

Dear Editor,

Please consider for publication in Peer community in Environmental Toxicology and Chemistry the revised version of our paper entitled "Ivermectin resistance in dung beetles exposed for multiple generations". In the new version we carefully addressed the comments of three reviewers. We hope you consider that the new version is suitable for publication, but we will be happy to address any further comments.

On behalf of all co-authors,

Dr. Daniel González-Tokman

INECOL, Mexico

Reviewed by anonymous reviewer, 25 Jul 2023 12:32

The authors present a study in experimental evolution, in which one population of *Euoniticellus intermedius* were exposed or not to ivermectin for 18 generations. Moreover, authors carried out toxicity experiments in generation F1, F2, F3, F6 and F18 for both lines (exposed or not to ivermectin across multigenerational experiment). In the context of ecotoxicology, the questions posed in this study seem very relevant to me, by studying the long-term responses to a pollutant. The authors rely on evolutionary biology to characterize the response of *Euonicellus intermedius* populations to chronic exposure to ivermectin, in particular through genetic adaptation. This study is on the whole clear and well carried out, despite some technical problems related to a mortality of individuals regardless of treatment. I particularly appreciated the good contextualization of the subject and the fact that the hypotheses were well posed. The article is not too long, allowing not to get lost in the information. In addition, the statistics seem to me rather well done and rigorous. Finally, I think it is important to emphasize that such a long multigenerational experiment (18 generations) on arthropods is rather rare and requires significant work.

I would like ask about the following points to improve the manuscript.

RESPONSE: Thank you very much for your positive view of our paper and your enriching comments.

Line 24: it would be interesting to add the life history traits studied, for example "We compared reproductive success (total brood balls, emerged beetles, proportion emerged and days to emergence)".

RESPONSE: We agree and added the studied traits.

Line 22: I was wondering if the term "line" was the most relevant. Basically, you have a line that you place in two different conditions.

RESPONSE: We used the term “line” consistently with other studies that start with a single population that is separated into different regimes. Similar wording has been used, for example in the context of evolution in response to diet regimes (e. g. Warbrick-Smith et al. 2006. Proc. Nat. Acad. Sci), pathogen exposure regimes (e. g. Dubovskiy et al. 2013 PLOS ONE) or temperature/diet regimes (Alton et al. 2024 Phil Trans Royal Soc B). We believe that by being consistent with our wording, we can still use the term line in our context.

Line 33: I don't understand why talking about “pre-adaptation” here, it seems outside the subject of the article. Moreover, even if the authors were not able to observe adaptation during their multigenerational experiment, nothing says that in natural conditions, populations are not able to respond, whether by phenotypic plasticity or longer-term adaptation. However, it is possible that I did not understand the meaning of the sentence, can the authors clarify this notion of pre-adaptation in this sentence?

RESPONSE: We agree that plasticity and long-term adaptation are still possible. We re-worded this section of the abstract for clarity.

Line 34: in keywords, “maternal effects” doesn't seem very appropriate. On the other hand, I would have added “evolution experimental” or “multigenerational experiment”.

RESPONSE: Thank you for your suggestion. We removed “maternal effects” and included “experimental evolution” in the keywords, as suggested.

Line 47 – 49: I find the wording of the sentence a bit "utilitarian", where only the money a species brings in can decide whether or not to keep it. I would modify the sentence as follows:

Before modification:

“The economic value of dung beetles in cattle pastures is calculated in up to \$423 USD per cow and, therefore, their conservation is urgent to maintain their ecosystem services”

After modification:

“In addition, the economic value of dung beetles in cattle pastures is calculated in up to \$423 USD per cow and, therefore their conservation is urgent to preserve these ecosystems and associated services”

RESPONSE: We appreciate your suggestion, and it was considered in the new version.

Line 55 – 56: clarify what the “reproductive disadvantages” are.

RESPONSE: DONE. We meant reduced body size and sexual traits.

Line 71 – 74: the explanation on why “ivermectin resistance seems to take longer and be less effective than resistance to insecticides” is not clear, it seems to me necessary to clarify the sentence, perhaps by providing examples.

RESPONSE: We agree and provide an example to clarify the sentence in the new version.

In the experimental lines and the toxicity experiment part, the number of couples (replicates) maintained during all the experiments lacks clarity. Ideally, without mortality, there were 20 couples (replicates) in each generation and each condition?

RESPONSE: Thanks for pointing out the lack of clarity. In the new version, we indicate that the number of replicates depended on the number of emerged beetles and the timing of emergence.

In view of the significant mortality problems, is it possible that a strong genetic drift could have prevented or slowed down the demonstration of an adaptive response (improved resistance)? This aspect could have been addressed in the discussion.

RESPONSE: We agree that high mortality in advanced generations could be due to genetic drift and acknowledge this in the new version of the discussion.

Line 95 – 100: given its rapid expansion, I was wondering whether *Euoniticellus intermedius* was considered an invasive species or not? Why not study a local species instead?

RESPONSE: Despite being exotic, there is no evidence showing that *E. intermedius* is invasive in Mexico (Del Val et al. 2017 *FoI Entomol Mex*; González-Tokman et al. in press. *Entomol Exp et Appl*). Unlike other dung beetles (*Scarabaeinae*), which have been difficult to breed in the laboratory, *E. intermedius* is easily grown in the laboratory, is highly fecund and has relatively short generation time, making it an ideal system to study the effects of agrochemicals in dung beetles. We added this information in the new version of the manuscript.

Line 108: it would be interesting to specify the generation time of *Euoniticellus intermedius*.

RESPONSE: We agree. We added this information in the new version.

Line 118: were the male-female crosses totally random?

RESPONSE: Crosses of newly emerged beetles were random, although certainly some individuals emerge earlier and were not mated with individuals that emerged several days later.

Line 121: I don't get the impression that this part is about “toxicity experiments”, I think that can be removed from the title.

RESPONSE: Thank you for your observation. We agree and removed this from the title.

Line 146: it would be interesting to add if possible the cause of the high mortality of the control line in F13?

RESPONSE: Although we do not know for sure, we recognize that mortality might be caused by genetic drift, as we acknowledge in the new version of the discussion section.

Line 164 – 165: it could have been interesting to add and discuss the results on the change in sex ratio mentioned in line 164 – 165.

RESPONSE: Changes in sex ratio were not analyzed in this study. We removed some lines in the methods that generated the confusion that we measured this variable.

Line 168 – 169: if this experiment was not considered in the analyses, it is perhaps preferable to delete this sentence: “As an additional experiment, five couples emerged from IVM62 in F18 were exposed to the same ivermectin concentration (62 µg of ivermectin per kg of fresh dung), but not a single individual emerged in the new generation, which was not considered for statistical analyses.”

RESPONSE: Despite this additional experiment was not analyzed, we believe it is worth reporting results with this additional generation.

Line 173 – 174: it may be interesting to better detail the method for estimating heritability and to show the regression curves.

RESPONSE: We added more details in the methods section. The Table S1 now included contains the regression estimates for each trait.

Overall this part seems good to me, I was just wondering if some insertion was not in the order of discussion. For example, “surprisingly” (line 238), “giving a potential indication of resistance” (line 239 240), “ivermectin resistance ratios (RR) indicated lack of resistance and only small tolerance to the contaminant in generation F3” (line 241 – 242) or “indicating some tolerance” (line 251).

RESPONSE: As we define resistance and tolerance based on the values of the resistance ratios, we believe that these are descriptions of the results, so we made no changes in this section.

Line 266 – 267: there appears to be an amplification of adverse effects of ivermectin on traits measured. These results were not discussed.

RESPONSE: Thank you for your observation. We included discussion of these results in the new version of the manuscript.

Reviewed by anonymous reviewer, 05 Nov 2023 14:54

The work carried out by Daniel González-Tokman and colleagues seeks to detect the possible occurrence of resistance to ivermectin in a line of *Euoniticellus intermedius* fed with droppings containing the famous antiparasitic. The experiment was conducted over 18 generations (+1 with a complementary test). Several biological parameters are taken into account, and the line is compared with a parallel-bred line fed without ivermectin. The choice of *E. intermedius* is an interesting one, as it is a species that seems to show great ecological plasticity. It is an abundant and frequent species in pastoral systems in Africa, America and Australia (it has been introduced in the latter 2 areas). Maintaining a breeding program over such a large number of generations is not without its risks, and the authors have encountered a few difficulties. But these appear to be minor and do not detract from the study. Consequently it seems to be a nice work, well designed and well described. I must point out that I am not fully competent to judge the relevance of the analyses carried out. In any case, they seem appropriate to me. The main result is the non-appearance of resistance. Given the potential capacities of this generalist species and the rearing conditions (systematic feeding with IVM), it is reasonable to assume that the absence of resistance is representative of the process at work within dung beetles under real-life conditions. This result is of main importance. I think this study deserves to be published. I have no conceptual or methodological criticisms to make.

RESPONSE: We appreciate your positive view of our manuscript and your enriching comments.

I have only a few minor remarks:

a) in Materials and Methods:

- line 172: you should precise "Figure 3a".

RESPONSE: We agree and changed it, as suggested.

- line 218: it is written "were significant for most analyzed variables" but in Table 1 ALL the results are in bold (even with $P=0.051$). Either you consider $P=0.051$ is significant and change your text in "were significant for all analyzed variables" or you apply a strict interpretation of $P=0.05$ threshold and, in the Table 1, this result must be unbold.

RESPONSE: Thank you for noticing this. We unbolded the $P=0.051$.

b) in results :

- table S1 (announced line 259) is lacking.

RESPONSE: Sorry about that. We include table S1 in the new version.

- the titles of tables 1 to 3 may be improved : they are too similar (for example Table 3 presents the results for 5 generations among 18, it could be precised) and I think that it is not correct to write "18 generations of exposure" beacuse the line "control" is out of IVM exposure. Same remark for the legend of figures 2 and 3.

RESPONSE: We agree. We precised table and figure legends where suggested.

c) references :

- Messina (1993), Price & Schluter (1991), Ritz et al. (2016) and Young et al. (2003) do not figure in the text.

- conversely: Hlina 2020 is cited line 199-200 but is not listed in the references.

RESPONSE: Sorry about these mistakes, which were corrected in the new version.

Reviewed by Marcel Amichot, 10 Nov 2023 15:38

Dear Authors, I spent a lot of time making sure I understood everything and, in fact, I didn't understand everything. I have two major concerns about this manuscript, the first one is the selection protocol for resistance and the second one is the concept of insecticide resistance as used by the Authors.

RESPONSE: Thank you for your comments on our manuscript. Below we provide answers point by point.

First the the selection protocole.

-Why use such a low dose when it is known that ivermectin concentrations in dung are higher in livestock facilities ? In addition, when resistance to insecticide needs to be selected for, the selection pressure (i.e. the dose/concentration of insecticide) applied to the insects must be gradually increased throughout the generations.

RESPONSE: The used ivermectin dose is considered moderate, as previous evidence in our studied species shows that it can reduce adult emergence by up to 50% (Baena et al. 2018). We considered very risky to use a higher dose for our experiment, as higher mortalities would have not allowed to give rise to the next generations. In the new version of the manuscript, we try to make clear that our experiments represent realistic conditions of cattle pastures. We agree that increasing ivermectin concentrations throughout generations would have increased the selection pressure and would have been a good protocol to evaluate selection in response to ivermectin. However, given that our experiment was performed with a low and constant ivermectin dose, the present study is an evaluation of transgenerational effects of ivermectin in concentrations similar to those found in treated farms; we therefore cannot discard that adaptation could have occurred with higher concentrations or by selecting the fittest families.

-Secondly, it is not clear whether the treatments were applied to pairs (20?) placed in separate containers or whether the pairs were all placed in a single container. In the latter condition, it seems difficult to be sure of avoiding inbreeding.

RESPONSE: Thank you for this observation. In the new version we clarify that, starting in the F1, beetles were maintained in randomly formed pairs of a male and a female in 1L plastic containers.

-Third, at lines 140-141, the Authors state " In generations F6 and F11-F17 we were not able to register emerged beetles in the IVM lines ". So my question : if you have no progeny, how were you able to pursue the selection process ?

RESPONSE: Despite we did not register the number of brood masses, emerged beetles, development time, etc., we had progeny, and it was used to form the next generation. We clarified this in the new version.

Second, the resistance to insecticides

All along the manuscript, the Authors, from my point of view, mix the insecticide resistance concept with the effects that ivermectin may have on life traits as fecundity for instance. In brackets, I mention the definition of insecticide resistance as proposed by the IRAC (irac-online.org) " When insect population can no longer be controlled by a dose of insecticide which used to provide control of c insects must be able to pass on the ability to resist the insecticide to their offspring". In the text of the manuscript, the Authors refer to insecticide resistance although they describe effects on larvae or adult emergence from brood balls or developmental time. I would like the Authors to be more selective in their wording.

RESPONSE: We agree that the term resistance in some sections of the results and discussion could be misleading, so they were carefully corrected in the new version. We interpreted resistance based on resistance ratios, as suggested by Byford et al. (1999): LC50 in the IVM line / LC50 in the Control line. Therefore, resistance only refers to the lethal effects of ivermectin and is not related to other variables such as developmental time.

Additional comments

-The difference between figure 2 and figure 3 is difficult to address. Does the figure 2 present the result of tests done with the parents submitted to 10µg of ivermectine/kg of dung and does the figure 3 present the results of tests done with the progeny submitted to other ivermectine concentrations (10 ; 31 or 62 µg/kg) ? Please clarify.

-There is a wide variability for the number/proportion of emerged insects in figure 3 as compared to figure 2, please discuss that point.

RESPONSE: As you noticed, figure 2 shows the result of tests done with the parents submitted to 10µg of ivermectin/kg of dung and figure 3 presents the results of 'toxicity experiments', done with the progeny exposed to different ivermectin concentrations in both experimental lines. We refer to toxicity experiments in figure 3 legend to avoid confusion. As figure 2 and figure 3 illustrate different generations, it is expected that some differences in reproduction and development time are observed. We believe that such differences fall in a natural range and find no argument to expect the same results in figures 2 and 3. This also applies, for example, to generations F1, F2 and F3 in the Control line, which somehow differ in their reproductive output and development times.

-The Authors collected insects and dung from two different ranches in which ivermectin is not used. What about other antiparasitic, antibiotic or other drugs ? And why two ranches ?

RESPONSE: We work in two ranches for logistic reasons, as we needed a constant supply of dung for more than a year. The ranches needed to be easily accessible from our laboratory. In these sites, *E. intermedius* beetles are not available (given the high altitude), so we decided to collect them in another ranch that also does not use ivermectin. Regarding the presence of other contaminants, we cannot discard that other contaminants are present (i. e. herbicides, antibiotics, other antiparasitics) at some time of the year, but we were not aware of any other chemical application. Cattle ranches in Mexico are almost never free of contamination (see, for example, Villada-Bedoya, et al. 2019. Dung beetle body condition: a tool for disturbance evaluation in contaminated pastures. *Environmental Toxicology and Chemistry*, 38: 2392-2404.), therefore, we chose the ranches mainly based on ivermectin use and location.

Conclusion

In fact, this manuscript provides interesting results as far as I understand them in the current version of the manuscript: the reproductive capacities or development times of control and treated beetles are not so different in several situations. Having said that, the interest of the results also depends on the condition that the dose of ivermectin used here, 10 µg/kg of excreta, can be realistic under certain environmental conditions. I suggest that the authors completely reconsider the structure of the manuscript and build it around these physiological characteristics.

RESPONSE: We appreciate the proposed suggestions. In our study the, the used dose resembles the concentration of ivermectin found in dung of a treated cow four weeks before, representing a realistic condition in cattle pastures around the world, as we specify in the new version of the manuscript (citing Wohde et al. 2016). We have considered all the reviewers' comments, which helped to substantially improve the new version of the manuscript. We think that the experiment

is now clearly explained and that the manuscript addresses the relevance of the results in the context of contamination of cattle pastures.