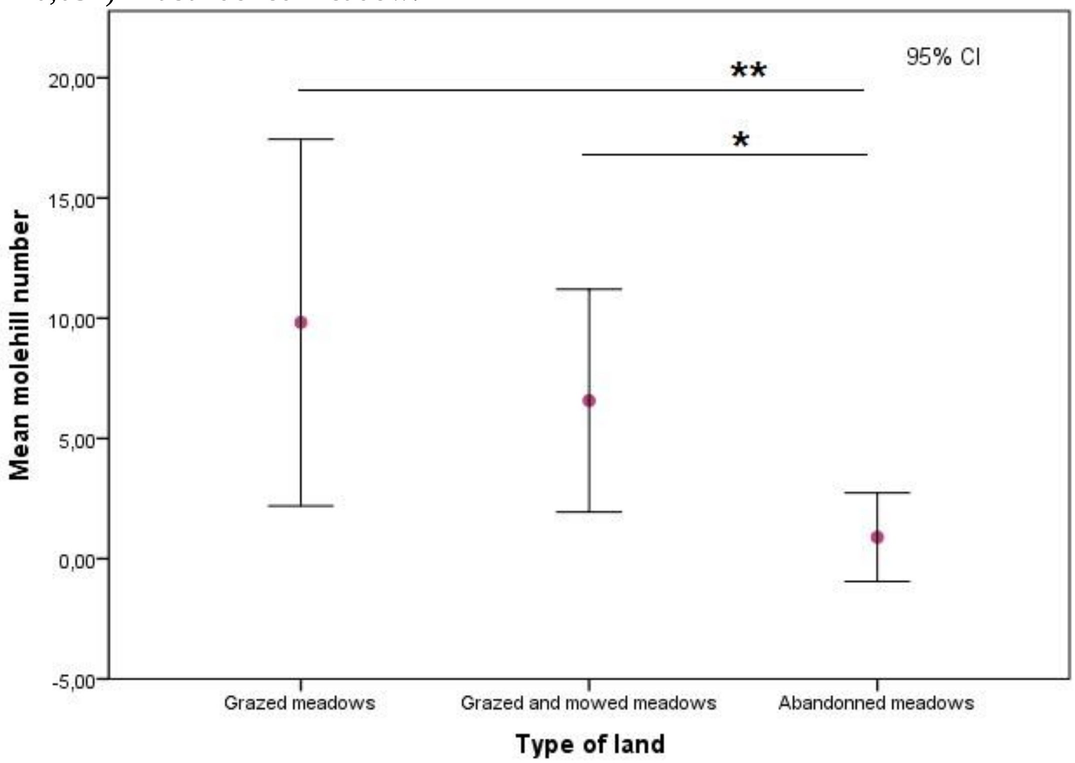

Fig.5 Mean molehill number found on different land types.

Discussion

Our study showed that mole activity differs across various land use type. Grazed meadows, as well as mowed and grazed meadows seem to offer more favorable habitats for the mole than abandoned meadows, where the smallest activity of the mole was noticed. Such results can be connected with the fact that the moles are associated with drained mineral soils of pH greater than about 4.0 and abandoned meadows are sensitive to soil pH changes (Critchley et al. 2002). The significant correlation between soil moisture and molehill numbers (Scheafer 1981) may indicate that moles dig more when soil structure displays less mechanical resistance. In our study plant cover on abandoned meadows was more dense in comparison to other land types so we can expect that underground parts of plants could prevent moles from digging their tunnels and creating molehills. Moles can also create less molehills in densely grown areas, because the root structures in such area may provide enough air for the mole tunnels. Additionally it appears that moles do not dig tunnels on steep slopes as it was shown by us for abandoned meadows. Moles cannot be found in soils with a very stony matrix which would inhibit the construction of an effective tunnel system (Milner & Ball 1970). At steep slopes soil erosion is heavier therefore we can expect more stony matrix that restrains mole activity.

Another important factor for moles is earthworm abundance (Funmilayo 1970). Each land use type provides various limiting factors for earthworms (pH, soil type, heavy metal contents, presence of organic matter) (Eijsackers 2011). We may expect that studied land use types display various earthworms abundances. Knowing that there is strong correlation between earthworm and mole number (Edwards et al. 1999) we may conclude that abandoned meadows are comparatively poor in earthworms. This could be an interesting perspective for the further study.

Mole is considered to be a pest species as it forms multiple molehills on its territory causing visible, significant alterations in its habitat, negatively perceived by people. However, it has also a very important function for the meadow ecosystem, mainly improving soil structure through aerating it. Therefore it is essential to protect the mole as valuable species for ecosystem functioning. Our study may contribute to developing adequate land management strategy for moles protection. Nowadays, more and more people abandon land and our research allowed to conclude that this implicates reduction of habitat available for the mole. Keeping grazing areas in the landscape can be suggested to protect this species.

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Supplementary materials

Tab.1 Correlation in pairs between measured parameters

			type	hills	elevation	slope	exposure
Spearman's rho	type	Correlation Coefficient	1,000	-,631**	,527**	,142	-,260*
		Sig. (2-tailed)	.	**,000**	**,000**	,196	**,017**

	N	84	84	84	84	84
hills	Correlation Coefficient	-,631**	1,000	-,337**	-,269*	,328**
	Sig. (2-tailed)	**,000**	.	**,002**	**,013**	**,002**
	N	84	84	84	84	84
elevation	Correlation Coefficient	,527**	-,337**	1,000	,092	-,381**
	Sig. (2-tailed)	**,000**	**,002**	.	,403	**,000**
	N	84	84	84	84	84
slope	Correlation Coefficient	,142	-,269*	,092	1,000	-,008
	Sig. (2-tailed)	,196	**,013**	,403	.	,944
	N	84	84	84	84	84
exposure	Correlation Coefficient	-,260*	,328**	-,381**	-,008	1,000
	Sig. (2-tailed)	**,017**	**,002**	**,000**	,944	.
	N	84	84	84	84	84

Tab.2 Generalized linear model

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	156,641	1	,000
type	4,510	2	,105
slope	167,452	23	**,000**
type * slope	57,335	12	**,000**

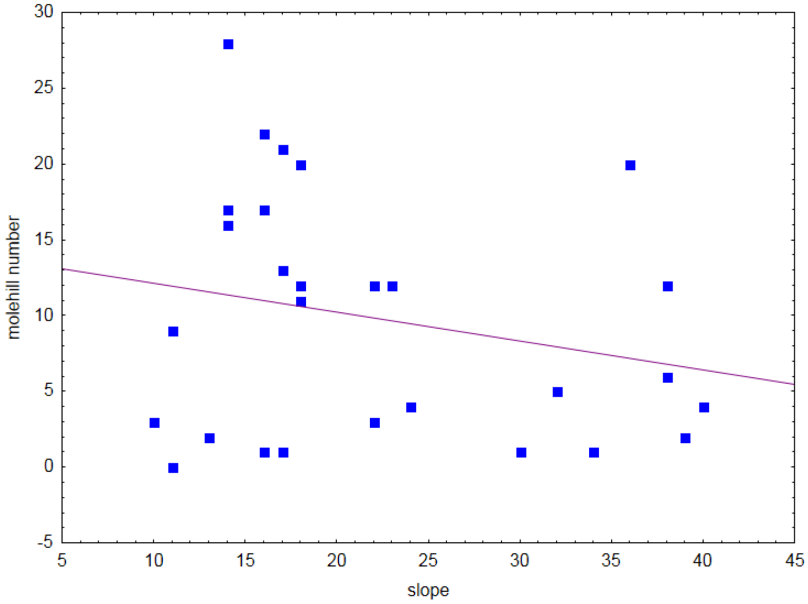

Fig. 1 Correlation between molehill number and slope for **grazed meadows**, r=-0,115, P>0,05

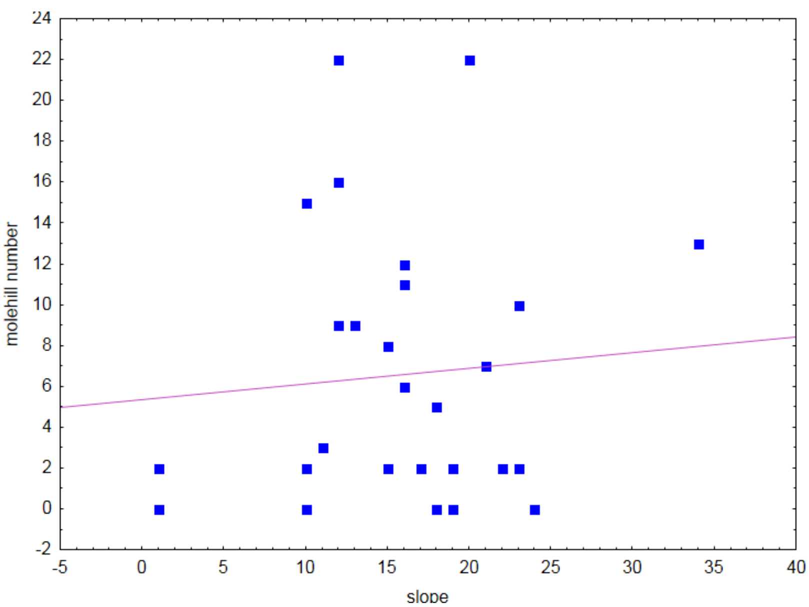

Fig. 2 Correlation between molehill number and slope for **grazed and mowed meadows**, r=-0,025, P>0,05

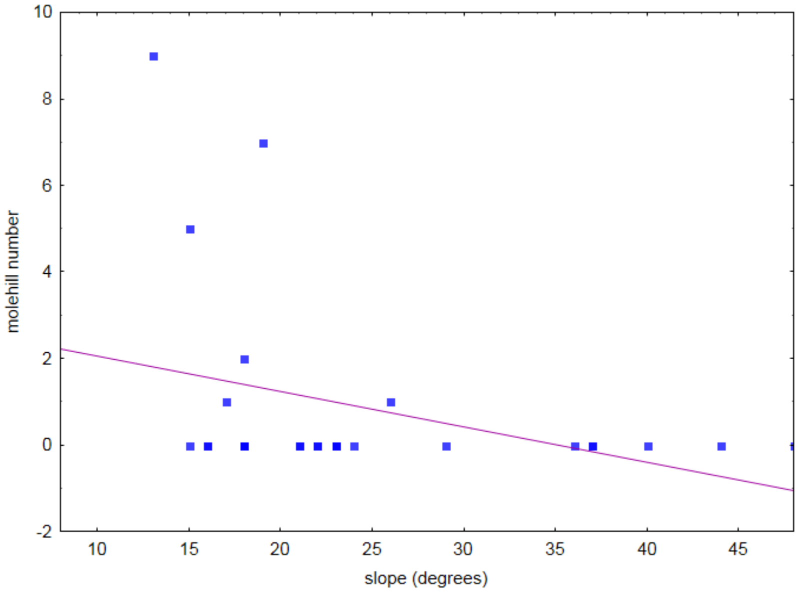

Fig. 3 Correlation between molehill number and slope for **abandoned meadows**, r=-0,409, **P=0,031**

MORE ~~PHOTOS~~ DOCUMENTATION OF HARD WORK ON PROJECTS

Field work...

Measurements and discussions in Station...

... and finally presentation of our breathtaking results

Asia and her birthday *Mentha* drink

...and *nejczer* was there.