Institute of Environmental Sciences

Jagiellonian University

Methodological Workshop in Evolutionary Biology for PhD students – practical part

Ochotnica Górna, 23-28th June 2018

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1. Workshop's participants

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4. Research topics suggested by participants

- How *Pinus* species are affected by human activity over a period of time in Jaszcze stream valley? (AKB)
- How does surface structure affect speed of snails? (MP)
- What light color is the most attractive for insects? (MP)
- Biodiversity of soil fauna in different habitats. (KZ)
- Does plant composition affect diversity of invertebrate species? (KT)
- How does plant height affect diversity of carabid beetles? (JM)
- How does floral diversity affect movements of grasshoppers? (JM)
- Does human activity affect distribution of flowering plants? (MGG)
- How is awareness of local flora affected by education and age? (EB)
- Snail preferences for different types of habitat. (AKB)
- What type of food is the most attractive for houseflies? (MP)
- The influence of temperature on feeding activity of selected gastropod species. (KZ)
- The influence of leave size of *Petasites* on the species richness of gastropods (KZ)
- Food preferences of Roman snails (KT)
- The effect of temperature on movement of carabid beetles (JM)
- Are basal rosette plants more common in pastures than in non-exploited fields? (MGG)
- Do lichens prefer more humid habitat? (MGG)
- Diversity of insects on the surface of cow's dung depending on the breed (EB)
- Can cell-phone software be used for plant species identification? (EB)
- How does temperature affect mating behavior of Roman snail? (KT)
- Is there a difference in using natural fertilizer depending of type of farming (vegetables versus crops)? (AKB)

5. Topics selected by participants

- How does surface structure affect speed of snails?
- Can cell-phone software be used for plant species identification?
- How does temperature affect mating behavior of Roman snail?

6. Topics finally developed by participants

- Fast and furious: the locomotor activity of the invasive slug *Arion vulgaris* on three different surfaces
- A comparative assessment of smartphone applications for the plant identification in the field
- High temperature speeds up mating behaviour in Roman snail *Helix pomatia* (L., 1758)

7. Research projects, reports, reviews and cover letters

7.1. Fast and furious: the locomotor activity of the invasive slug *Arion vulgaris* on three different surfaces

Miriam Gonzalez Gonzalez[1], Monika Prus[2]

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7.1.1. Project

Title: How does surface structure affect speed of slugs?

Summary

Invasive species are a worldwide problem that causes forfeiture of millions of dollars to the local governments. In particular, *Arion vulgaris* also known as Spanish slug is one of the most invasive species. Nowadays, very little is known about why some species have more capacity to become invasive than others which represents a risk for the integrity of the biodiversity. The locomotor activity is an interesting trait to be studied because of its direct role in the capacity of spreading. Because of that, the movement rate of Arion vulgaris individuals will be tested in different surfaces to assess its influence, and shed light about their adaptation capacity to different environments.

Aim / hypothesis

The main aim of the study is to assess how the structure of the surface affect the speed of invasive Spanish slug, *Arion vulgaris*. The experiment will be conducted on 3 types of the surface: grass, road, gravel.
Hypothesis:
1. On the road the movement rate will be the highest.
2. On the gravel the movement rate will be the lowest.

Methods

Arion vulgaris individuals will be collected in the Jaszcze valley in Ochotnica Górna. The movement rate will be checked for a randomly collected 20 individuals. Each individual will be tested on all three surfaces in a time interval between each trial to reduce the influence of stress or tiredness on the results. The speed of a snails will be calculated as the distance covered by each individual in 10 minutes.
The dataset will be analysed with one-way ANOVA with speed as a dependent variable and type of the surface as an explanatory variable.

Impact of results

Arion vulgaris is an invasive crop pests, thus the knowledge about the speed (movement rate) allow us to understand the patterns of migration and potential to dispersion. Results may also help in creating some alternative protection plans and in calculation of plant damage done by those snails.

7.1.2. Report – first version

Fast and furious: the locomotor activity of the invasive slug *Arion vulgaris* on three different structures.

Summary

Invasive species are a worldwide problem that causes forfeiture of millions of dollars to the local governments. In particular, ***Arion vulgaris*** (also known as *Arion lusitanicus*) is one of the most invasive mollusks. Nowadays, very little is known about why some species have more capacity to become invasive than others which represents a risk for the integrity of the biodiversity. In this study, we used the **locomotor activity** as a measurable trait for understanding this phenomenon, because of its direct role in the capacity of spreading and colonizing new areas. We analyzed 30 individuals of *A.vulgaricus* and measured their **speed** (cm/min) in three different **surfaces** (grass, gravel mixed with sand and pavement). We found that the slugs movement rate were the highest on the pavement and mixture of gravel and sand, and were the lowest on grass. Moreover, on pavement tested slugs behaved more erratic, what suggests that they were stressed out inasmuch is not their natural environment. In conclusion, the structure of the surface can be determinant of how mollusks and other invertebrates interact with the environment and more importantly, this can affect how they move and spread, potentially explaining why some species become more invasive than others.

Introduction

Arion vulgaris (Moquin-Tandon, 1855) or *Arion lusitanicus* [1] also referred as Spanish slug has become a pest of great magnitude in all over Europe during the last fifty years [2]. However, the origin of this particular specie as well as its taxonomic status is controversial. The newest investigations relate them from south-western Europe [3]. It has been quickly spread with transport of food products and by short distance active dispersal, and in the particular case of Poland genetic studies show that was introduced by different ways [4]. It should be noted that is an important pest to potato [5], oilseed rape [6] and to legume [7]. One of the biggest problems is its high resistance to different molluscicides [8].

Behavioral traits are extremely important for determining why some species have more capacity to become pests than others [9]. In other invasive species have been shown that the exploration activity is an important trait for explaining the invasiveness [10,11]. Recent investigations demonstrated that there are several important factors that control the movements of slugs as light intensity, air humidity and soil moisture [12,13]. In addition, in *A. vulgaris* body size determines the dispersal and temporary colonization of crops growing in arable fields [14].

Grimm and Schaumberger carried out a series of interesting experiments in Spanish slug. They observed that the soil surface and environmental conditions influences the slug activity. Locomotion activities of *A. vulgaris* were maximal in the morning and in the

evening, and minimal in afternoon. What is more, they showed the existence of direct correlation between body mass and locomotor activity [15].

Nowadays, there is the great necessity to understand and develop new targeting strategies that fight against crop pests, without affecting the beneficial species and polluting the environment. Some of these potential new strategies can be a combination of techniques mixing chemical or biological approaches with physical barriers. The deep understanding of how *Arion vulgaris* spreads and locomotes in the environment will make possible to establish predictions about their distribution and it would help to control the populations.

Here, we decided to check whether the structure of the surface has an impact on the movement rate (speed) of a Spanish slug, *Arion vulgaris.* The experiment was conducted on 3 types of the surface: grass, pavement mixed with sand. We tested following hypothesis: 1) On the road the movement rate will be the highest, 2) On the gravel the movement rate will be the lowest, 3) Movement rate will correlate with body mass.

Material and Methods

Study species

As a model in our study we used the Spanish snail, *Arion vulgaris* which is an invasive species.[16]. It is thought that the species appeared in Poland somewhere between 1980-1990 [17]. Nowadays it is spread throughout the whole country [18] and can occupied variety of habitats [4].

Experimental design

30 individuals of *Arion vulgaris* were collected from the meadow near to the stream in Jaszcze valley in Ochotnica Górna – a small village in Lesser Poland Voivodeship. Slugs were recollected on the early morning and late evening before the experiment and left to calm down before performing the experiment. The slugs had enough food and water supplies during the duration of all the tests. We tested three types of surface: short grass (grazing area), gravel mixed with sand and pavement with minimal slope. 10 randomly chosen individuals were measured on only one type of surface .Each individual was tested just once and after performing the experiment they were released.

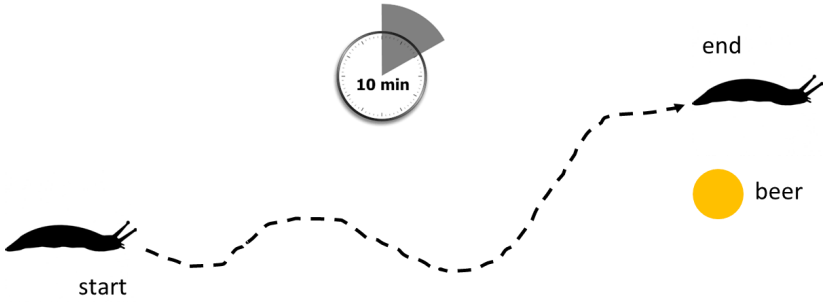

Figure 1. Basic diagram of the experimental setup. Slugs were left to move freely during 10 minutes, and after that the total trajectory travelled was measured using flexible thread. Beer was used as attractant.

Before each test, we put individuals separately on the chosen surface and gave them a minute or two to acclimatize. After that period of time we marked the starting point and started to count down 10 minutes (see Figure 1). Slugs were allowed to move freely, however we were trying to attract them with beer samples to follow the straight line. The usage of beer as an attractant for this species is described by few paper e.g. by Piechowicz et al. [19]. The trajectory of a slug was marked with a flexible thread, and after each test we measured its length. Because of that we were able to record the traveled distance even if they did not follow straight line. Additionally, after testing each individual was weighted.

The measurements were divided into two blocks – one in the evening and one in the morning, due to the temperature fluctuations (optimal temperature for this species is 15ºC). In each block we performed 5 measurements on each surface in a randomized order. Such design also allowed us to test slugs at the time of their greatest locomotor activity [15].

To calculate speed (movement/locomotion rate) we used a formula:

$$v = \frac{s}{t},$$

$$where\ v\ is\ a\ speed\ \left[\frac{cm}{min}\right], s - the\ distance\ [cm], t - time\ [min]$$

Statistical analysis was performed in RStudio [20]. To analyze the influence of the surface structure on the movement rate we did ANCOVA with a model *speed~mass+surface*, where mass of the individual is a covariate and a type of surface is an explanatory variable. To check for differences between groups we ran post-hoc test using of *multcomp* package.

Results

Mean movement rate within each surface was: on the grass – 4.261 ± 0.804687 cm/min, o n the pavement – 6.16 ± 1.819334 cm/min, on the mixture of gravel and sand – 5.468 ± 1. 753509 cm/min. We observed statistically significant influence of the surface' structure o n the movement rate of Spanish slug, *Arion vulgaris* (F=5.767, p= 0.00845) and no effect of mass of the individual on the movement rate (F= 0.733, p=0.39969). The post-hoc test s howed that there is a difference between gravel mixed with sand and grass (t= 2.570, p= 0 .04132) as well as between pavement and grass (t=3.241, p=0.00878), however there is no difference between pavement and gravel mixed with sand (t=0.275, p=0.95914).

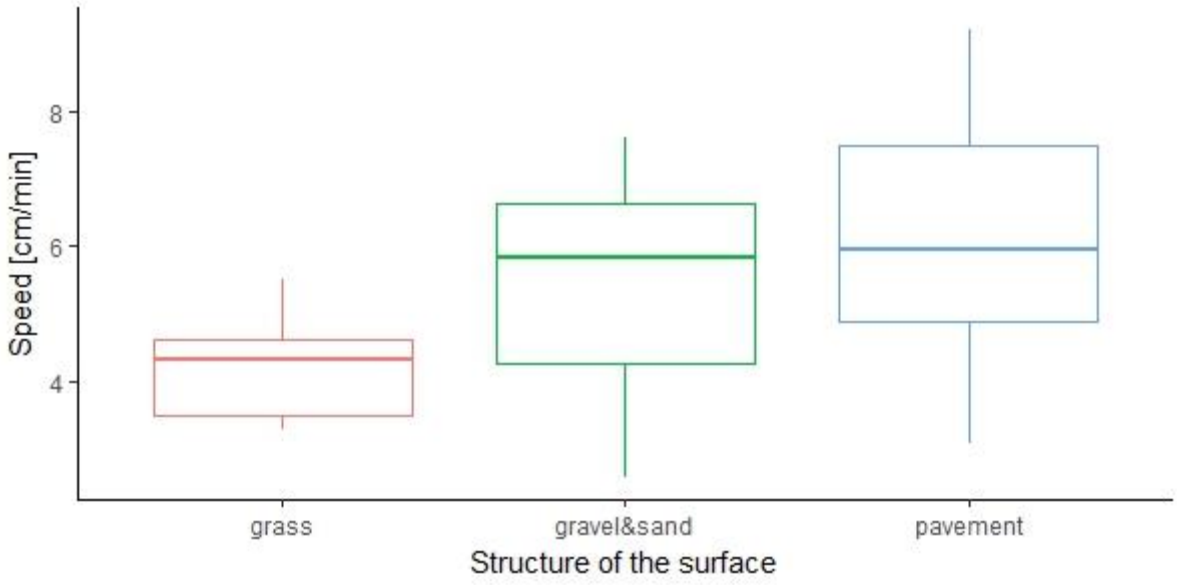

Figure 2. Effect of surface on speed [cm/min] of *A. vulgaris*. The boxplot represents median for each surface with a lower (Q1) and upper (Q2) quartile. Pavement showed the greatest slug speed.

Additional behavioral observations

We did not find any confirmation that beer acted as an attractant for our experimental individuals - only 3 slugs out of 30 tested followed the beer. We also observed that the movements of tested individuals were more unpredictable on the pavement – they were moving erratically, often around the circle. Contrary, those on the grass, after choosing direction they were moving forward without changes.

Discussion

In the current study we have tested the effect of three different surfaces on the locomotor activity of *Arion vulgaris*. What is more, we took into consideration the possible role of the body mass in their movement capacity. Recent studies have shown that the bigger the slug is, the faster it moves [14], but contrary to that, we did not find this phenomenon in our experiments. This suggests that other factors could condition the fast movement in slugs.

In addition, three different surfaces were studied: gravel mixed with sand, short grass (grazing area) and pavement surface (road). In all the situations the flat ground was chosen for minimizing the slope which could derivate in making slugs to move slower. Our predictions were right inasmuch that we predicted faster movement in road because of its smooth surface. What is more; slugs showed faster movement on the pavement compared with grass and gravel mixed with sand. An interesting observation was performed on the pavement, despite moving faster the slugs were performing more erratically movements which can be explained as stressed behavior; they moved faster but without any order trying to escape from possible predators.

In contraposition, slugs were slower in grazing area perhaps they were seeking potential source of food and they did not feel exposed to potential predators. With it remains clearly important, slugs live in meadows and areas with vegetation which remains logical that they would behave more naturally in them.

Another interesting finding was that beer is not as good attractive substance as postulated [19]. Anyway, it remains possible that slug were too stressed to be looking for nourishment and they just tried to hide in a secure place.

Our results shed light about some intriguing locomotor behaviors of the pest *A. vulgaris*. These results can be interesting to develop new strategies for preventing their spreading. Other authors also checked the speed of slugs but in laboratory conditions and they reached 0.75 cm/min in a period of 24 hours without taking into consideration periods of resting (average value) [15]. Moreover our experimental setup was designed for analyzing their locomotor behavior in a more natural environment, which may explain the different outcome.

Which remains interesting is why the erratic movement was only observed in pavement and interesting hypothesis can be extracted from these results for future experiments. Because this invasive species moves faster in pavements, they could distribute faster in big urbanized areas and then colonize fields. Because of that, it could be tremendously interesting to test the locomotor activity of the Spanish slug and autochthonous species and see if there are any differences in their speed movements.

Acknowledgements

The authors thank all the members of the course for helping recollecting slugs. Specially, we would like to thank Kamila Zając for her advice and technical contributions. We also would like to thank Dr. Joanna Rutkowska for her comments and advices.

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7.1.3. Reviews

7.1.3.1. Ulf Bauchinger:

Comment to the editor (highly confidential)

As outlined under the comments to the authors I do think that the topic of this study is of high interest, but in order to warrant publication in biology letters the authors need to perform major corrections. Such corrections seem possible if the authors do hold some data at hand that are not presented in the ms, but are nevertheless discussed and interpreted as result. These data must be provided and properly statistically analysed in order to hold the promise of the title and the formulated claims. The only reasons why I do not reject this ms right away is that I think it is possible that the authors may have used smart phone recordings or imaging technique and thus may indeed be able to quantify and qualify the data with respect of being erratic or not.

To use two figures is warranted, however, I am not sure if the authors are aware of the publishing prices and that they will be charged for a colour page only because of the yellow used to indicate the location of the beer in figure 1. This is even more ironic as the presence of a beer as attractant did not have any impact on the study.

Major revision required

Comment to the authors

The ms *Fast and furious: the locomotor activity of the invasive slug Arion vulgaris on three different structures* reports presents data on the speed of movement for an invasive slug species on different surface structures. The authors claim that speed of movement and the degree of erraticness may help to understand ‘why some species become more invasive than others’. The topic itself is very exciting and also of great interest to a wide audience in general and to the vegetarian audience in particular. Also the title is very appealing, creates a great tension and thus could attract readers to read this article, but while the ‘Fast’ component is supported by data, the ‘furious’ element is not supported by data.

Major comments:

The statement of the slugs moving more erratic over one kind of surface compared to the others is of high relevance for the conclusion that appears in the abstract, the discussion and, as already outlined, also in the title. While the speed, i.e. the distance travelled over time (here limited to a ten minute interval) is estimated by use of a flexible thread, the degree of erraticness is not supported by any data presented in the ms. This has of course fundamental influence on the interpretation of the invasiveness of this species: Being faster with less directed movement pattern could result in that those slugs will never reach anywhere. Or, in alternative may be interpreted in the complete opposite way that undirected movement is the actual key element of rando range expansion, and thus invasiveness. In order to discuss this exciting trade-off however, the data that are used to make statements like 'after choosing direction they were moving forward without changes (line 121)' or 'moving erratically (line 120) **must** be provided and statistically analysed.

The authors argue that movement over different surface structures may be a key element that determines if a slug species is more invasive than other species. In order to indeed draw such conclusions one would have to compare the movement pattern of different species over the different structures. With the current single species approach a discussion of invasiveness is still possible, but rather along the lines that this species can expand its range into areas heavily populated by humans. In such areas gravel and pavement may support movement compared to movement on grass. Invasiveness as such could be demonstrated in this ms based on the comparison between structures and not between species!

Given that some citations show an influence of size on dispersal the interaction term between body mass and surface type should be considered. Was body mass accounted for when randomly assigning the slugs to the treatments? Was body mass different between treatments. The authors have such data and should expand the ms on a potential role of body mass in determining speed or movement patterns.

Minor comments

The manuscript should be read and corrected by somebody native to English.

City and Country are missing in the affiliations.

Why are some terms in bold face in the abstract?

Line 19: the use of the word 'stressed' is not justified since no stress specific measurements are firstly determined and performed. Change wording please.

Line 20: 'and other invertebrates' this statement seems not justified to me, or even the opposite, why should this statement be restricted to invertrebrates and not also applies to vertebrates?

Keywords are missing

Line 25 delete ‘in’

Line 27 strange sentence: do you mean that the most recent studies consider that this species originates from the South-West Europe? Or alternatively, the south-western part of Europe. Consider rewording the entire sentence.

Line 31 ‘one of the biggest problems’: for whom?

Line 33 awkward sentence: either ‘for other invasive species it has been shown that’ or ‘Other invasive species have been shown to’. Also please consider to name the other invasive species since this information could be quite interesting for this ms.

Line 36f could the authors give a quality to this statement? In which direction? Larger is more, or larger is less?

Line 39 if the authors excite me with a statement like ‘carried out a series of interesting experiments’ for these experiments, then the authors should also inform about these experiments.

Line 40 this seems to be key for this manuscript. Please give more details on how soil surface influenced the slug activity

Line 50 ‘Here we decided to check’: Please consider that the reader is not so interested in your decision process and secondly to use a more scientific language. I typically check if my car has still some oil in the engine, but I try to test a scientific hypothesis.

Line 52 three types of surface, but only two are listed

Line 65 give precise coordinates. This could be quite interesting especially for work on invasive species.

Line 67 ‘enough’. How did you assure that enough was enough?

Line 84 you can use a weighted mean, but here it is weighed

Line 96f did you test for normality of distribution and homogeneity of variances?

Figure 2 please give indicate statistical differences in the graph

Line 102ff the speed if measured to the thousands of a centimetre? Did the authors use a calliper to measure the thread? The error is even given with a higher precision. Also the decimal places in the p-values is inflationary.

Line 130 if truly a flat ground was chosen then the slope should be 0 and not only minimized

Line 133 remove ‘;’

Line 134ff not supported by data, see also major comment

Line 151f awkward sentence, please reword. Interesting, interesting. What instead of which. Also this statement is not supported by data so far

7.1.3.2. **Agnieszka Bednarska:**

This article describes a well-designed experiment on the effect of surface types (grass, gravel mixed with sand and pavement) on the locomotor activity of the invasive alien species A. vulgaris. The results obtained by the authors are pretty clear, so the conclusions can be well supported by the data (but see below!).

As far as general comments, it is not clear why (based on which criterion) only 10 minutes were chosen to follow the movement of slugs? Moreover, the authors conclusion on the lack of correlation between the body mass and locomotion activity is not clear. What was the range of the body mass of slugs used in the experiment? If all individuals were just too similar to each other in terms of their body mass, the effect of body mass could not be found by definition (i.e., the experiment was not designed to answer that type of question).

Which measure of variance (SE, SD?) is reported together with mean values? If the same method was used in tests on all three surfaces, why some values are given with different accuracy? Please use the accuracy for recorded values adjusted to the accuracy of the tape you used to measure the distance.

Good and adequate detail is provided on the statistical analyses. Thank you! I appreciate the actual p values being reported, rather than just noting $p < 0.05$. But in some cases it would be even nicer to round a bit. For example $p=0.00845$ could safely be rounded to $p = 0.008$. Ditto for the extra places.

Although the text is reasonable well written and easy to follow, the manuscript might benefit from a more condensed writing, as the wording used is rather lengthy and not to the point. Please, use simple, direct sentences where you can and don't use long words or phrases (e.g., "we decided to check", "after that period of time", "we were able to record") when as shorter word/phrase will do ("we checked", "after that time", "we recorded"). The manuscript should be also corrected linguistically. I included some corrections and suggestions below, but I do not think that this is sufficient.

Please see below my more specific comments. Line numbers refer to the numbers inserted on the left-hand margin of each page.

Line 9: Invasive alien species are commonly regarded as a major threat to the biological diversity on a global scale, second only to habitat loss, so this should be especially stressed rather than only economic issues connected with the invasive species.

Line 14: "this phenomenon"? Which? The locomotor activity? Please rephrase.

Line 15: Is A. vulgaricus yet another name for A. vulgaris?

Line 17: The highest can be only one. Even if there was no significant differences between pavement and gravel mixed with sand, it does not mean that on both surfaces movement was the highest. Please rephrase.

Line 18: Moreover, slugs tested on pavement behave more erratic than …. ?

Line 27: species

Line 28: Is that sentence needed here?

Line 28-31: Please divide into two sentences.

Lines 31-33: I suggest to move this just after the first sentence of the paragraph in which you already mentioned that A. vulgaris is an important pest in Europe.

Line 35: To become pest or maybe to become invasive species? Being a pest does not mean that the species is invasive.

Line 35: In other species, such as ….(please give an example, so the reader knows to which species you refer without the need of checking the reference list).

Line 38: such as light intensity …

Line 40: colonization of arable fields

Line 41: Please delete the part starting with "carried out". It is enough to say that "Grimm and Schaumberger observed that …"

Line 47: "strategies for fighting against crop pests" rather than "strategies that fight"

Line 49: Again, enough to say "a combination of chemical or/and biological approaches …"

Line 50: Please replace "locomotes" with "moves"

Line 51: … "its distribution and it may help to control its population size."

Line 53: We checked …

Line 55: Some part of sentence is missing

Lines 55-58: Please use the present, not future time for describing hypothesis

Line 57: How? It is not enough to hypostatize that movement rate correlate with body mass? Do you expect positive or negative correlation?

Lines 62-65: I would move this section to the Introduction - it is a general information about the species, similar to those already provided which does not fit to Methods section.

Line 70: Why "recollected", not just collected (or sampled)?

Line 70: How much time before the experiment the animals were collected and for how long they were left to "calm down"?

Line 71-72: Better reads: "The slugs were provided with food and water ad libitum." But did you really feed them DURING all the tests? The measurements lasted 10 minutes, so I don't think that the individuals required food supply.

Line 73: It is not clear if the information about the minimal slope concern all surface types or only pavement? What was the value?

Line 74: 10 out of how many (if they were randomly chosen)? And I guess, the individuals were tested not measured on different surfaces.

Line 75: each individual …. was released.

Line 79: Can you provide better, preferably more scientific name for ‘flexible thread’?

Line 82: An individual or individuals? It is not clear how many individuals were followed at the same time?

Line 83: “After that time …. and flowed the slug for 10 minutes.” Why 10 minutes were chosen? Should be justified.

Line 85: “you were trying” or you attracted them with beer? Why samples of beer? What do you mean by “samples” here?

Line 86: the usage … WAS described by Piechowicz

Line 88: Was marked …. and the distance was measured. Thus, we …

Line 90: “weighed (not weighted) to the nearest ?? mg (please provide the accuracy of the balance and its name)

Line 92: I guess the measurements were done (not divided) into two blocks

Line 103: Did you check the distribution of your data before doing statistical analysis? Wouldn’t be simpler to say “with a type of surface as a factor and body mass as a covariate”?

Line 105: The type of post-hoc test (LSD, Tuckey, Sheffe, Bonferroni) is more important information than the one on which package you use to do this test. Please provide information on the type of post-hoc test.

Line 108: See my previous comment on accuracy of reported values and round them properly in all places in the manuscript where needed.

Line 110: We found, not observed.

Lines 112-115: The information written in such a way is not much interesting as it does not explain what kind of differences you found. The same sentence can be written as follows: “The slugs being place on grass moves significantly slower when those on pavement and grass, which did not differ between each other.”

Line 119: No need to place Q1 and Q2 in brackets, as you never refer to this acronyms in the text. Please delete.

Line 119: I guess not pavement but slugs showed any speed?

Line 124: Followed the beer or the beer trace?

Line 125: More unpredictable on the pavement than what?

Line 126: “after choosing one direction they were moving forward without changes of that direction.”

Line 130: “present” rather than “current”

Line 132: What do you mean by “movement capacity”?

Line 133: Should be “the bigger the faster”

Line 133-134: Please see my general comment on body mass issue.

Line 137: Why just slower, not faster? The sentence should be rewritten to indicate that the flat ground was chosen to minimize the effect of slope on the speed of movement.

Line 139-140. Not true! See the result section in which you showed no difference between pavement and gravel mixed with sand.

Line 141: add “as” before “despite”

Line 143: …. trying to escape from possible predators for which they were an easy prey on the smooth surface.”

Line 145: “in contrary”

Line 146: “… source of food without being easily exposed to potential predators”

Line 147: Please rephrase the sentence.

Line 149: Which type of beer did you use and which was used by other authors (e.g., non-alcoholic, alcoholic, black, lager)? Do you think that the slugs can have some beer preferences? □

Line 156: What “(average value)” means here?

Line 158: “remains not explained” reads better. Then, “Testing this hypothesis is, however, out of the scope of this study, but can be the aim of the future experiment.”

Line 161: Because the studied invasive species moves faster …. They may distribute faster in urbanized (delete big) areas and then colonize neighboring arable fields faster.

Line 166: Author contributions is not included. Please provide the proper statement to the revised version of the manuscript.

7.1.3.3. <u>Jaya Sravanthi Mokkapati</u>:

After my review of the manuscript **entitled** ***Fast and furious: the locomotor activity of the invasive slug Arion vulgaris on three different structures,*** below given are my comments It is very well written and is well balanced between the different topics, such as experimental design, results and discussion. Hence, this paper has a potential to be accepted, but some minor points have to be clarified or fixed before proceeding further and a positive action can be taken.

Here are the summarized points:

1. Key words are missing.
2. Summary can be renamed as Abstract.
3. Line 19 – what is the natural environment of collected species? Why not the locomotion was tested in their natural environment may be as a control?
4. There are a few slips in simple expressions and sentence structures especially at the beginning of the paper. You may want to refine the English usage. For example, in title – remove dot, in lines 18-19 - the sentence need to be rephrased for better understanding, line 26 - "specie" s missing. Lines 27-30, complex sentence need to be rephrased, line 59 – remove dot, line 125 – is the possible role etc.
5. It would be significant if the locomotion activity was checked for at least 60 mins in each habitat (Moriss et al., 2018).
6. Lines 50-52, objectives should be mentioned precisely, of course, hypotheses are pretty clear.
7. Line 84, why the species were weighed after experiment? May be it is worth checking the locomotion activity in terms of energy spent by weighing before and after. Also, the size of the slugs may result in different locomotion activity.
8. Lines 102-103, numbers could be up to three decimal points.
9. Line 113-114, "Pavement showed the greatest slug speed" need to be justified.
10. Line 135-137, give reference for stressed behaviour.
11. As possible additional recent background reference materials you may want to check out the following:

    Morris, A., Green, M., Martin, H., Crossland, K., Swaney, W. T., Williamson, S. M., & Rae, R. (2018). A nematode that can manipulate the behaviour of slugs. Behavioural processes, 151, 73-80.

    van Grunsven, R. H., Jähnichen, D., Grubisic, M., & Hölker, F. (2018). Slugs (Arionidae) benefit from nocturnal artificial illumination. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology.

If the revisions can be made, I conclude that this manuscript is according to the guidelines and scope for publication. Most of all, it is pretty clear what authors want to give as a take-home message.

7.1.3.4. Katarzyna Toch:

The manuscript by Gonzalez Gonzalez and Prus focuses on the effect of the surface's structure on the slug's *A. vulgaris* ability to move. The authors tested three surfaces that are common in the slug's habitat and likely to encounter by the animal. They report that structure of the surface can affect the movement of the Spanish slug. The experiment was well planned and obtained results seems to be very clear. However, the manuscript has several drawbacks which I list below.

1. Authors measured the activity of slugs during 10 minutes. One might argue that this time is not enough. In first 10 minutes animals might have been only adjusting to the environment and their movement did not reflect their long-distance activity when encountering such surfaces.
2. In the last paragraph of introduction authors pointed three hypothesis which they were testing in their experiment. However, they did not explain what are those hypothesis based on. It would be good to first explain how certain surfaces may affect the movement – why particular textures might slow down animals and others facilitate their activity.
3. I am not convinced with authors' usage of beer. Despite whether it worked in this case or not, it is hard to infer about their possible migrations and invasiveness since in nature they spread without being attracted to beer smell. If authors seek for applications of their results I would suggest conducting such experiment in a way that imitates natural conditions.
4. The manuscript has some minor issues. Line 68: sentence "The slugs had enough food and water supplies **during the duration** of all the tests." should be linguistically improved

Overall, I would like to emphasise that authors' experimental design was well established and the manuscript is written logically and interestingly. Also, I really appreciate the humorous title.

7.1.4. Report – final version

Fast and furious: the locomotor activity of the invasive slug *Arion vulgaris* on three different surfaces

Abstract

Invasive alien species are commonly regarded as a major threat to the biological diversity on a global scale. In particular, *Arion vulgaris* (also known as *Arion lusitanicus*) is one of

the most invasive mollusks. Nowadays, very little is known about why some species have more capacity to become invasive than others which represents a risk for the integrity of the biodiversity. In this study, we used the locomotor activity as a measurable trait for understanding this phenomenon, because of its direct role in the capacity of spreading and colonizing new areas. We analyzed 30 individuals of *A.vulgaris* and measured their speed (cm/min) in three different surfaces (grass, gravel mixed with sand and pavement). We found that the slugs movement rate were the highest on the pavement and the lowest on the grass. In conclusion, the structure of the surface can be determinant of how mollusks interact with the environment and more importantly, this can affect how they move and spread, potentially explaining why some species become more invasive than others.

Keywords: invasive species, *Arion vulgaris,* movement rate, structure of the surface

Introduction

Arion vulgaris (Moquin-Tandon, 1855) or *Arion lusitanicus* [1] also referred to as Spanish slug has become a pest of great magnitude all over Europe during the last fifty years [2]. It should be noted that is an important pest to potato [3], oilseed rape [4] and to legume [5]. However, the origin of this particular species as well as its taxonomic status is controversial. The newest investigations relate them from south-western part of Europe [6]. It is thought that the species appeared in Poland somewhere between 1980-1990 [7]. Nowadays it is spread throughout the whole country [8] and can occupied variety of habitats [9]. It has been quickly spread with transport of food products and by short distance active dispersal. In the particular case of Poland, genetic studies show that was introduced by different ways [9]. One of the biggest problems for controlling the population of that pest, is its high resistance to different molluscicides [10].
Behavioral traits are extremely important for determining why some species have more capacity to become invasive species than others [11]. For other invasive species have been shown that the exploration activity is an important trait for explaining the invasiveness (e.g. in mosquitofish and crayfish) [12,13]. Recent investigations demonstrated that there are several important factors that control the movements of slugs such as light intensity, air humidity and soil moisture [14,15]. In addition, in *A. vulgaris* larger individuals are able to more efficient dispersion and temporary colonization of crops growing on arable fields [16].

Grimm and Schaumberger observed that the environmental conditions influences the slug activity. Locomotion activities of *A. vulgaris* were maximal in the morning and in the evening, and minimal in the afternoon. What is more, they showed the existence of positive correlation between body mass and locomotor activity [17].

Nowadays, there is the great necessity to understand and develop new targeting strategies for fighting against crop pests, without affecting the beneficial species and polluting the environment. Some of these potential new strategies can be a combination of chemical or/and biological approaches with physical barriers. The deep understanding of how *A.*

vulgaris spreads and moves in the environment will make possible to establish predictions about its distribution and it may help to control its population size.

Here, we tested whether the structure of the surface has an impact on the movement rate (speed) of *A. vulgaris*. The experiment was conducted on 3 types of the surface: grass, pavement, gravel mixed with sand. We tested following hypothesis: 1) On the pavement the movement rate is the highest, 2) On the gravel mixed with sand the movement rate is the lowest, 3) Bigger individuals move faster.

Material and Methods

30 individuals of *A. vulgaris* were collected from the meadow near to the stream in Jaszcze valley in Ochotnica Górna (N 49.521975, E 20.222439) – a small village in Lesser Poland Voivodeship. Slugs were collected on the early morning and late evening before the experiment. They were kept in a plastic box filled with leaves and grass to imitate its natural habitat and left for ca. half an hour to calm down before performing the experiment. We used three types of flat surface: short grass (grazing area), gravel mixed with sand and pavement. 10 out of 30 randomly chosen individuals were tested on only one type of surface. Each individual was tested just once and after performing the experiment they were released.

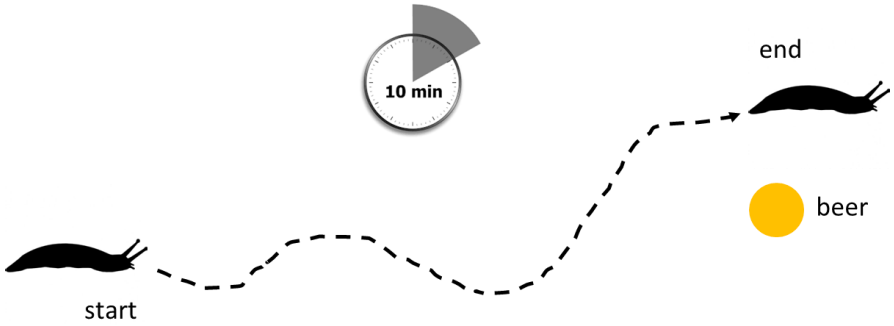

Figure 1. Basic diagram of the experimental setup. Slugs were left to move freely during 10 minutes, and after that the total trajectory travelled was measured using the string. Beer was used as attractant.

Before each test, we put two individuals on the chosen surface and gave them a minute or two to acclimatize. After that period of time we marked the starting point and started to count down 10 minutes (see Figure 1). Kozłowska and Kozłowski [18] showed that during the first 10 minutes *A. vulgaris* are the most active, thus this period of time enabled us to record their maximal speed since they were not tired nor distracted. Slugs were allowed to move freely. We used ca. 5ml of beer (Kasztelan) as an attractant, because we wanted them to follow the straight line. The usage of beer as an attractant for this species was described by few papers e.g. by Piechowicz et al. [19]. The trajectory of a slug was marked with a string and the distance was measured using measuring tape to the nearest 0.01 cm. Thus we were able to record the traveled distance even if they did not follow the straight line. Additionally, after testing each individual was weighed to the nearest 0.01g with using a pocket balance KERN CM 60-2N.

The measurements were done in two blocks – one in the evening and one in the morning, due to the temperature fluctuations (optimal temperature for this species is 15ºC). In each block we performed 5 measurements on each surface in a randomized order. Such design also allowed us to test slugs at the time of their greatest locomotor activity [17].
To calculate speed (movement/locomotion rate) we used a formula:

$$v = \frac{s}{t},$$

$$where\ v\ is\ a\ speed\ \left[\frac{cm}{min}\right], s - the\ distance\ [cm], t - time\ [min]$$

Statistical analysis was performed in RStudio [20]. To analyze the influence of the surface structure on the movement rate we did ANCOVA with a model *speed~mass+surface*, with a type of surface as a factor and body mass as a covariate. To check for differences between groups we ran Tukey post-hoc test using of *multcomp* package.

Results

Mean movement rate (mean ± SD) within each surface was: on the grass – 4.26 ± 0.80 cm /min, on the pavement – 6.16 ± 1.82 cm/min, on the mixture of gravel and sand – 5.47 ± 1 .75 cm/min. The range of the body mass of the individuals in the whole experiment varied from 0.31 g to 4.46 g. We found statistically significant influence of the surface' structure on the movement rate of *A. vulgaris* ($F_{2,26}$=5.78, p= 0.008) and no effect of mass of the in dividual on the movement rate ($F_{1,26}$= 0.73, p=0.40). The Tukey post-hoc test showed that slugs moves significantly slower on the grass than on the gravel mixed with sand (t= 2.57, p= 0.041) or on the pavement (t=3.24, df=18, p=0.009). However there is no difference in its speed between pavement and gravel mixed with sand (t=0.27, df=18, p=0.96).
We did not find any confirmation that beer acted as an attractant for our experimental individuals - only 3 slugs out of 30 tested followed the beer trace.

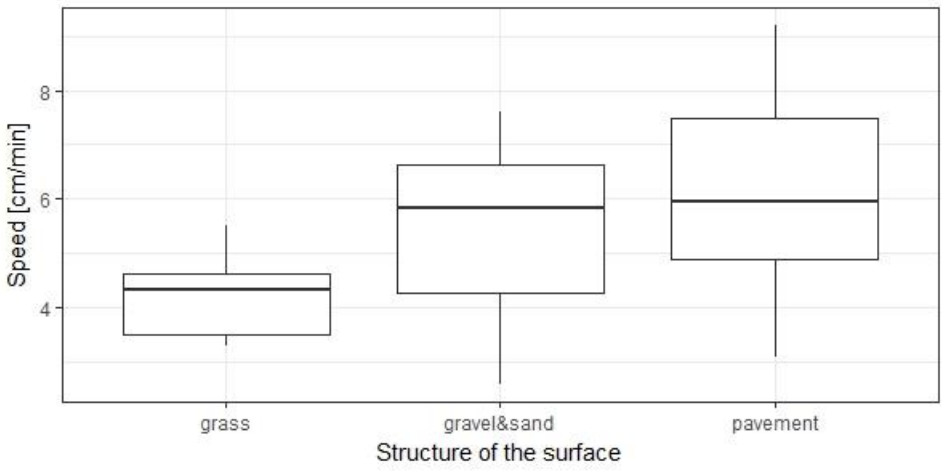

Figure 2. Effect of surface on speed [cm/min] of *A. vulgaris*. The boxplot represents median for each surface with a lower and upper quartile. Speed of slugs (cm/min) ± SD on grass (4.26±0,80), pavement (6.16±1.80) and gravel mixed with sand (5.47±1.75) were obtained. On the pavement slugs showed the greatest speed.

Discussion

In the present study we have tested the effect of three different surfaces on the locomotor activity of *A. vulgaris*. What is more, we took into consideration the possible role of the body mass in their movement rate. Recent studies have shown that the bigger slugs move faster [16], but contrary to that, we did not find such relationship in our experiments. This suggests that other factors could condition the fast movement in slugs.

In addition, three different surfaces were studied: gravel mixed with sand, grass (grazing area) and pavement (road). Our predictions were right inasmuch that we predicted faster movement on the pavement, because of its smooth surface. What is more slugs showed faster movement rate on the pavement and gravel mixed with sand compared with grass. In contrary, slugs were slower in grazing area - perhaps they were seeking potential source of food without being easily exposed to potential predators. This behavior can be explained by their natural habitat: slugs live in meadows and areas which remains logical that they will behave more naturally in them.

Another interesting finding was that beer (lager beer, Kasztelan) is not as good attractive substance as postulated [19]. However, it remains possible that slug were just too stressed to be looking for nourishment and they just tried to hide in a secure place.

Our results shed light about some aspects of locomotor behaviors of *A. vulgaris*. Grimm and Schaumberger observed in laboratory conditions that *A.lusitanicus* travelled the distance of 10.8 m in a period of 24 hours without taking into consideration periods of resting (average movement rate – 0.75 cm/min) [17]. Our experimental setup was designed for analyzing their locomotor behavior in a more natural environment, which may explain the different outcome.

Based on the results, we concluded that this invasive species moves faster on pavements and gravel mixed with sand. In consequence they may distribute faster in urbanized areas and then colonize neighboring arable fields faster. Because of that, it could be tremendously interesting to test the locomotor activity of the Spanish slug and autochthonous species and see if there are any differences in their speed movements.

Acknowledgements

The authors thank all the members of the course for helping recollecting slugs. Specially, we would like to thank Kamila Zając for her advice and technical contributions. We also would like to thank Dr. Joanna Rutkowska for her comments and advices.

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7.1.5. Cover letters

7.1.5.1. Miriam Gonzalez Gonzalez:

Dear Dr D.G. Reid
Editor in Chief
Journal of Molluscan Studies

On behalf of me and co-authors we send you as an attachment the manuscript entitled "Fast and Furious: the locomotor activity of the invasive slug *Arion vulgaris* on three different surfaces".

Since our scientific study responses to the requirements for this prestigious journal, we would like to ask for considerating it for publication. Our research shed light to the locomotion behaviour of the invasive slung *Arion vulgaris* and provides valuable data to the field.

Invasive species are a worldwide problem and ***.Arion vulgaris*** (also known as *Arion lusitanicus*) is one of the most invasive mollusks that attack gardens and crops as potato. Unfortunately, very little is known about why some species have more capacity to become invasive. In this study, we used the **locomotor activity** (expressed as cm/min) as a **measurable trait** in three different surfaces (grass, gravel mixed with sand and pavement. Our findings suggested that the slug's movement rate were the highest on the pavement being the lowest on grass. Moreover, on pavement tested slugs behaved more erratic. In conclusion, the structure of the surface can be determinant of how mollusks and other invertebrates move, which is especially important for understand the behavior of invasive species.

We believe that our findings have important implication in the mollusks field. Therefore we consider our manuscript as a valuable contribution.

Yours sincerely,

Miriam Gonzalez Gonzalez
Institute of Zoology and Biomedical Research, Jagiellonian University
Gronostajowa 9, 30-387 Kraków, Poland
miriam.gonzalez.gonzalez@doctoral.uj.edu.pl

7.1.5.2. Monika Prus:

26th June 2018

The Editor
Annals of Applied Biology

Dear Sir or Madame,

Please find attached manuscript entitled *"Fast and furious: the locomotor activity of the invasive slug Arion vulgaris on three different structures."* accompanying this letter.
The main aim of our study was to assess the effect of surface's structure on the movement rate in *Arion vulgaris* slugs. This species is widespread and highly invasive and thus it is really important to understand its ability to migrate and colonize new territories. It is believed that nowadays this species is one of the most invasive molluscs. Such knowledge may help us to create some alternative methods of protection which are safer than conventional and really useful while those conventional with a usage of chemicals failed. In our study we tested three structures of the surface: short grass (grazing area), gravel mixed with sand and pavement. We found out that slugs were the fastest on the pavement, so on the most smooth surface out of 3 tested, and were the slowest on the grass. Moreover, we did not find any support for effectiveness of the popularly used attractant (here: alcohol samples). To our knowledge, this is the only study so far, that was done under natural, not laboratory conditions, and thus took into consideration different structures of the surface which the animal encounters.
We believe that this paper will be suitable for publication in the Annals of Applied Biology.
We declare no conflict of interests and all authors accepted the manuscript and its submission. Manuscript is written based on original data and it is not under consideration of any other journal.

Yours sincerely,
Monika Prus

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7.2. A comparative assessment of smartphone applications for the plant identification in the field

Arpan Kumar Basak[1,] , Jaya Sravanthi Mokkapati[1], Evgenii Baiakhmetov[2]

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[2]*Jagiellonian University, Institute of Botany, Krakow, Poland*

7.2.1. Project

Title: Can cell-phone software be used for plant species identification?

Summary

Plant identification has been considered as mostly exclusively a prerogative of botanists for three centuries. However, it is of interest to much wider audience including not only professionals (e.g. ecologists, foresters, landscape architects), but also the general public (e.g. ecotourists, nature lovers paysagists). Traditional approaches relied on morphological characters may be time consuming and claim skilled subject matter experts. In addition, an expert on one genus/family may be unfamiliar with another. This leads to an increasing interest of using computer algorithms for species identification. However, there are only a few literatures available on testing smartphones applications for plant species identification (PSI) in the field. Therefore, our project aims to verify three open source smartphone applications as potential useful tools for PSI. Three current PSI applications based on different machine learning algorithms will be compared for their reliability in identifying species in the field. The experimental design consists of data collection and testing in the applications. The results will be analyzed in logistic regression model. We expect that the current datasets may not have sufficient information to support the application, in order to identify plant species enriched in the field.

Aim / hypothesis

Our main aim is to test the reliability of smartphone applications based on different computer vision algorithms for plant species identification in the field. Here, we hypothesize that the current smartphone applications for plant identification may not be the reliable sources in terms of their top-2 accuracy.

Methods

Observation and data collection

A test dataset will be collected from the images of plants located in the Ochotnica Górna province by a smart phone device. The plant images will be taken using mobile phone equipped with a prime lens of 28 mm equivalent focal length and a face beauty sensor giving 4920 x 3264 resolution (Model – Vivo1718 with Android v7.1 Nougat operating system with 1.8GHz Snapdragon 450 MSM8953 octa-core processor). The test dataset will contain minimum of 10 species representing plants from each of three different habitats - meadows, forest and residential areas. From each species, images of 20 plants will be captured in both solid leaf green background and natural background conditions for each plant.

Testing of the images

Testing will be conducted on the collected images, with the aid of smartphone applications specifically PlantNet, Picture This Plant Identification (PTPI) and PlantSNAP. These

applications are based on different machine learning image classification algorithms, such as, Convolutional Neural Network (CNN), Fuzzy Algorithm and Genetic Algorithm respectively. A binary identification code will be assigned for the images identified by the application, if the application succeeds in recognizing the plant species within top-2 hits, we score them 1 (as identified) or 0 (as not identified).

Statistical analysis

In the statistical analysis, we are aiming to put recognition of the plant species as a dependent variable with binary levels, and three different smartphone applications as independent variable with image background as a mixed factor with two levels. This type of model can be fitted with logistic regression in order to answer the question of reliability.

Impact of results

Results of our project will give a clear idea about the current usage of smartphone plant identification applications, consequently, the demand for new or updated databases for plant species identification.

7.2.2. Report – first version

A comparative assessment of smartphone applications for the plant identification in the field

Abstract

Knowledge of species identification is demandable for nature conservation as well as for the public awareness of biodiversity and nature value. Traditional approaches relied on morphological characters is complex, time consuming, and can be challenging in field. Nowadays, notable progress in process of imaging and pattern recognition with relevant technologies, such as mobile devices, increases interest in automating the procedure of species identification. This paper is the first attempt to test open source smartphone applications in the field if different image recognition algorithms affect reliability of plant identification. We analyzed the three most popular applications in the Google Play Store and established that the most reliable application PlantNet (79.5%) uses an image recognition algorithm based on biological databases with morphological and geographical parameters, while PlantSnap and PictureThis relies on an image recognition algorithm without referring to biological databases and having reliability 46.5% and 35.5% respectively. We determined that a solid green screen decreases reliability of all the three applications: PlantNet (78%), PlantSnap (41%) and PictureThis (34.5%). We state that none of the checked applications can be used as a powerful tool for rapid plant identification in the field. We claim that current databases should be complemented with biological data.

Keywords: plant identification, smartphone applications, computer algorithms

1. Introduction

Current estimates of biodiversity range between 10 million and 14 million [1], of which only about 1.2 million have been documented [2]. For land plants there are 392,630 accepted species, and of those approximately 95% are flowering [3]. Annually botanists discover new species, for instance, according to The State of the World's Plants Report [3] 1730 new plants were described in 2017.

Traditional process of plant identification by using identification keys allows through series of answered questions to recognize the desired species. Nevertheless, such procedure in the field requires a pre-botanical experience and knowledge of plants, that puts it away from reach for most nature lovers. Same deal with identification by DNA sequencing, which may be a more precisely tool, but it is still time-consuming and costly for the general public.

Thus, traditional identification of plants is almost impossible for a wide audience and challenging even for many botanists that focused only on one genus/family and may be unfamiliar with another. The situation is further aggravated by the increasing deficiency of skilled taxonomists [4] that leads to the co-called taxonomic crisis [5]. As a consequence, nowadays knowledge connected with plant identification is restricted to a small group of people.

At the same time, taxonomists have been making efforts to develop more efficient approaches for species identification, including digital image processing and pattern recognition techniques [5]. The last decade resulted in an abundance of researches in the field of computer vision and machine learning that applied for automated identification of plant [6–10].

The steadily growing use of portable devices (e.g. smartphones, tablets) and their ongoing technical development bring ideas of species identification by photo-imaging closer for the usage in field studies. However, in the literature, there are no reported articles on testing automated plant identification applications for the portable devices in the conditions of field work.

Consequently, this article specifically focuses on checking open source smartphone applications on the reliability of plant identification in the field. The study tests the three most popular apps in the Google Play Store (https://play.google.com/store): PlantNet (developed by Cirad, INRA, Inria, IRD, and the Tela Botanica network; over 1,000,000 installations), PlantSnap (PlantSnap Inc.; over 500,000), PictureThis – Plant Identification (Hangzhou Dana Technology Inc.; over 500,000). We address the following hypotheses:

1. Image recognition algorithms using biological databases with morphological and geographical data for plant identification may be more reliable than the sole structure image databases.
2. The current smartphone applications for plant identification may not be the reliable sources in terms of their top-1 and top-2 accuracy.
3. A solid green screen can increase the accuracy of the plant identification applications.

Here, we predict that plant genera can accurately be identified only when the databases from which the applications are developed have rich scientific data of biology, morphology and geography of the plants. In addition, we predict that strong algorithms like

deep neural networks, together with artificial intelligence, are necessary in order to identify plants correctly.

2. Material and Methods

(a) Experimental design

A test dataset includes images of plants located in the Ochotnica Górna province. The plant images were captured using a smartphone Vivo1718 (with Android v7.1 Nougat operating system with 1.8GHz Snapdragon 450 MSM8953 octa-core processor) equipped with a prime lens of 28 mm equivalent focal length and a face beauty sensor giving the 4920 x 3264 resolution. Each photo was taken from a focal length of approximately 16 cm at the same period of the day in order to reduce the variability. The images were taken at the same location of the province. The test dataset contains 50 genera of flowering plants. From each identified genus, images of four plants have been captured both in a solid green screen and natural background conditions (figure 1) for each plant (the total number of images is 400). Taking into account that plant identification up to species level in the field may be challenging even for experienced botanists, we score results through all the applications only to genus level.

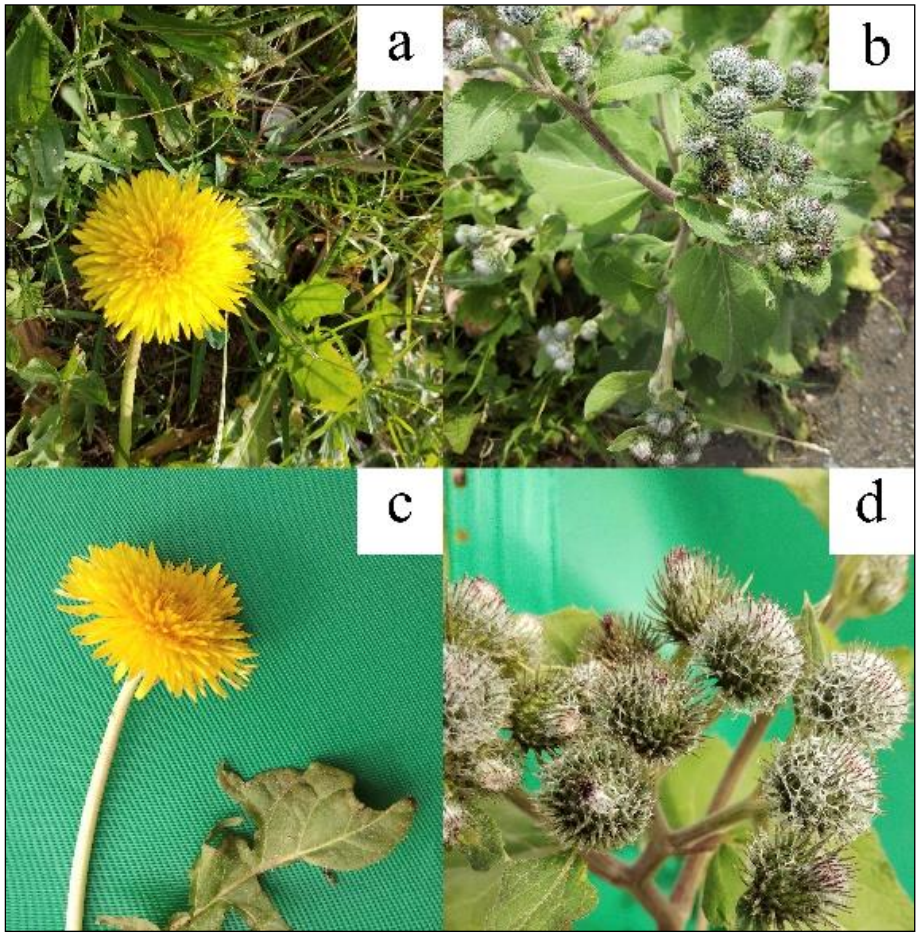

Figure 1. Representative images of plants with natural background (a, b) and the solid green screen (c, d).

(b) Image Processing

All images captured are edited as per guidelines of the smartphone applications and image processing is conducted with the aid of specific open source smartphone applications PlantNet (accuracy 99%), PlantSnap (98%), PictureThis – Plant Identification (90%), available in the Google Play Store. These applications are based on different machine learning image classification algorithms, such as, Convolutional Neural Network (CNN) [11], Fuzzy Algorithm [12] and Genetic Algorithm [13] respectively.

In order to reduce the technical variability we conducted the image processing with two different smartphones having similar configuration, assuming the smartphone configuration causing no variability in identification of the plants. A binary identification code is assigned for the images identified by the application, if the application succeeds in recognizing the plant species within top-2 hits, we score them 1 (as identified) or 0 (as not identified).

(c) Statistical Analysis

We generated a reliability matrix for samples as observations and genera as features. Considering the identification index of the applications as response variable for each genera, with respect to different applications with the effect of background screen as independent variable. Accuracy of each plant applications was estimated based on their ability to identify the plant genus from the image. This computation was estimated by calculating the number of true positives (as 1) for each applications with background and without background respectively.

Results

The test dataset containing a total of 400 images from 50 known plant genera were applied to each of the three plant identification applications, PlantNet (PLN), PlantSnap (PLS) and Picture This-Plant identification (PLT). For each image, the correct or incorrect prediction in each application was scored in binary and the data was analyzed by three models, distance matrix, accuracy percentage and pairwise student t test on the accuracy ratios using R programming.

The distance matrix was analyzed by computing the Jaccard distance for binary response estimating how distant each application is located from plant genera prediction. From distance matrix, we obtained three clusters by computing Multi-dimensional Scaling each for PlantNet, PlantSnap and Picture This (figure 2).

We observed that plantNet is indeed a reliable application when compared to other applications vailable (table 1).

We attempted to compute pairwise t.test and observed for different images captured for each genus. The accuracy for these applications when compared is statistically significant ($p < 0.001$).

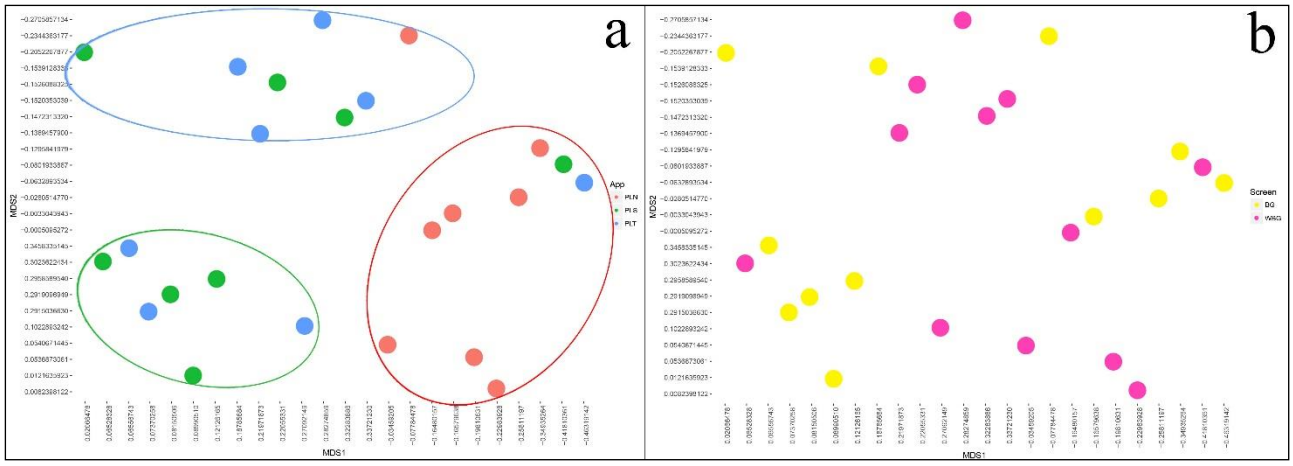

Figure 2. Distance matrix for (a) the tested smartphone applications and (b) background effects.

Table 1. Details of tested applications and observed accuracy

Application	Dataset	Algorithm	No. of plant images	Claimed accuracy	Observed accuracy	
					With natural background	With a solid green screen
PlantNet	PlantCLEF	CNN	113,205+	99%	79.5%	78.0%
PlantSnap	PlantSnap	FA	71000+	98%	46.5%	41.0%
PictureThis	PictureThis	Genetic Algorithm	4,000+	90%	35.5%	34.5%

Discussion

Despite intensive and elaborate research on automated plant species identification, only very few studies resulted in approaches that can be used by the general public, such as PlantNet, PlantSnap, Picture This etc. These smartphone applications use computer vision techniques for identifying the plant species in either natural and/or plain background.

In this study, the comparative assessment of the global plant identification performance of the three smartphone applications demonstrated the better plant recognition with natural background by PlantNet (79.5%) followed by Picture This (46. 5%) and PlantSnap (35.5%). The comparatively high observed accuracy of PlantNet is in corroboration with the fact that it uses additional metadata such as topographic characteristics, taxonomy, climate factors, soil type, land-use type, and biotope to the visual content in the identification process [8]. Moreover, separate indexes for each visual feature were applied by a multi-organ, multi-image and multi-feature fusion strategy in PlantNet along with the integration of cross-languages functionalities [4]. However, in order to process an image by several features, it requires a powerful algorithm with extensive training.

A few smartphone applications using deep learning technologies such as CNN and train their classifiers on large plant image datasets in automated plant species identification systems. However, these algorithms were not tested in real world particularly for plants in the field or mountain areas. In this study, the identified distance matrix data yielded a separate cluster for the CNN based PlantNet application from the other two explaining the power of deep learning techniques together with artificial intelligence in complex image processing (figure 2a). For the other two applications, although there were separate visible clusters, they seem overlapped explaining the algorithmic interlinking in image processing. Further, no significant effect of plain background in plant recognition when compared to natural environment was observed for all three tested applications (figure 2b) suggesting the image recognition not solely relayed on texture but involved deep learning [4].

Based on the statistical significance in terms of p-values it can be stated that PlantNet is comparatively better biological tool to identify plants accurately. Therefore, this study concludes that although the plant identification by smartphone applications is convenient, but the better prediction is only possible if the application uses biological data and deep learning algorithms with artificial intelligence.

Authors' contributions. All authors designed the experiment; all authors performed experiments. All authors wrote, edited and revised the manuscript. All authors gave final approval for publication and agree to be held accountable for all aspects of the work performed.

Competing interests. We declare we have no competing interests.

Acknowledgements. We thank Prof. Joanna Rutkowska for her comments that improved the scientific content of the paper.

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7.2.3. Reviews

7.2.3.1. Ulf Bauchinger:

Comment to the editor (highly confidential)

The editorial work has been better for biology letters: in formers times a ms without line numbers would not have been sent out for review:)

The topic is of great interest for a wide audience, but requires some major improvements. Specifically, the formulation of the hypotheses and how these are tested needs attention.

Figure 2 is not explained, not understandable, and also printed in a way that nobody could ever understand the axis. This figure is in the current form and presentation not justified.

I do request that a statistician is consulted to evaluate the used statistical methods.

Major revision required

Comment to the authors

To receive a manuscript without line numbers makes it extremely difficult and time consuming to provide a detailed review. I was tempted to return the review without reading.

The ms *A comparative assessment of smartphone applications for the plant identification in the field* aims to test the success rate with which plant species can be determined to the genus level through the use of commonly available aps for the smart phone. I am grateful that somebody addresses this important issue of the "co-called taxonomic crisis" (please see line 41 and correct to so-called). Honestly, after reading this manuscript I had a sleepless night about having been unaware of such an impactful crisis and the 'increasing deficiency of skilled taxonomists; thank you. Give the dimension of such a crisis I would have hoped that our society is much more prepared and the available aps indeed help to solve this crisis, thanks to this ms I now know that it is not. Nevertheless, I have some concerns about the methodological approach and the data presentation that make it difficult for me to accept the paper in the current form. Because all material has been obviously stored on the electronic devices I do hope the issues outlined below can be addressed.

Major comments:

Authors claim that hypothesis 1 'Image recognition algorithms using biological databases with morphological and geographical data for plant identification may be more reliable than the sole structure image databases.' is addressed in the ms. However, it is not clear where are the respective data to be found and where these data are interpreted to address the hypothesis.

Hypothesis 3 formulates that a solid green screen used as background can increase the accuracy of the plant identification application. Either I am so simple minded and do not recognise the genius in this approach or the authors directly qualified for an Ig Noble award. Why the hell would a solid green background increase the recognition pattern of GREEN plants? Were there no white or black paper sheets available?

Figure 2 and also the description of this figure in the text and also in the legend raises many questions. What does the figure actually show? What do we actually learn from and see on this figure? What do the data points symbolise? Why are there different numbers in figure a and b? What explains the numbers of the data points?

Minor comments

The manuscript should be read and corrected by somebody native to English.

Line 13 ff complicated sentence, consider rewording.

Line 30 what means documented? Pictures taken? Described as species? Be more precise here.

Line 31 'Annualy' sounds strange do you mean every year?

Line 53 move information reference

Line 61 explain terms used. What means top-1 and top-2 accuracy. Not every reader may be aware of this

Line 75 ‘plants’ I guess you mean individuals

Line 99 ‘reliability matrix’ is introduced here and does not reappear again? Why? Where are the results for this reliability matrix? What is a reliability matrix? Do you use distance matrix interchangeably?

Line 101 ‘Accuracy’ are you sure this is the right term to be used for something that is either right or wrong?

Lines 107-113 should be moved to materials and method section

Lines 116f Indeed reliable? 1/5 of the plant species not recognised and this can be called reliable? What do other applications achieve or even humans? Just for the sake of comparison?

Line 118 move to materials and methods. Also, what do you mean by attempt? Were you not successful?

Table 1 Does the statistical comparison take into account the number of plant images known by each app?

Lines 125 -128 move to introduction. This is discussion here.

Lines 134f ok, but what does this mean? What is the point the authors want to make here?

Lines 143f ‘For the other two applications, although there were separate visible clusters, they seem overlapped explaining the algorithmic interlinking in image processing.’ I am sorry, but I really don’t understand what the authors want to say?

Line 147. Please can the authors explain better how the obtained result and the figure 2b actually may allow such an interpretation? How do the obtained data allow conclusion about the involvement of deep learning? Unclear.

7.2.3.2. <u>Adam Łomnicki</u>:

It is an extremely important report which should be published in Polish in “*Wiadomosci Ekologiczne*” or in *Chrońmy Przyrodę Ojczystą* or abroad in English. At the beginning I should express my deep believe that the printed reports are not to show only how well educated and bright are their authors in comparison with the authors’ colleagues and professors but to improve the general knowledge of the readers, One has to take into account that in Poland most of plant taxonomists have a very limited knowledge of R programming and smartphones in spite of being a very good taxonomists and very useful in estimating plant diversities. I will return to this problem when discussing the details of this report. My believe is based on the numerous references which should allow the authors to understand the mechanism of taxonomic identification. On the other hand my

knowledge of smartphones is limited and it may be a case that the knowledge how the application work is not available and the authors are not able to teach the readers what are the procedures which make species or genus to be identified by the smartphones. If this is the case it should be explicitly written in the report.

When mentioning current estimate of biodiversity (page 1) one should be more specific whether it includes all microorganisms not only bacteria but also viruses. Instead of "apps" it should be written "applications" (page 2) One should explain also what does it mean "deep neural network" and "face beauty sensor"

One should be more clear what does it mean that two different smartphones have similar configuration (page 5). The "reality matrix" is not a standard statistical procedure and it should be clearly explained for biologists. "Accuracy ratio using R programming also should be explained in full details. Are accuracy tests statistically significant on the same level for all applications? (page 4)..

In page 5 observed differences between natural and solid green ground are not compared with t test and its significance. On the same page is comparison of species but we are concened her with genus only.

Concluding, it is very important text but it should be more clearly explained and published for the use of other field biologists.

7.2.3.3. <u>Monika Prus</u>:

I find received manuscript very interesting. The study concerns interesting issue on usefulness of available smartphone applications for the plant identification in the field. I agree with the authors that such assessment is needed, because nowadays we more and more rely on technology.

In the introduction authors clearly presented state of the art, knowledge gap and need for such studies. They truly convinced me that their work is novel. Unfortunately, the hypothesis have to be reformulated. We have to clearly state them, so "*will be/they are more reliable*" instead of "*may be more reliable*". Methods in *Material and Methods* section are explained very well and due to that it is very easy to reproduce the study. I think that the number of tested genera was sufficient. In my opinion it was also a good idea to test those applications with picture with and without background, because it enabled to check whether they are truly useful in the field, where it is not always possible to exclude the natural background. However I missed the explanation how the authors decided whether the application is reliable or not. What percentage of accuracy the application should get to state that? Is it 90%? Is it the same for all three apps? As I understood, the values in brackets are just accuracy declared by the founders of each application. I have a problem with *Result* section. I think that two first paragraphs should be moved into *Statistical analysis* part in *Material and Methods* section, because the authors described there the procedure of dataset testing. *Table 1* is clear and self-

explanatory, unfortunately I can not write the same about *Figure 2*. It needs to be improved, because now the legend is completely invisible. It will be also good, if the authors explained it more details in the *Result* section, because it is done for the first time in *Discussion* part. *Discussion* itself is written very good – it is clear, it explains results and presents conclusions.

From technical point of view, received manuscript fulfill almost all technical requirements – only pages and lines are not numbered. The structure of the manuscript is preserved and each necessary part is present. I found only two typo *co-called, vailable.*

7.2.3.4. Kamila Zając:

The manuscript deals with the comparison of usefulness of smartphone applications for the plant identification in the field. In my opinion authors conducted a very interesting work which may have significance even for people not connected with science (nature lovers, foresters, students), who want to recognize and identify plant species, which may not be so easy even for botanists. It should be emphasized here that presented study is innovatory and tries for the first time to investigate the issue of testing open source smartphone applications in the field if different image recognition algorithms.

Based on the results published in the manuscript it is able to say what kind of smartphone application is the best for plant identification. I would recommend publication, after some revision of comments presented in this review.

The most important think, in my opinion is that hypothesis should be clearly stated, so I would recommend to avoid statements “may be”/”may not be”. I suggest to reformulate hypotheses, because researchers during performing experiment had some predictions. In the results part there are some information that should be placed in material and methods section, probably it is due to the authors being overlooked (lines 107-115). Additionally, pages should be numbered and line numbers should be included, as it was required. This would make it easier for the reviewer to refer to particular parts of the manuscript.

Figure 2 is unreadable and needs to be corrected. For me, and I think also to other people reading the legend and axes descriptions is almost impossible.

Despite the minor comments, I recommend the manuscript entitled “A comparative assessment of smartphone applications for the plant identification in the field” for publication after sending the corrected version.

7.2.4. Report – final version

A comparative assessment of smartphone applications for the plant identification in the field

Abstract

Nowadays, plant identification in the field is becoming one of the most challenging tasks due to the increasing deficiency of skilled taxonomists that leads to the so-called taxonomic crisis. Traditional approaches to plant identification relied on morphological characters, which is difficult and time consuming. In this context, identification of plants based on images is considered as a promising solution that can help cover the taxonomic gap and be a useful tool for nature conservation as well as for the public awareness of biodiversity and nature value. This paper is the first attempt to test open source smartphone applications in the field. We aimed to examine reliability of different image recognition algorithms on 50 plant genera collected in Lesser Poland. We analyzed the three most popular applications from the Google Play Store and ascertained that the most reliable application PlantNet (79.5%) uses an image recognition algorithm based on biological databases with morphological and geographical parameters, while PlantSnap and PictureThis rely on an image recognition algorithm without referring to biological databases and having reliability 46.5% and 35.5% respectively. We determined that a solid green screen has no or very less reliability percentage: PlantNet (78%), PlantSnap (41%) and PictureThis (34.5%), when compared to natural environment. Therefore, the study concludes that although plant identification by smartphone applications is convenient, in order to get better recognition, the current databases should be complemented with biological data.

Keywords: plant identification, smartphone applications, computer algorithms, reliability

1. Introduction

Current estimates of biodiversity is ranged between 10 million and 14 million [1], of which only about 1.2 million have been reported [2]. For terrestrial plants there are 392,630 accepted species, and of those approximately 95% are flowering [3]. Every year, botanists discover new species, for instance, according to The State of the World's Plants Report [3] 1730 new plants were described in 2017.

Traditional process of plant identification by using identification keys allows through series of answered questions to recognize the desired species. Nevertheless, such procedure in the field requires a pre-botanical experience and knowledge of plants that puts it away from reach for most nature lovers. Dealing with identification of species by DNA sequencing, which is considerably a precise tool, yet time-consuming and not economical for the general public.

Thus, traditional plant identification tools are almost impossible to reach wider audience and challenging even for many botanists that focused specifically on one genus/family and may be unfamiliar with another. The situation is further aggravated by the increasing deficiency of skilled taxonomists [4] leading to the so-called taxonomic

crisis [5]. As a consequence, nowadays knowledge connected with plant identification is restricted to a small group of people.

At the same time, taxonomists have been making efforts to develop more efficient approaches for species identification, including the flavors of emerging technology such as, digital image processing and pattern recognition techniques [5]. The last decade resulted in an abundance of researches in the field of computer vision and machine learning that applied for automated identification of plant [6–10].

The progressive use of portable devices (e.g. smartphones, tablets) and their ongoing technical development bring ideas of species identification by photo imaging closer to the usage in field studies. However, in the literature, there are no reported articles on testing automated plant identification applications for the portable devices in the conditions of field work.

Consequently, this article specifically focuses on checking open source smartphone applications on the reliability of plant identification in the field. This study observes the three most popular apps in the Google Play Store (https://play.google.com/store): PlantNet (developed by Cirad, INRA, Inria, IRD, and the Tela Botanica network; over 1,000,000 installations), PlantSnap (PlantSnap Inc.; over 500,000), PictureThis – Plant Identification (Hangzhou Dana Technology Inc.; over 500,000). Therefore, we address the following hypotheses:

1. Smartphone plant identification applications based on biological databases with morphological and geographical data can more accurately predict the name of a plant genus than the applications based on sole structure image databases.

2. The current smartphone applications for plant identification cannot be reliable sources in terms of their correct prediction in the top-2 search results.

3. A solid green screen can increase the accuracy of the plant identification applications.

Here, we predict that plant genera can accurately be identified specifically when the databases from which the applications are developed have rich scientific data of biology, morphology and geography of the plants. We presume that the robust algorithms in deep neural networks, supported with emerging artificial intelligence, are necessary in order to identify plants accurately. Moreover, the natural premises of the plant with noise from other plant species can interfere in image recognition to some extent. Hence, we assume that the use of artificial solid background, preferably green in color, will increase the accuracy of the applications.

2. Material and Methods

(a) Experimental design

Taking into account that plant identification up to species level in the field may be challenging even for experienced botanists, hence we analyzed results through all the applications only to genus level. A test dataset includes 50 previously identified plant genera. The images from the test dataset were applied to each of the three plant identification applications: PlantNet (PLN), PlantSnap (PLS) and Picture This-Plant identification (PLT).

The plant images were captured in the Ochotnica Górna, Lesser Poland province using a smartphone Vivo1718 (with Android v7.1 Nougat operating system with 1.8GHz Snapdragon 450 MSM8953 octa-core processor) equipped with a prime lens of 28 mm equivalent focal length and a face beauty sensor giving the 4920 x 3264 resolution.

Each photo was taken from a focal length of approximately 16 cm at the same period of the day in order to reduce the variability caused by light. From each identified genus, four different individuals have been captured both in a solid green screen and natural background conditions (figure 1) (the total number of images is 400).

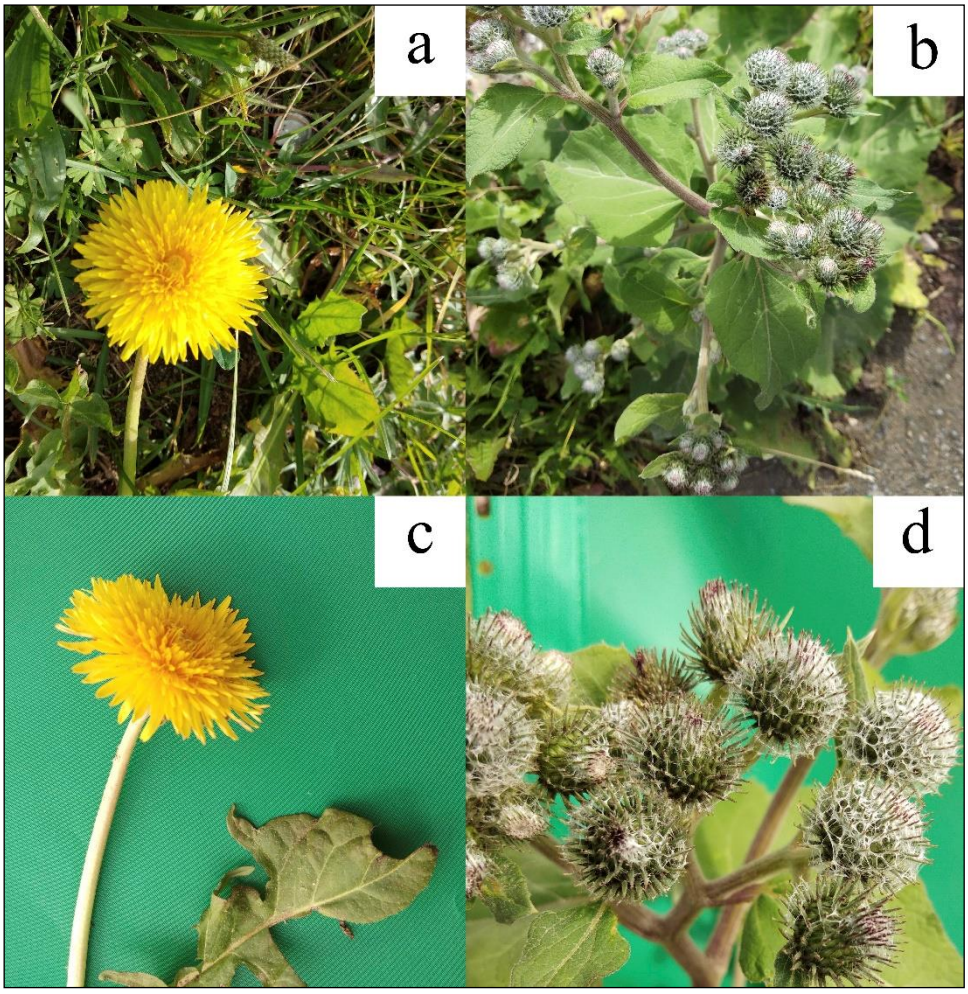

Figure 1. Representative images of plants with natural background (a, b) and the solid green screen (c, d).

(b) Image Processing

All images captured are edited as per guidelines of the smartphone applications and image processing is conducted with the aid of specific open source smartphone applications PlantNet (accuracy 99%), PlantSnap (98%), PictureThis – Plant Identification (90%), available in the Google Play Store. These applications are based on different machine learning image classification algorithms, such as, Convolutional Neural Network (CNN) [11], Fuzzy Algorithm [12] and Genetic Algorithm [13] respectively.

In order to reduce the technical variability we conducted the image processing using two different smartphones having similar configuration, affirming that the smartphone

configuration causes no variability in identification of the plants. A binary identification index is assigned for the images identified by the application, if the application succeeds in recognizing the plant genus within top-2 hits, we score them 1 (as identified) or 0 (as not identified).

(c) Statistical Analysis

We generated a reliability matrix that can be defined as a binary matrix: for samples as observations and genera as features. The accuracy of each application was estimated based on calculating the odds ratio that is the ability to identify the genus from the total images processed by the application. Further, a distance matrix was generated by computing Jaccard distances between the observations of the reliability matrix.

From the distance matrix we computed multi-dimensional scaling components (MDS1 and MDS2) and observed the distance of the clusters obtained for each application. Following the accuracy, we have computed the binary response considering the fixed effect of application usage under which we have a mixed effect of using a background screen. The package lme4 in R was used to compute the Generalized Linear Mixed Model (GLMM).

3. Results

From the distance matrix, we obtained three clusters by computing Multi-Dimensional Scaling each for PlantNet, PlantSnap and Picture This, considering the first two components MDS1 and MDS2 (figure 2). We computed GLMM, from the binary response of the applications identifying the images. It was observed that the applications have identified the plant genera without the effect of background ($F = 49.895$, $df = 2$, $p < 0.001$) as observed from the accuracy table (table 1). However, the effect of background mixed with application was not statistically significant ($F = 1.4$, $df = 2$, $p > 0.5$).

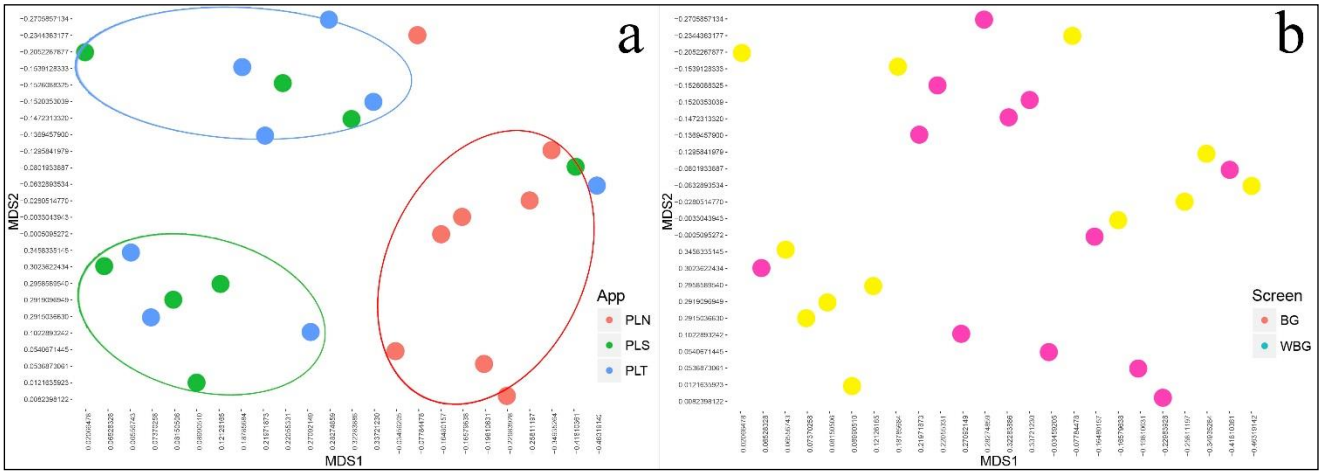

Figure 2. Distance matrix for (a) the tested smartphone applications and (b) background effects. Legends: PlantNet (PLN), PlantSnap (PLS), Picture This-Plant identification (PLT), Application (App), background (BG), without background (WBG), multi-dimensional scaling components (MDS1 and MDS2).

We observed that PlantNet is comparably a reliable application than the others. However, the claimed accuracy for all three applications was considerably less than the observed accuracy (table 1).

Table 1. Details of tested applications and observed accuracy

Application	Dataset	Algorithm	No. of plant images	Claimed accuracy	Mean observed accuracy	
					With natural background	With a solid green screen
PlantNet	PlantCLEF	CNN	113,205+	99%	79.5%	78.0%
PlantSnap	PlantSnap	FA	71000+	98%	46.5%	41.0%
PictureThis	PictureThis	Genetic	4,000+	90%	35.5%	34.5%

4. Discussion

Despite intensive and elaborate research on automated plant species identification, very few studies resulted in approaches that can be used by the general public, such as PlantNet, PlantSnap, Picture This etc. These smartphone applications use computer vision techniques for identifying the plant species in either natural and/or plain background.

In this study, the comparative assessment of the global plant identification performance of the three smartphone applications demonstrated a better plant recognition with natural background by PlantNet (79.5%) followed by Picture This (46.5%) and PlantSnap (35.5%). A comparatively higher observed accuracy of PlantNet is in corroboration with the fact that it uses additional metadata such as topographic characteristics, taxonomy, climate factors, soil type, land-use type, and biotope to the visual content in the identification process [8]. Moreover, separate indexes for each visual feature were applied by a multi-organ, multi-image and multi-feature fusion strategy in PlantNet along with the integration of cross-languages functionalities [4]. However, in order to process an image by several features, it requires a powerful algorithm with extensive training.

A few smartphone applications using deep learning technologies such as CNN, train their classifiers on large plant image datasets in automated plant species identification systems. However, these algorithms were not tested in the field or wild. In this study, the MDS data projected a separate cluster for the CNN based on PlantNet application from the other two explaining the power of deep learning techniques together with artificial intelligence in complex image processing (figure 2a). For the other two applications, although there were visible clusters, they seem to overlap, therefore explaining the confluence of algorithm in image processing. Further, no significant effect of the solid screen background was observed for all three tested applications (figure 2b) suggesting the image recognition not robustly relying on texture but involved deep learning [4].

Based on the statistical significance in terms of p-values computed from GLMM, it can be stated that applications are performing better without the solid background. This is in contrast to the previous reported literature where the noise from premises of the plants

can affect the image recognition [15]. Moreover, PlantNet is comparatively a better biological tool to identify plants accurately. Therefore, this study concludes that although plant identification by smartphone applications is convenient, but the better prediction is only possible if the application refers to biological data and deep learning algorithms encapsulated in the field of artificial intelligence.

Authors' contributions. All authors designed the experiment; all authors performed experiments. All authors wrote, edited and revised the manuscript. All authors gave final approval for publication and agree to be held accountable for all aspects of the work performed.

Competing interests. We declare we have no competing interests.

Acknowledgements. We thank Prof. Joanna Rutkowska for her comments that improved the scientific content of the paper.

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7.2.5. Cover letters

7.2.5.1. <u>Arpan Kumar Basak</u>:

26th June 2018

The Editor
Trends in Plant Science, Cell

Dear Sir or Madam,
I have enclosed an abstract of our paper entitled *A comparative assessment of smartphone applications for the plant identification in the field* accompanying this letter.

Knowledge of species identification is progressively demanding for nature conservation as well as for the public awareness of biodiversity and nature value. Traditional approaches rely on morphological characters, which are complex, time consuming, and can be challenging in field. Very recently, notable progress in process of imaging and pattern recognition with relevant technologies, such as mobile devices, increases the application of machine learning and artificial intelligence in automating the procedure of species identification. This paper is the first attempt to test open source smartphone applications considering the usage of different image classification algorithms affecting the reliability of plant identification especially in the field. We analyzed the three most popular applications available in the Google Play Store and established that the most reliable application PlantNet (79.5%) uses an image recognition algorithm based on biological databases with morphological and geographical parameters, while PlantSnap and PictureThis relies on an image recognition algorithm only, without referring biological features and having reliability 46.5% and 35.5% respectively on selected species. Also, we determined that a solid green screen decreases the reliability of all the three applications: PlantNet (78%),

PlantSnap (41%) and PictureThis (34.5%) behaving like a noise. We state that none of the checked applications can be used as a powerful tool for rapid plant identification in the field. We claim that current databases should be complemented with biological data.

We appreciate the ease of technology in the application of gathering data in Plant Science. However, it will be interesting to study their reliability in a broader aspect in parallel with emerging technology. Therefore, we would like to report these findings and their importance making this research suitable for publication in your esteemed journal Trends in Plant Science. The paper is not under the consideration in any other journal, assuring that the data has not been published as of now. I would like to mention that, the co-authors, Evgenii Baiakhmetov and Jaya Sravanthi Mokkapati are aware of the manuscript being sent for publication.

Yours sincerely,

Arpan Kumar Basak

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7.2.5.2. Evgenii Baiakhmetov:

26 June 2018

The Editor
Frontiers in ICT | Mobile and Ubiquitous Computing

Dear Sir or Madame,
Please find enclosed a manuscript of our paper entitled *A comparative assessment of smartphone applications for the plant identification in the field* accompanying this letter. Nowadays species identification in the field is becoming one of the most challenging tasks due to the increasing deficiency of skilled taxonomists that leads to the co-called taxonomic crisis. Traditional approaches relied on morphological characters is complex and time consuming. In this context, identification of plants based on images is considered as a promising solution that can help cover the taxonomic gap and be a useful tool for nature conservation as well as for the public awareness of biodiversity and nature value. This paper is the first attempt to test open source smartphone applications in the field if different image recognition algorithms affect reliability of plant identification. We analyzed the three most popular applications in the Google Play Store and established that the most reliable application PlantNet uses an image recognition algorithm based on

biological databases with morphological and geographical parameters, while PlantSnap and PictureThis relies on an image recognition algorithm without referring to biological databases and have almost twice less reliability than the first one. We determined that a solid green screen decreases reliability of all the three applications. Thus, we state that none of the checked applications can be used as a powerful tool for rapid plant identification in the field. We claim that current databases should be complemented with biological data.

We think that our findings, their novelty and broad interest makes this work suitable for publication in the Frontiers in ICT. The paper is not under consideration in any other journal and the data has not published previously. The co-authors, Arpan K. Basak and Jaya S. Mokkapati are aware that this manuscript is being sent for publication.

Yours sincerely,

Evgenii Baiakhmetov

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7.2.5.3. Jaya Sravanthi Mokkapati:

27 June 2018

The Editor
PLOS Computational Biology

Dear Sir or Madame,

Please find the enclosed a manuscript of our study entitled *A comparative assessment of smartphone applications for the plant identification in the field* accompanying this letter.

Plant identification is not only important to botanists and plant ecologists but also required or useful for large parts of society, from professionals (such as landscape architects, foresters, farmers, conservationists, and biologists) to the general public. But the identification of plants by conventional means is difficult, time consuming, and (due to the use of specific botanical terms) frustrating for novices. In recent years, computer science research, especially image processing and pattern recognition techniques, have been introduced into plant taxonomy to recognize the plant and eventually many smartphone applications hit the market. However, to what extent we can rely on mobile applications in identifying plants in real world is still in question. Therefore, here we

report the first study to assess the widely used smartphone applications for their reliability in plant identification in the field. Specifically, we studied three different smartphone applications which are using different algorithms and based on different databases in order to produce scientifically rich data in comparison for their respective performance levels. We found that PlantNet, a Convolutional Neural Network (CNN) based mobile application developed using database containing biological, taxonomical and geographical data of plants is comparatively accurate than the other two applications, PlantSnap and Picture This which solely work by image recognition from their own plant image databases. Moreover, this study also focused on background effect of images while processing for plant identification with different algorithms. The results showed the power of algorithms used by different applications and ensued that strong deep learning algorithms are definitely required for higher accuracy.

We hope you find the paper sufficiently interesting within the scope of the journal and worthy of publication in the PLOS Computational Biology. The paper is not under consideration in any journal and the data has not been published or presented previously. The co-authors, Arpan Kumar Basak and Evgenii Baiakhmetov are aware that this manuscript is being sent for publication and are aware of the order of authorship. The submitting author shall be solely responsible in case any dispute arises. All authors have no conflicts of interests.

We await your decision with interest.

Yours Sincerely,

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7.3. High temperature speeds up mating behaviour in Roman snail *Helix pomatia* (L., 1758)

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7.3.1. Project

Title: The effect of temperature on the mating behaviour in *Helix pomatia*

Summary

Helix pomatia is a terrestrial gastropod distributed across the Europe. Its period of reproduction begins in late spring and ends in early autumn, therefore it encounters wide range of temperatures. In this project we would like to check whether the temperature can affect mating behaviour of Roman snails. We put forward a hypothesis that increased temperature will cause snails to initiate sexual interaction faster in comparison to lower temperature. Experiment will be conducted in two suboptimal temperature treatments: 10°C and 30°C.

Aim / hypothesis

The main aim of this study is verification of the effect of suboptimal temperature on mating behaviour in Roman snail *Helix pomatia* (Linnaeus, 1758). Temperature is known to increase reproductive traits (such as spermatogenesis and oviposition) in many gastropods (Jess and Marks, 1998, Gomot et al, 1990, Benbellil-Tafoughalt and Koene, 2015). We hypothesis that snails from higher temperature treatment will initiate mating behaviour faster than those from lower temperature.

Methods

Roman snail *Helix pomatia* is a terrestrial gastropod distributed across the Europe. It can be found in forests, gardens and vineyards, usually in close proximity to rivers. It requires high humidity and loose soil for eggs laying. Its optimal temperature oscillates around 20°C, however they are active from the end of May to an early September (Welter-Schultes, 2012) , so they might experience high temperature fluctuations . All individuals of *H. pomatia* are simultaneous hermaphrodites which do not self-fertilize (Fretter and Peake, 1975). For the scope of this project we will collect 40 individuals from Jaszcze stream valley, Ochotnica Górna, Poland. To assure sexual maturation of all individuals only those with appropriate size of shell (approx. 5cm) will be gathered.
Experiment will be conducted in two temperature treatments: 10°C and 30°C. This temperatures are suboptimal for *H. pomatia*, nevertheless they still represent the range of temperature which snail experience in nature during mating season. Each pair of snails will be placed in containers set up to imitate their natural habitat. Mating behaviour will be assessed as the time needed for each pair to initiate mating. Observations will be conducted simultaneously for both treatments in two blocks. Animals will be checked every 15 minutes for 8 hours (or until mating is observed).
Data gathered from the experiment will be analysed using ANOVA test.

Impact of results

Roman snail feeds on variety of fruits, vegetables, flowers and leaves (Fretter and Peake, 1975). Since it often appears in gardens it is considered to be a pest which damages crops (Barker, 2002). Knowledge about rate of its reproduction in respect to seasonal temperature fluctuations could be meaningful for European farmers. What is more, *H. pomatia* is cultivated and eaten as a food, therefore the results from our project could be directly used in food industry.

Literature

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7.3.2. Report – first version

The effect of temperature on mating behaviour in Roman snail *Helix pomatia* (L., 1758)

Summary

For every animal allocation of resources is important and affects their life history evolution. However, every strategy is dependent on the environmental conditions. Animals which are exposed to seasonal fluctuations of biotic factors particularly depend on the optimal allocation strategies. Among many features reproductive traits are extremely important since they directly affect the fitness of the animals. We conducted an experiment which examined the effect of the higher than optimal temperature on the mating behaviour in terrestrial gastropod, *Helix pomatia.* We discovered that according to our expectations, increased temperature speeds up the initiation of mating.

Introduction

Reproductive strategies are important life history traits which can be affected by seasonality [1]. Changing environmental conditions are influential especially for temperate ectothermic animals since their allocation of resources must consider harsh seasons and terrestrial gastropods is a group particularly affected by external factors [1]. Among environmental factors temperature is one of the most important because it can significantly either stimulate or delay reproductive traits (e.g. spermatogenesis, oviposition) in many gastropods [1, 2, 3]. After insects, molluscs are the second most numerous phylum in animal kingdom. Among them, gastropods are the most diverse class in terms of species richness. The vast majority of gastropods are sequential or simultaneous hermaphrodites [4] which has its advantages, such as possibility of reproduction by self-fertilization because some species have difficulties in finding a partner to copulate. Also hermaphrodites have higher probability of meeting a potential partner as every encountered individual can be mated with and increased productivity due to the discharge of their functions and division of resources [6]. Mating behaviour is a complicated process which may differ depending on the species. In stylommatophoran terrestrial species courtship and copulation may be done unilaterally (one partner plays a specified role: male or female, while the other individual plays a reverse role during copulation, and usually, after one round of copulation the roles change), or reciprocally (both individuals play the male or female role at one time during copulation) [7]. Mating can be done in different positions: unilaterally or face-to-face, but within stylommatophorans face-to-face and simultaneous reciprocal behavior is the most common mating behavior [8].

In our study we have decided to examine the effect of temperature, one of the most important abiotic factor for ectotherms, on the mating behaviour of Roman snail, *Helix pomatia* L., 1758. Since temperature increases other reproductive-related traits, we put forward a hypothesis that higher temperature will also facilitate faster mating activity.

Roman snail feeds on variety of fruits, vegetables, flowers and leaves [9]. It often appears in gardens and is considered to be a pest which damages crops. Knowledge about speed of its reproduction in respect to seasonal temperature fluctuations could be meaningful for European farmers. What is more, *H. pomatia* is cultivated and eaten as a food, therefore the results from our project could be directly used in food industry.

Materials and methods

Roman snail *Helix pomatia* is a terrestrial gastropod widely distributed across the Europe. Usually it occurs in forests and open habitats, especially along the rivers. It requires high humidity and loose soil for eggs laying. Its optimal temperature oscillates around 20°C, however they are active from the end of May to an early September [10], so they might experience high temperature fluctuations. All individuals of *H. pomatia* are simultaneous hermaphrodites which do not self-fertilize [9]. Their copulation is characterized by face-to-face mating behaviour. Mating process consists of several steps: 1) snails circle each other with heads up and touch one another with their tentacles, 2) stimulate the partner, 3) inject love dart into the sole of the snail (*H. pomatia* produces love dart which stays in body

of dart receiver, then dart is rebuilt by dart shooter [11]), 4) rest, 5) genitals opening overlap, 6) they twist bodies around each another, one individual receives a spermatophore which is a sperm packet (this step may take from 4 to 7 minutes), 7) penis is removed, but animals can remain attached together with their feet for couple of hours [9].

The experiment was conducted in two temperature treatments: 20°C and 27°C. Each pair of animals were placed into plastic containers filled with moist soil and moss to assure proper humidity and a leaf of *Taraxacum officinale* as a food source. Our initial plan was to measure the time required for each pair to start copulation. However, pilot study showed that during 3 hours of observations we were able to observe only first step of mating behaviour, also called introductory behaviour. Due to that we have decided to asses the rate of mating as the time till the initiation of first step. Experiment was conducted simultaneously in two temperatures. In total, 48 individuals used in this process were collected in Jaszcze stream valley, Ochotnica Górna, Poland. To assure sexual maturation of all individuals only those with appropriate size (approx. 5 cm) will be gathered. Obtained data was analyzed using Kruskal-Wallis non-parametric test.

Results

We observed statistically significant influence of temperature on the speed of mating initiation (chi-squared=16.625; $p<0.05$). Higher temperature showed decrease in the time needed for snails to engage in mating process (Fig. 1). Median value for 20°C was 23.5 minutes, whereas in higher temperature median was 7 minutes.

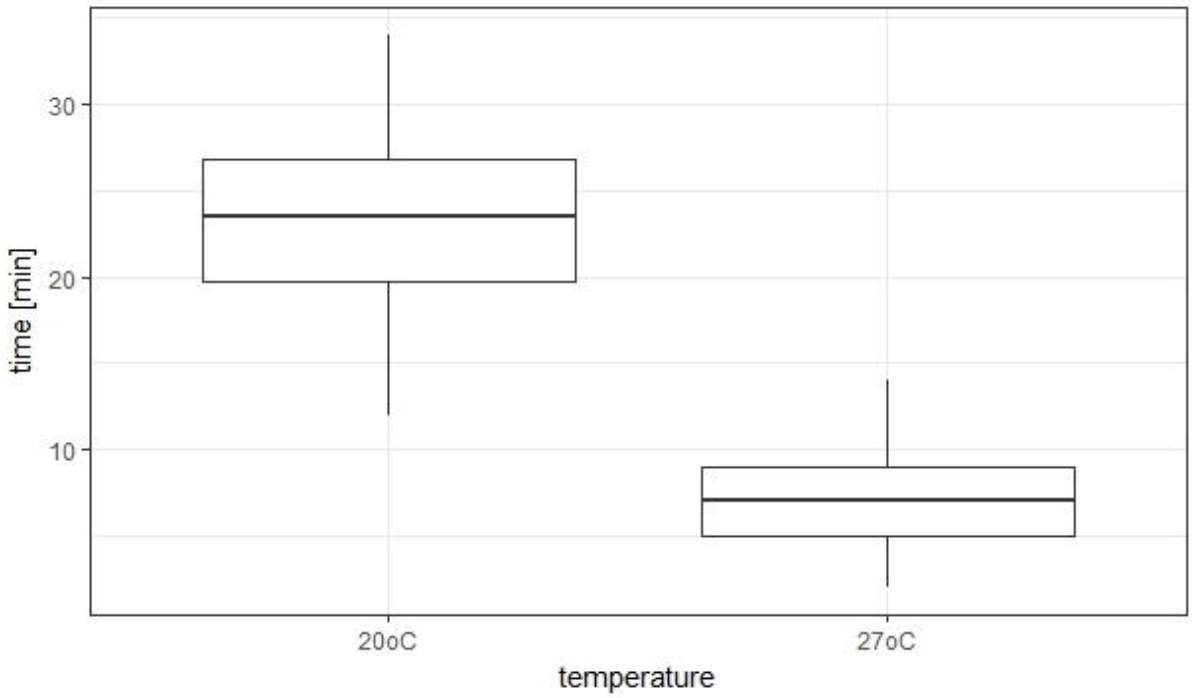

Figure 1. The effect of temperature on time needed to initiate mating process in *Helix pomatia.* Horizontal bold line represents median value, whereas vertical 95% confidence intervals.

During the experiment, we have observed first step of mating behaviour. Interestingly, we were also able to observe circling behaviour, which had – according to literature data – not been noted before in this species [16].

Discussion

Temperature is known to affect reproductively-related traits in gastropods as well as in other ectotherms. According to this findings we assumed that the speed of mating initiation in *H. pomatia* will increased in higher, suboptimal temperature. We found that time needed for snails to start mating is significantly lower in 27°C than in optimal conditions of 20°C. Studies about influence of temperature on reproduction in gastropods (e.g. mating, courtship, copulation) are carried out mostly in the context of exploring species biology or ecology and not in framework of analyzing their life history evolution. Nevertheless, data about *Arion vulgaris*, invasive slug, show pattern which is comparable to our finding. *A. vulgaris* commonly occurs in Europe and is able to survive in different temperature regimes which seems to affect its duration of mating process. In Poland, where the average temperature during its mating season (July – October) is about 14.7ºC copulation time ranges from 240 to 330 min [12], whereas in Norway (average temperature during mating season: 11.7°C) it takes 240 to 600 minutes [13]. However we cannot interpret this results directly as an influence of temperature since *A. vulgaris's* mating season in Poland and in Norway differ in others climatic factors as well. The same pattern can be observed in many terrestrial gastropods which are characterized by wide geographic range of distribution [10]. Also in freshwater gastropods temperature can affect the egg laying capacity but the pattern seems to be opposite. For *Helisoma duryi*, *Biomphalaria alexandrina* and *Bolinus truncatus* optimum temperature is 26-28ºC in which growth and egg-laying are observed. Raising the temperature to 33ºC causes *H. duryi* to postpone egg-laying from 4-5 weeks to 14 weeks. For *B. alexandrina* and *B. trunculus* 33°C induces 100% mortality [14].
To summarize, we want to emphasize that temperature seems to be an important abiotic factor influencing reproductive traits. We can hypothesize that climate change may in future affect the biology of terrestrial gastropods, hence the knowledge about possible changes in their biology is crucial especially since *H. pomatia* is a common pest across the whole Europe.

Authors' contributions. K.T. designed the study, K.T. and K.Z. performed the experiments and analysed the data, K.T. and K.Z. wrote the manuscript, all authors revised the manuscript, gave final approval and are accountable for the work herein.

Acknowledgement. We would like to thank Monika Prus and Miriam Gonzalez Gonzalez for their invaluable help during field work.

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7.3.3. Reviews

7.3.3.1. **Agnieszka Bednarska:**

Climate change and the average global temperature increase is an issue receiving increasing attention. From the point of view of evolutionary biology, changing temperature conditions are important as they may affect the way organisms deal with the energy allocation to growth, maintenance and/or reproduction. This manuscript describes a study that aims at contributing to increasing our insight into the way temperature affects mating behavior of snails. The choice of test organisms, *H. pomatia* is relevant, as according to the authors fairly little work has been done on the effect of temperature on this species and "knowledge about speed of its reproduction in respect to seasonal temperature fluctuations could be meaningful for European farmers" as this species "is considered as pest of many crops".

There are, however, quite a number of problems with this manuscript. First of all, although the authors stress in Summary and Introduction the importance of "fluctuations of temperature" and/or "seasonal variations in temperature" on "the optimal allocation strategies" or "allocation of resources", they, in fact, do not study this issues in their paper. They just compared how long does it take to mate for snails at two different constant temperatures. Therefore, they should not put their study in the wider context of temperature fluctuation or seasonal variability, as such context does not fit their study.

There is no enough information about the methodology which was used, e.g., at which conditions snails were kept after collection, how long they were stored before being used in the experiment? Did the authors acclimatize snails to studied temperatures before the mating experiment had been started? How exactly the observations of mating behavior were done? The authors mentioned that "experiment was conducted simultaneously in two temperatures", but how? Does it mean that one observer followed all snails (replicates) at one temperature and the other one observed all snails at the other temperature, or maybe the authors designed the observations in a way which allowed them to exclude the observer effect? This should be clarified as then one can evaluate the correctness of the experimental design. It is also not clear if all collected individuals were used, how snails were divided into two different temperatures, how many replicates was used in each temperature, how many times each individual (or pair) was observed? Did the authors observed snails in different or always in the same container(s)? If the same container(s) was used, could it affect the mating behaviour? How long did it take to complete the experiment – hours or rather days? How snails which 'waited' for their turn in the experiment were kept – separately or in groups? It is crucial to know such information for the proper evaluation of the experimental design and statistical test used for data analysis. How many days, and at which temperature snails were stored while waiting for their turn in the experiment? Were they used only once or repeated measurements were done on the same pair (or the same individuals)?

The text need quite some work before the article can be indeed considered for publication seriously - I strongly advise that the manuscript is thoroughly corrected linguistically before resubmission and all formatting requirements are followed - the use double line-spacing was highlighted in format requirements of the journal, but was not followed by the authors.

Following the line numbers in the border of the manuscript, I offer some comments and suggestions for improving the manuscript.

Line 1: “Temperature effect” sounds better.

Line 11: “every strategy” - which strategy do you mean? I guess strategy of energy allocation to different life-history traits, but it should be clearly written.

Line 10: “animals exposed to seasonal fluctuations” or rather those living at such conditions?

Line 11: It is not clear what do you mean by saying “many features”? Do you mean different life-history traits?

Line 13: “to study” instead of “which examined” sounds better

Line 14: We found that increased temperature …. sounds better

Line 19: I would delete this sentence as you did not study the effect of seasonality on reproduction strategy.

Line 20: “influential” or just “important”?

Line 23: Please delete “significantly”?

Line 27: “has advantages such as “ and “in case of difficulties …”… sounds better

Line 30: What do you mean by “productivity” here?

Line 31: Please replace “which may differ” by “differs”

Line 35: add “either” in front of ‘male or female role”

Line 38: It is rather obvious that in your study you studied; the phrase “we studied” is enough

Line 40: Other than what? I would say “other reproductive-related traits than just mating behaviour”

Line 41: It is not clear what you define as “mating behaviour” and what as “mating activity”. Should be clarify.

Line 43: “pest of many crops”. Also, I am not convinced if “speed of reproduction” is the proper phrase to be used here. Please think if “speed of breeding” wouldn’t be better expression here?

Line 45: It is rather obvious that if it is eaten, then, it is a food ☺. Please rephrase into e.g., " it is bred as a food source".

Line 46: "may be useful" instead of "could be directly used"

Line 50: "can be easily found" sound better than "occurs", especially in case of common species

Line 51: Optimal temperatures for what? Growth? Reproduction?

Line 53: Daily fluctuations or seasonal fluctuations in temperature?

Line 53: If "all individuals are simultaneous hermaphrodites" then it simply means that the species is simultaneous hermaphrodites, doesn't it?

Lines 55-61: Please describe all steps of mating process in the same style (passive or active form).

Line 62: What was the size of the plastic container? I guess, the containers were not filled with moist soil as there would be no room for the snails :) So just say that the boxes contained X cm of soil. Or if you think the X cm is too precise to be correct, you could say "about X cm".

Line 65: Replace "Due to that we have decided" with "Therefore, we decided … the first step of mating"

Line 67: The way of making observations by to observers should be clarified (see my general comment)

Line 68: Which process? Do you mean experiment?

Line 69: were gathered

Line 70 data WERE analyzed

Line 73: You observed snails, not results, so "we found" is the proper expression here

Line 74: Offer precision and detail whenever possible, especially in terms of statistics. Is always more useful to know the exact *p* value than just that it was < 0.05.

Line 74: It is not temperature which showed anything! This are snails which showed a particular behaviour in different temperatures. I would avoid using a word "decrease" here – the only what you can say based on the statistical analysis of the data is that snails at 27°C needed significantly less time to start the first step of mating than those from 20°C.

Lines 79-80: As far as graph itself, please provide unit for temperature together with description of x axis (temperature [°C]) rather than with values. As far as figure caption, the graph shows box-and-whisker plots, but the rectangular part of the plot is not explained. Moreover, it would be useful to have information about statistical test used for analysis of the data presented on that graphs or included in figure caption together with *p* value.

Line 82: How many time, in how many pairs of snails?

Line 88: Why not just “reproduction traits”?

Line 90: Based on which criteria you decided that 27 is already suboptimal temperature for that species?

Lines 91-94: Please provide reference(s) to support that statement?

Line 101: Which pattern do you mean? The same like for *A. vulgaris* in Poland and Norway, or the same as found in your study for *H. pomatia*?

Line 108; Why “seems”? You found a clear effect, so it should be “is”.

Line 109: Rather possible changes in mating behaviour as only that parameter (and in fact only one particular step of mating behaviour) was studied, so be careful with over-conclusions.

7.3.3.2. <u>Adam Łomnicki</u>:

I am afraid it is not the most ambitious an outstanding report, nevertheless it was properly made. One striking feature of Roman snail when looking for it in the field is its dependence not only on external temperatures but also on humidity, so that this molluscs refrain from any activity at the time of very low humidity. It would be nice to learn how the mating behaviour of these animals is affected by two factors simultaneously: temperature and humidity.

The only shortcoming of the report is the lack of the sizes of the samples on which chi test was based. The number of snails refraining from reproduction and the numbers of those with initiate it should be given for two different temperatures. The final result of chi test and its significance is of importance on it too, especially in order to estimate the ability of the test to reject the II type of statistical two errors. When describing the Fig. 1, I am not sure whether 95% confidence interval is present by vertical line or vertical dimension of two rectangles.

7.3.3.3. <u>Arpan Kumar Basak</u>:

The Editor

Biology Letters

Dear Sir or Madam,

I was given the opportunity to review the research manuscript entitled *The effect of temperature on mating behaviour in Roman snail Helix pomatia (L., 1758)* submitted to your esteemed journal. I will appreciate author's work in attempting to answer an interesting question of science. In general, the research question happens to be interesting.

Even though the paper is short, it provides a sequential flow to the reader. The statistical analysis is simple and comprehensible, providing a clear understanding of the effect of temperature on mating behavior, as author attempts to show.

Therefore, I have explained my comments briefly describing them below:

Line Numbers	Comments
9 -11	The structure of the sentence needs a correction.
13 – 14, 18, 30, 42, 44, 49, 52, 88, 97, 99	This sentence is grammatically incorrect
34 – 36	This sentence is not represented represent properly.
54 - 59	The mating behavior is not explained clearly, lack of choice of words.
86	Not appropriate for the meaning the author attempts to project
101	The word seems is subscripted
103	Egg laying animals are called oviparous, recommended terminology
107 - 109	The structure of the sentence needs a correction

However, I would like to mention that the overall quality of writing is average; author should reconsider correcting the grammatical errors before submission.

To summarize, I liked the structure of the manuscript explaining the scientific findings. However, I would prefer if the author could reconsider these minor corrections before publishing in your esteemed journal.

Thanking you,

Yours sincerely,

Arpan Kumar Basak

7.3.3.4. <u>Miriam Gonzalez Gonzalez</u>:

The manuscript provides findings about mating behaviour of an important commercial (and pest) specie; *Helix pomatia* or common snail. The deep understand of how the roman snail reproduces could be useful in "snail's farms" that constitute a particular interesting sector in Southern Europe, where these animals are eaten as delicatessen. So far, the global charming is increasing the temperatures in all Europe and efforts should be made for analyzing how this is affecting animals. In this particular case, *Helix pomatia* is considered a pest in common gardens and crops of vegetables which could mean that as the temperatures get raising this particular pest could be more persistent. **This article could be interesting for experts on molluscs, researchers that work on environmental protection and "snail" farmers.**

The major claims are that in higher temperatures snails start mating behaviour sooner that in lower temperatures. In addition, they provide information about "circling behaviour" in mating, being the first authors to notice it. Unfortunately, I do not find great novelty in the findings, and as far I know it is not clear why this finding is in the context of life history evolution. It would be interesting to see more than data about the novel finding about the circling behaviour. What is more, with some additional experiments on other range of temperatures and with longer time-points it would be relatively simple to obtain very valuable data. For example, it would be highly recommendable study low for example 15ºC, 20ºC, 25ºC and 30ºC. In addition, more information about relative humidity would be very valuable inasmuch that these animals depend greatly on it.

In particular I would like to point at this potential improvements:

- Materials & Methods section: more schematically and in some parts is mixed with introduction. This section should be descriptive in order that someone should easily follow it for performing the experiment.
- Figure 1. More information about the figure itself should be provided. I cannot look at the figure and understand it (not explanatory).
- Greater description about the "new circling behaviour".
- In the experimental setup I consider highly important the relative humidity and it has not been measured.
- The aim of the study and possible application of the study should be clearer.

7.3.3.5. <u>Evgenii Baiakhmetov</u>:

The manuscript presents a very interesting and valuable study on temperature effects on the mating behavior in terrestrial gastropod. Authors hypothesize that higher temperature will facilitate faster mating activity. The study establishes that at 27°C snails

need significantly lower time to start mating in comparison with optimal conditions at 20°C.

Generally the article is good written and interesting. However, my main concerns are with the fact that the experimental part may be improved. The authors refer, for instance, to the study by El-Emam and Madsen (1982) that carried out experiments in a range of temperature: 18°C, 26–28°C, 33°C. Thus, I would recommend conducting the experiment at the lower temperature. On top of that, why did you choose 27°C as a top temperature?

Furthermore, according to Ligaszewski et al. (2007) the optimal humidity for *Helix* breading is 75-85%. Did you check humidity during the experiment? May it affect the results? I also suggest to add more information about sampling (see figure 1 and 2 in Lind, 1990) due to in a good-quality habitat most snails are resident, probably for life, and restrict their movements to a small part of the habitat (Pollard, 1973; 1995). Could snails from different parts of Jaszcze stream valley have different mating propensity irrespective to temperature?

I have no doubt of scientific accuracy, including statistical analysis. Except the mentioned remark, the research methods are appropriate, and evidence is provided for the conclusions drawn. I found the writing style easy to read and appropriate for a wide audience. The authors use the suitable figure to illustrate results. In addition, the manuscript is of appropriate length and includes required parts.

Therefore, I suggest that the manuscript is returned to the authors with a possibility for re-submission after rearranging the experiment and minor revisions.

Minor comments for the authors

[Line 36] Spelling should be British English. Thus, *behaviour* instead *behavior*.

[Line 61] *was* instead *were*

[Line 61] *were* instead *was*; using *the*

[Line 68] "*will be gathered*". Probably should be past tense.

[Line 94] The references order should be revised.

[Line 94] Remove double space.
[Line 97] "*this results*"
[Line 126; 148] You do not refer to these articles in your manuscript.

References

Ligaszewski M, Łysak A, Mach-Paluszkiewicz Z. 2007 Reproductive performance of *Helix pomatia* (Gastropoda: Pulmonata: Helicidae) and survival of its hatchlings under farm conditions. *Am. Malacol. Bull.* **22**, 1– 6.
Lind H. 1990 Strategies of Spatial Behaviour in *Helix pornatia. Ethology* **86**, 1– 18.
Pollard E. 1973 Growth classes in the adult Roman snail (*Helix pomatia* L.). *Oecologia* **12**, 209– 212.

Pollard E. 1975 Aspects of the ecology of *Helix pomatia* L. *J. anim. Ecol.* **44**, 305– 329.

7.3.4. Report – final version

High temperature speeds up mating behaviour in Roman snail *Helix pomatia* (L., 1758)

Summary

For every animal allocation of resources is important and affects their life history evolution. However, every strategy is dependent on the environmental conditions. Animals which are exposed to seasonal fluctuations of abiotic factors particularly depend on the optimal allocation strategies. Among many features reproductive traits are extremely important since they directly affect the fitness of the animals. We conducted an experiment to study the effect of the higher than optimal temperature on the mating behaviour in terrestrial gastropod, *Helix pomatia*. We found that increased temperature speeds up the initiation of mating.

Introduction

Changing environmental conditions are important especially for temperate ectothermic animals since their allocation of resources must consider harsh seasons and terrestrial gastropods is a group particularly affected by external factors [1]. Among environmental factors temperature is one of the most important because it can either stimulate or delay reproductive traits (e.g. spermatogenesis, oviposition) in many gastropods [1, 2, 3]. After insects, molluscs are the second most numerous phylum in animal kingdom. Among them, gastropods are the most diverse class in terms of species richness. The vast majority of gastropods are sequential or simultaneous hermaphrodites [4] which has advantages, such as possibility of reproduction by self-fertilization in case of difficulties in finding a partner to copulate. Also hermaphrodites have higher probability of meeting a potential partner as every encountered individual can be mated with [6]. Mating behaviour is a complicated process differs-depending on the species. In stylommatophoran terrestrial species courtship and copulation may be done unilaterally (one partner plays a specified role: male or female, while the other individual plays a reverse role during copulation, and usually, after one round of copulation the roles change), or reciprocally (both individuals play the either male or female role at one time during copulation) [7]. Mating can be done in different positions: unilaterally or face-to-face, but within stylommatophorans face-to-face and simultaneous reciprocal behavior is the most common mating behavior [8].

We studied the effect of temperature, one of the most important abiotic factor for ectotherms, on the mating behaviour of Roman snail, *Helix pomatia* L., 1758. Since temperature increases other reproductive-related traits than just mating behaviour, we put forward a hypothesis that higher temperature will also facilitate faster mating behaviour.

Roman snail feeds on variety of fruits, vegetables, flowers and leaves [9]. It often appears in gardens and is considered to be a pest of many crops. Knowledge about speed of breeding in respect to seasonal temperature fluctuations could be meaningful for European farmers. What is more, *H. pomatia* is bred as a food source, therefore the results from our project may be useful in food industry. What is more, we postulate that knowledge about the reproductive strategies in response to temperature will be an important information to those who study life history evolution of ectotherms.

Materials and methods

Roman snail *Helix pomatia* is a terrestrial gastropod widely distributed across the Europe. Usually it can be easily found in forests and open habitats, especially along the rivers. It requires high humidity and loose soil for eggs laying. Its optimal temperature for growth, activity and reproduction oscillates around 20°C, however they are active from the end of May to an early September [10], so they might experience high temperature seasonal fluctuations. *H. pomatia* is simultaneous hermaphrodite which do not self-fertilize [9]. Their copulation is characterized by face-to-face mating behaviour. Mating process consists of several steps. First, snail circle each other with heads up and touch each other with their tentacles. Then, they stimulate the partner and one of individual injects love dart into the sole of another (*H. pomatia* produces love dart which stays in body of dart receiver, then dart is rebuilt by dart shooter [11]). After that, they rest. When their genitals openings overlap they twist their bodies around each other and one individual receives a spermatophore (this step may take from 4 to 7 minutes). At the end of the copulation penis is removed but animals can remain attached together for couple of hours [9].
The experiment was conducted in two temperature treatments: 20°C and 27°C. Each pair of animals were placed into plastic boxes containing about 15 cm of moist soil and moss to assure proper humidity, however we were not able to control it precisely. After each completed observation soil and moss was discarded and boxes were prepared again for next pair of snails. Leaf of *Taraxacum officinale* was also added as a food source. Our initial plan was to measure the time required for each pair to start copulation. However, pilot study showed that during 3 hours of observations we were able to observe only first step of mating behaviour, also called introductory behaviour. Therefore, we assessed the speed of mating as the time till the initiation of the first step. Experiment was conducted simultaneously in two temperatures by single observer. In total, 48 individuals were used in the experiment, 12 pairs of snails were randomly assigned to each treatment. Before measurements animals were kept together in big boxes, to assure low individual density. They were acclimatized in experimental temperatures for ca. 10 minutes. During this time they did not exhibit any mating behaviour. Animals were collected in Jaszcze stream valley, Ochotnica Górna, Poland. To assure sexual maturation of all individuals only those with appropriate size (approx. 5 cm) were gathered. Obtained data was analyzed using Kruskal-Wallis non-parametric test.

Results

We found statistically significant effect of temperature treatment on the speed of mating initiation (chi-squared=16.625; df=1; p=0.0307). Snails at 27ºC needed significantly less time to start the first step of mating than those from 20ºC (Fig. 1). Median value for 20°C was 23.5 minutes, whereas in higher temperature median was 7 minutes.

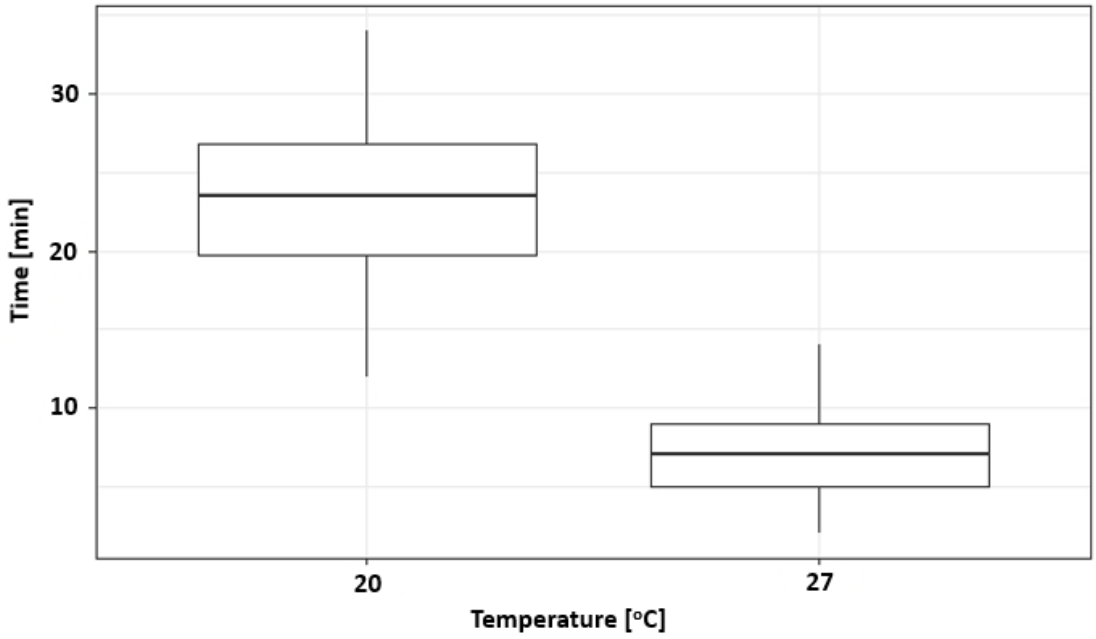

Figure 1. The effect of temperature on time needed to initiate mating process in *Helix pomatia.* Horizontal bold line represents median value with upper and lower quartile in boxes.

During the experiment, we have observed first step of mating behaviour. Interestingly, we were also able to observe circling behaviour in all pairs of snails for both temperatures, which had – according to literature data – not been noted before in this species [16].

Discussion

Temperature is known to affect reproductively-related traits in gastropods as well as in other ectotherms. According to this findings we assumed that the speed of mating initiation in *H. pomatia* will increased in higher, suboptimal temperature. We found that time needed for snails to start mating is significantly lower in 27°C than in optimal conditions of 20°C. Studies about influence of temperature on reproduction traits in gastropods (e.g. mating, courtship, copulation) are carried out mostly in the context of exploring species biology or ecology and not in framework of analyzing their life history evolution, so rarely temperature manipulation is conducted. Nevertheless, data about *Arion vulgaris*, invasive slug, show pattern which is comparable to our finding. *A. vulgaris* commonly occurs in Europe and is able to survive in different temperature regimes which seems to affect its

duration of mating process. In Poland, where the average temperature during its mating season (July – October) is about 14.7°C copulation time ranges from 240 to 330 min [12], whereas in Norway (average temperature during mating season: 11.7°C) it takes 240 to 600 minutes [13]. However we cannot interpret this results directly as an influence of temperature since *A. vulgaris's* mating season in Poland and in Norway differ in others climatic factors as well. The same pattern can be observed in many terrestrial gastropods which are characterized by wide geographic range of distribution [10]. Also in freshwater gastropods temperature can affect the egg laying capacity but the pattern seems to be opposite. For *Helisoma duryi*, *Biomphalaria alexandrina* and *Bolinus truncatus* optimum temperature is 26-28°C in which growth and egg-laying are observed. Raising the temperature to 33°C causes *H. duryi* to postpone egg-laying from 4-5 weeks to 14 weeks. For *B. alexandrina* and *B. truncatus* 33°C induces 100% mortality [14].

In our experiment we were not able to properly control for humidity which is said to be an important factor affecting biology of gastropods [17]. Nevertheless, we think that for the duration of the assay humidity level was sufficient. What is more, if there were to be any differences in the level of humidity between treatments, higher temperature regime would be the one with lower moisture. Our predictions are that lower humidity will be stressful for animals and will cause postponement of mating. Since we were able to find increased speed of mating in 27°C we conclude that for the duration of the experiment humidity was not a limiting factor.

We want to emphasize that temperature is an important abiotic factor influencing reproductive traits. We observed that higher temperature speeded up the mating behaviour which can have a consequences in case of resources allocation in *H. pomatia.* We can hypothesize that climate change may in future affect the biology of terrestrial gastropods, hence the knowledge about possible changes in their biology is crucial especially since *H. pomatia* is a common pest across the whole Europe.

Authors' contributions. K.T. designed the study, K.T. and K.Z. performed the experiments and analysed the data, K.T. and K.Z. wrote the manuscript, all authors revised the manuscript, gave final approval and are accountable for the work herein.

Acknowledgement. We would like to thank Monika Prus and Miriam Gonzalez Gonzalez for their invaluable help during field work.

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7.3.5. Cover letters

7.3.5.1. <u>Katarzyna Toch</u>:

26th of June 2018

The Editor
Journal of Experimental Biology

Dear Sir,

Please find enclosed a manuscript of our paper entitled “The effect of temperature on mating behaviour in Roman snail, Helix pomatia (L., 1758)”.
Allocation of resources is important for every organism and it affects their life history evolution. Nevertheless, every strategy depends on the environment that it develops in. Experiencing seasonal fluctuations of biotic and abiotic factors extorts the need of optimal allocation. Among many features, reproductive traits are extremely important since they directly affect the fitness of the animals. Yet, no study so far directly analysed the influence of suboptimal temperature on mating behaviour of molluscs. We conducted an experiment which examined the effect of 27°C degrees on the mating behaviour on terrestrial gastropod, Helix pomatia. We report, that according to our expectations, increased temperature speeds up the initiation of mating.
We believe that our results are appropriate for publication in Journal of Experimental Biology. The paper is not under consideration in any other journal and data has not been published previously.

Yours sincerely,

Katarzyna Toch

Institute of Envrionmental Sciences, Jagiellonian University
Gronostajowa 7, 30-378 Kraków, Poland
katarzyna.toch@gmail.com

7.3.5.2. <u>Kamila Zając</u>:

27 June 2018

The Editor
Journal of Thermal Biology

Dear Sir or Madame,
Please find in attachment the manuscript entitled "The effect of temperature on mating behaviour in Roman snail *Helix pomatia* (L., 1758)".
For animals allocation of resources is important and affects their life history evolution. However, every strategy depends on the environmental conditions (temperature, humidity, photoperiod). Animals which are exposed to seasonal fluctuations of abiotic factors particularly depend on the optimal allocation strategies. We conducted an experiment which examined the effect of the higher temperature (27^{o}C) than optimal (20^{o}C) on the mating behaviour in terrestrial gastropod, *Helix pomatia*, which is widely distributed species in Europe. Based on our results we discovered that increased temperature can cause faster initiation of mating behaviour in this species, which was in line with our expectations.
We believe that manuscript with our results is suitable for publication in Journal of Thermal Biology. This paper is not under consideration in any other journal and the data has not been published previously.

Yours sincerely,

Kamila Zając

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