

Dear Dr Niels Verhulst,

Thank you for reviewing our manuscript. We greatly appreciate the feedback from the reviewers. We have improved our manuscript based on their recommendations, and we hope that you will find the current version to be acceptable for publication. We have replied to the comments below.

We look forward to hearing from you.

Sincerely,

Reviewer 1: *Amendments for the revised version appear in blue in the revised version of the manuscript.*

The manuscript by Poda and collaborators focuses on a long-debated topic: the existence of communication signals potentially mediating mating in *Anopheles gambiae* and *An. coluzzii* mosquitoes. The manuscript is mostly focused on replicating the study by Mozuraitis and collaborators and extending this with additional experimental approaches. Based on their results, male mosquitoes of the two species would lack volatile signals promoting female attraction, contradicting the previous report. Therefore, authors have shown that the results and conclusions by Mozuraitis and collaborators should be considered as potentially flawed and require further replication by third parties.

We wish to inform you that we performed our behavioral, chemical and electrophysiological experiments first. Meanwhile, the work by Mozuraitis et al was published. Because their results were very different from ours, we replicated their work to be sure we had not missed anything.

Comment #1: I suggest the authors have the English language style checked in order to avoid some slight distractions caused by small issues here and there.

Done.

Specific comments follow below:

Introduction

Comment #2: Authors should avoid dedicating such a long part of this section to point out what they perceive as flaws of a single previous study covering mating signals used during swarming in the same mosquitoes. Instead, they should give more attention to describing their own work with better clarity in the last paragraph, as usually done when closing the introductory text. Please keep most of your perspectives on the methodological flaws of Mozuraitis and collabs. for the discussion section.

The two paragraphs pointing out to the methodological flaws of Mozuraitis et al were summarized and moved to the discussion section (now lines 533-553) and we described our work in greater detail in the last paragraph (now lines 113-120).

Material and Methods

Comment #3: According to the authors “Colonies originated from wild gravid females collected in inhabited human dwellings from Burkina Faso, in 2017 in Bama for *An. coluzzii* and in 2015 in Soumouso for *An. gambiae*.” but they were “repeatedly refreshed with F1 from mosquito females caught in the wild”. What does “repeatedly” mean? Once a year? It would be desirable to offer readers a clearer definition.

We refreshed the colonies twice a year. This is now specified line 136 in the new version of the manuscript.

Comment #4: Line 370: please be specific and avoid ambiguous expressions such as “At “mosquito sunset time””.

Done. We replaced “mosquito sunset time” by “mating time” (line 352 in the revised version of the manuscript).

Comment #5: Line 371, the phrase “Males were checked for erected antennae, ensuring that change in light and dark rhythm, transport and electrophysiology laboratory conditions did not alter

their physiological state for mating.“ needs edition. Firstly, instead of “light and dark rhythm” use the more adequate “light-dark cycles”. Secondly, erected antennae should not be referred to as being equivalent to “physiological state for mating” as this is a mere proxy of what the authors look for that may indicate a motivation for mating, but would not grant unaltered mating physiology. For instance, you can state that this was used as a proxy for mating motivation without referring to whether the physiological state for mating was altered or not in mosquitoes.

We agree with the reviewer. The sentence was reworded as suggested (now lines 353-354).

Results

Comment #6: This section can be improved by making it more direct. There are many instances in which lack or presence of a compound in a particular sample are referred to, making the reader lose a clear path. As this reviewer understood, the results of the different experiments trying to sample male swarm-associated VOCs have shown that no significant chemical pattern could be established, suggesting that no VOCs are emitted by male swarms in these two species of *Anopheles*. Results should be better organized, so that the above conclusion could be stated at the beginning of the discussion, avoiding unnecessary details.

This section was reworded as suggested. Some details were removed from the manuscript (lines 457-461, 466-473, 483-485 in the revised version of the manuscript).

Discussion

Comment #7: Line 525: this phrase needs to be edited to make it straightforward: females were not attracted to air currents passed over male swarms.

The sentence was reworded as suggested (lines 500-501 in the revised version of the manuscript).

Comment #8: Line 538: authors should tone this phrase down as negative EAD traces are by no means definitive proof of lack of olfactory sensory neurons (OSNs) in antennal sensilla. It should be noted that few OSNs may not be detectable in the complex signal recorded in EADs, but could

very efficiently inform the brain of the presence of a certain ligand. As always in science, lack of response should not be strictly taken as a lack of signal, but suggested.

The sentence was reworded as suggested. It now reads "This suggests a lack of response by females to any volatile chemicals present in our swarm extracts" (lines 513-514 in the revised version of the manuscript).

Comment #9: Line 574: I suggest toning down this phrase, as the authors themselves have said in the previous paragraph that the absence of presence does not prove a lack of male swarm pheromones.

Indeed! The sentence was changed as follows: "...our findings are of utmost importance. They suggest ..." (lines 571-572 in the revised version of the manuscript).

Reviewer 2: *Amendments for the revised version appear in red in the revised version of the manuscript.*

Main comments

I read the manuscript with great interest and I like the questions the authors addressed. I particularly like the olfactometer assay in which responses of female mosquitoes to swarms of males were tested. The authors investigated responses of two species of mosquitoes. I was impressed by the amount of effort placed in using different methodologies for volatile collection emitted by the male swarms. The overall goal of the study was to investigate if female mosquitoes respond to odours emitted by male swarms that would in this context be considered a sex pheromone(s).

Although I very much like the questions addressed and the olfactometer bioassay, I think some of the methodological details need to be clarified so we can better judge the quality of the research. Some of the conclusions are not expressed in the most accurate way in my view, and the manuscript needs to be largely improved prior to publication. The manuscript can benefit from a more streamlined introduction and the structure of M&M section needs to be largely improved. The

authors selectively analysed a few volatile compounds, and in this way the conclusions are limited. A more extensive analyses of the data I assume the authors have, and in combination with different statistical analyses could be of additional value for the manuscript, when details on the methods are clarified. I hope the authors will find my comments useful when reviewing their work.

We thank the reviewer for these general comments. We answered to all of them point by point in the detailed comments below.

Detailed comments

Introduction

Comment #1: Line 37-46: I would add all refs at the end of the sentence to facilitate the reading.

Done as suggested (lines 40-44 in the revised version of the manuscript).

Comment #2: Line 55-57: What do the authors mean with visual markers, are these land markers in this context?

Yes, these ground markers can be anything that visually contrast with the surroundings. "Visual markers" has been changed to "ground markers" (lines 56 and 61 in the revised version of the manuscript).

Comment #3: Line 60-71: this passage refers several times to markers, these markers, same markers. Can the authors please specify markers and the context? See earlier suggestion.

Done (see comment #2).

Comment #4: Line 72: Are there sex pheromones that are not volatile?

Yes. For example, some cuticular hydrocarbons are not volatile and can be used as contact sex pheromones at close range during courtship.

Comment #5: Lines 106-119: This paragraph could be significantly reduced and perhaps combined with the next one. The point here should be the connection of flight activity with the odour's cues, and nectar with odour cues. the text moves far away from the topic of the study.

These two paragraphs were removed from the introduction and moved to the discussion as suggested by Reviewer 1 (comment #2).

Comment #6: Line 118-119: This is a confusing reference to 5 compounds that I assume are not the same 5 compounds the authors refer to in line 89.

These were the same compounds. This is now clarified and references were added. This section was moved to the discussion as suggested by Reviewer 1 (comment #2) (lines 533-553 in the new version of the manuscript).

Comment #7: Line 120: see previous, best to complement sentence by add such as these and these compounds.

Done.

Comment #8: Line 130:” Bring to light the existence” should read “to investigate”.

Done (now line 109).

M&M

Comment #9: This section could be largely improved by using more specific heading as well as headings and subheadings.

We provided a more specific heading for the "Behavioral assays" section and subheadings for the "Bioassays" and "dynamic headspace in laboratory" sections (please see comments #13, 27).

Comment #10: Line 153: Please specify how often: yearly, X times throughout the season...

Done (now line 136 in the revised version of the manuscript). Also see comment #3 by Reviewer 1.

Comment #11: Line 164-166: Not clear why this information is relevant for this paper.

We performed several experiments using different colonies of mosquitoes. It is therefore relevant to specify for each experiment, what colony we used, where it is from and under what conditions it was reared. Moreover, most of our experiments were carried out with recently colonized mosquitoes, except for the replication of Mozuraitis' experiment for which we used an "old" laboratory colony that had not been refreshed in a long time. This is important because Mozuraitis also used similar laboratory colonies.

Comment #12: Lines 170-175: It would be great if dimensions could be added directly into the figure.

Indeed, it would make the text easier to read but on the other hand, it would overload the figure with information. Consequently, we decided to leave the figure and the main text as is.

Comment #13: Line 187: Bioassay is not sufficient. I suggest a general heading first referring to the objective of the experiment and then subheading on the set up, bioassay...

Done (lines 151, 172, 179, 203, in the revised version of the manuscript).

Comment #14: Line 188-191: How many replicates in total?

We did 12 replicates for each combination. This was specified in the previous version of the manuscript line 223 (now lines 206 and 435 in the "Test combinations" and "Results" sections, respectively, in the new version of the manuscript). The sentence was rewritten to improve clarity.

Comment #15: Lines 194-195. Glucose is not volatile thus there would be no odour bias. It is good that the authors add the glucose cup to the empty box as well so that humidity is similar in both boxes. I suggest to refer to these boxes as swarming boxes.

We thank the reviewer for this comment. As suggested, we specified the "humidity bias" but we also kept the "odor bias" with additional precisions. Indeed, glucose pads and the cup in which they were placed may contain volatile compounds due to manipulation. Moreover, the glucose may

contain bacteria, which can emit volatile compounds. This is now specified in the new version of the manuscript lines 177-178.

Comment #16: Line 197-201: This moving outside is not clear. Is that for natural light? So why there is need for additional artificial light?

Indeed, one of the reasons for moving the swarming boxes outside at sunset was to provide the mosquitoes with natural sunset light for swarming. It was also to avoid having the odor source in the same room as the olfactometer (females' side), and to avoid potential bias due to sounds emitted by the swarm (at that time, we did not know that males were unable to hear females at long range (see Feugère et al., 2021). However, the place where the boxes were setup was not appropriate for swarming. Indeed, it was too close to a wall, mosquitoes were not directly exposed to sunset light and no twilight horizon was visible from this place. Consequently, mosquitoes tended to fly randomly in the swarming boxes to try to escape and bounced on the ceiling of the boxes. To obtain consistent swarms, we took advantage of our previous work (Poda et al., 2019) and provided mosquitoes with the necessary stimuli to swarm within the boxes.

References:

*Feugère, L., Gibson, G., Manoukis, N. C., & Roux, O. (2021). Mosquito sound communication: are male swarms loud enough to attract females? *Journal of Royal Society Interface*, 18, 20210121. <https://doi.org/10.1098/rsif.2021.0121>*

*Poda, S. B., Nignan, C., Gnankiné, O., Dabiré, R. K., Diabaté, A., & Roux, O. (2019). Sex aggregation and species segregation cues in swarming mosquitoes: role of ground visual markers. *Parasites and Vectors*, 12(1), 589. <https://doi.org/10.1186/s13071-019-3845-5>*

Comment #17: Line 204: Previously to what?

Previously to the release of females into the olfactometer. We changed the structure of this section to fit better with the chronology and to make it easier to read (now lines 185-186).

Comment #18: Line 213: How many females were released at a time? Any evidences that females follow each other?

About 200 females were released at the same time (about 100 females of each species). This is now specified in the revised version of the manuscript (lines 192-193, 435).

To avoid impacting the females' choice, we did not stay in the room with the olfactometer and we did not observe the females' behavior. We are consequently unable to answer this question with certainty. However, as females did not make any choice between odor sources (50% of activated females in each arm), our guess is that females were not following each other and only responded randomly to air current.

Comment #19: Line 223: 100 females per replicate? The replication info is a bit too spread through the text. Hard to follow.

We improved the clarity of the text (please see lines 192-193, 206, 435, in the new version of the manuscript, and comments #14, 18 and 34).

Comment #20: Line 227: I guess the authors mean positional bias with “side effects”.

The sentence was rewritten (lines 209-211 in the new version of the manuscript).

Comment #21: Line 233: Not clear whether Burkina Faso is a lab, semi-field or field location/condition.

"Burkina Faso" was deleted from this section and additional information were provided in the mosquito section (lines 124-126 in the revised version of the manuscript).

Comment #22: Line 238-243: Not clear from where the air originated from. Did the authors used a cylinder with synthetic air, compressed air line or was air pumped air from the environment? It sounds like air was pumped from the environment, and this could explain why the authors do not see differences between treatments and controls. I don't think a charcoal filter is sufficient to eliminate compounds that are often present in the environment as are the compounds of interest here.

Air was pumped from the environment (ambient air from the room). This was clarified in the revised version of the manuscript (lines 228-229).

Charcoal filters are the most frequently used setup to "purify" air used in dynamic headspace VOC collection. This adsorbent has of course a limited efficiency, as does any other method, and is particularly useless for very small polar compounds such as formic acid, acetic acid or ethanol. It is, however, suitable to trap compounds of low to medium polarity and intermediate size to volatile compounds (Millar, 2002). In our case, the lack of both qualitative and quantitative differences in the VOCs contained in treatments and controls cannot be explained by this limitation. Mosquito volatile sex pheromones (if they exist) are not supposed to be present everywhere in nature and in quantities similar to those found in swarms.

Reference:

Millar, J. G. (2002). Sampling and sample preparation for pheromone analysis. In J. Pawliszyn (Ed.), Sampling and Sample Preparation for Field and Laboratory (Vol. 37, pp. 669–697). Comprehensive Analytical Chemistry. [https://doi.org/10.1016/S0166-526X\(02\)80057-5](https://doi.org/10.1016/S0166-526X(02)80057-5)

Comment #23: Table 1: From my experience with headspace collection the flow rate used is a too high. Did the authors do a breakthrough experiment, like placing a second tube just behind the tube used for collection to check if volatiles of interest a trapped in the second tube?

Indeed, two of our flow rates were relatively high and may have caused some losses that were not tested. We chose these flow rates as a trade-off between the large volume to be sampled and the shortest collection time as possible to avoid the dilution effect and pollution. However, these flow rates are not unusual and are frequently found in literature for both plant and insect VOC collection without any particular drawbacks (for examples, please see Kigathi et al., 2009; Kunert et al., 2005).

References:

*Kigathi, R. N., Unsicker, S. B., Reichelt, M., Kesselmeier, J., Gershenzon, J., & Weisser, W. W. (2009). Emission of volatile organic compounds after herbivory from *Trifolium pratense* (L.) under laboratory and field conditions. *Journal of Chemical Ecology*, 35(11), 1335–1348. <https://doi.org/10.1007/s10886-009-9716-3>*

Kunert, G., Otto, S., Röse, U. S. R., Gershenzon, J., & Weisser, W. W. (2005). Alarm pheromone mediates production of winged dispersal morphs in aphids. Ecology Letters, 8(6), 596–603. <https://doi.org/10.1111/j.1461-0248.2005.00754.x>

Comment #24: Line 253: It would be clearer for the general reader if the authors refer to the olfactometer swarming box.

The box used to extract VOCs was different from the swarming boxes of the olfactometer. These were two different setups especially designed for two different experiments. The extraction box was a 50 × 50 × 50 cm plexiglass box placed in a room during extraction (see lines 222-223, 238-239 in the new version of the manuscript), while the swarming boxes of the olfactometer were 60 × 60 × 120 cm plexiglass boxes (see lines 154-156 and 180 in the new version of the manuscript). To avoid confusion, we now call the box used for VOC extraction "extraction box". This is specified in the "Dynamic Headspace – extraction setup" section lines 222-223 and 238-239 in the new version of the manuscript.

Comment #25: Line 254 vs. table 1: Please be consequent with unit format.

We changed the unit format in Table 1; line 854 in the revised version of the manuscript.

Comment #26: Line 264: Carefully conditioned has no meaning for the general reader. Please, describe the procedure.

This section was rewritten (now lines 249-252 in the revised version of the manuscript).

Comment #27: Line 268: The entire section is about VOC collection - from line 231 through line 319. I suggest making subheadings more specific.

Done (see lines 221 and 238 in the revised version of the manuscript). See also comment #9.

Comment #28: Line 298-309: This passage could be reduced if the authors simply describe treatments and controls.

Done (now lines 285-295 in the revised version of the manuscript).

Comment #29: Line 312: Why is this necessary? Is the cleaning procedure not efficient?

The cleaning procedure was checked several times and was good enough to ensure clean fibers. We took this precaution to avoid side effects and uncontrolled variability due to minor differences in fiber sensitivity and to produce consistent extracts across replicates. Consequently, the variance observed between replicates for a given treatment can only be attributable to the sample and not to the method (fiber).

Comment #30: Lines 322-323: This should be the last sentence of the paragraph as it is not relevant for the general understanding of the procedure.

Done (lines 310-312 in the revised version of the manuscript).

Comment #31: Lines 358-363 and Lines 407-425: Everything related to identification should be combined in one section.

Done (please see lines 389-394 in the revised version of the manuscript).

Comment #32: Line 406: Would identification of volatile organic compounds be a better heading?

We changed the heading to "Identification of specific and active VOCs" instead (line 388 in the new version of the manuscript). We specified "specific and active" because we did not attempt to identify all the compounds present in the extracts and, consequently, "identification" alone could be confusing for the reader. Moreover, it would have been a waste of time as most of the chromatograms were very similar. Instead, we adapted our strategy to identify only compounds of interest. To that aim, we searched for qualitative and quantitative differences between controls and treatments. We revised this section and added a short description of our procedure to improve clarity (lines 389-394 in the new version of the manuscript).

Comment #33: Line 428: last sentence of the paragraph?

Done (lines 415-416 in the revised version of the manuscript).

Results

Comment #34: Line 451: How many per replicates and per species? This was already not clear with M&M section.

A concise sentence was added to summarize the nature of the replicates (lines 192-193 in the revised version of the manuscript). Please, also see comments #18 and #19.

Comment #35: Line 452: two decimals is overdone.

Done (lines 437 in the revised version of the manuscript).

Comment #36: Line 465-466: I think the authors mean that only quantitative differences were detected between controls and samples?

This paragraph was rephrased to improve clarity (now lines 449-454 in the new version of the manuscript).

Comment #37: Line 483-496: Was the air pumped from the environment and perhaps charcoal filter not sufficient to get it clean and clear controls?

Please see our answer to comment #22.

Comment #38: Results chemical analyses: It reads to me as if the authors selectively chose which compounds to quantify and identify. I think this approach limits the extent to which conclusions can be drawn. I expect the authors to have collected more VOCs in their blends. Even if not all compounds could be identified, I expect they could have been quantified. The authors also analysed VOCs individually. It would be interested to use some kind of multivariate analyses too, though the largest issue I see here is the presence of the compounds of interest in the controls.

Indeed! We did not find any qualitative differences between swarming mosquitoes and their respective controls. Only minor quantitative differences were found in some peaks and only compounds of interest were identified. Consequently, we think that it is not relevant to identify and to quantify the compounds present in samples when they did not show quantitative differences with

controls. They are undoubtedly not involved in mosquito chemical signaling. A deeper statistical analysis will not provide new results and would not be rational. Please see our answer to comment #32.

Discussion

Comment #39: Line 517: it should read ...evidence is available.

Done (line 492 in the revised version of the manuscript).

Comment #40: Line 531-533: Could it be that higher number of males was needed for females to detect the odour source in this set up?

We added this point to our experimental limitations (line 554-556 in the revised version of the manuscript).

Comment #41: Line 521: I think the conclusions here need to be more accurately stated. The authors found no evidence that the volatile organic compounds quantified in this study could be exploited by females as a sex pheromone. I expect the authors to have collected more VOCs in their blends than they have chosen to quantify. Even if not all compounds could be identified, I expect they could have been quantified. Multivariate approaches would also be to address differences in the blends.

Please see comment #32 and #38.

End of comments