nature portfolio

Peer Review File

Decoding 4-vinylanisole biosynthesis and pivotal enzymes in locusts

Corresponding Author: Professor Le Kang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
Guo et al have elucidated the biosynthetic pathway of the aggregation pheromone 4-vinylanisole (4VA) that plays a critical role in forming locust swarms and the resulting locust plagues. They identified the biosynthetic intermediates and the key enzymes controlling 4VA production. Two methyltransferases highly expressed in gregarious locusts are upregulated in response to increased population density and turn on and off 4VA synthesis. As a final twist, the authors identified 4-nitrophenol as a potent inhibitor of these methyltransferases and outline its potential use in the control of locust plagues.

This is an excellent study covering a multitude of aspects on the phenomenon and making use of appropriate and state-of-the-art techniques. The manuscript is generally well written and the presentation and reasoning is clear. I am impressed with the high quality of the work.

My few comments are all minor:

Title: “the production of aggregation pheromone and behavioral swarming of locusts” should possibly be “in locusts”?

Line 105 does not read well to me. “wheat seedlings” refer to “host plant” whereas lignin, amino acid etc refer to compound classes. Consider rephrasing to something like “The main compound classes in the wheat seedlings used as host plants include lignin, amino acids….”

Line 112-113: Can you be positive about that? Is there no lignin at all present in the artificial diet used as control? Or maybe the additional lignin fed to the locusts was not available to the insects?

Line 119-120: “Feeding of host plants sprayed with….. “. Consider replacing with “Feeding the locusts with host plants sprayed with…”

Line 128-130: Line 78: I don´t believe in the value of presenting long lists of raw data and automated identifications of various compounds (Tables S1 and S2). I would prefer a proper identification of the key compounds used for construction of the two pathways outlined in Fig 2A. The literature is full of misidentified compounds and these flaws easily live their own life when people refer to them and not confirm that the chemical identities are correctly assigned in the original study.

Line 198: It would not hurt to mention in what heterologous system the two enzymes were expressed (so that we do not have to look up Methods at this point). Please add this info!

Lines 405-408: What about the potential hazards of spraying with 4-nitrophenol? 4-Nitrophenol irritates the eyes, skin, and respiratory tract. It may also cause inflammation of those parts. It has a delayed interaction with blood and forms methaemoglobin which is responsible for methemoglobinemia, potentially causing cyanosis, confusion, and unconsciousness. When ingested, it causes abdominal pain and vomiting. Prolonged contact with skin may cause allergic response. Genotoxicity and carcinogenicity of 4-nitrophenol are not known. (See for instance Wikipedia for references https://en.wikipedia.org/wiki/4-Nitrophenol). I think that it would make sense to briefly mention (with a reference) any potential health hazards associated with large-scale spraying with 4-nitrophenol as the authors refer to it as a “sustainable control strategy”./ Christer Löfstedt

Referee #2

(Remarks to the Author)
The migratory locust Locusta migratoria exists in two phases, a solitary and a gregarious one, of which the latter forms swarms and is a pest of many crops. In their manuscript “Inhibiting biosynthetic enzymes blocks the production of aggregation pheromone and behavioral swarming of locusts” Guo et al. investigate the biosynthetic pathway of the locust aggregation pheromone 4-vinylanisole (4VA), which has been shown to be relevant for phase shift and the formation of swarms in the migratory locust Locusta migratoria. By performing different feeding experiments and gene expression analyses the authors identify 4-vinylphenol as a precursor that by two methyl transferases (VPMT1 and 2) becomes transferred into 4VA. Inhibiting the transcription of those transferases results in the loss of 4VA in the locusts, which then results in gregarious locusts exhibiting a more solitary behavioral phenotype. In a next step the authors investigate the potential bounding properties of the transferases and the precursor 4VP and based on that design another molecule (4 nitrophenol, 4NP) that even binds better and blocks the methylation process. In a last experiments the authors show that feeding gregarious locusts with 4NP again results in some kind of phase shift, with the locusts after feeding exhibiting more solitary-like behaviors.

To my knowledge this is the first time, that a phase shift in locusts from the problematic gregarious form towards the less problematic solitary form could be artificially induced by spraying a chemical. This is of high interest, as 4NP potentially could present and alternative strategy to the use of pesticides.

The manucript is well written and the data, their presentation and statistical analyses are convincing (I should mention, that due to lacking expertise I cannot judge the data on binding properties presented in Fig. 5).

I feel that the conclusions are robust and reliable.

I have only one major comment: as the authors state, spraying of NP4 could due to its potential to induce phase shift become a useful alternative to the use of pesticides. However, it would be very helpful if the authors include some analyses on the potential negative side effects of 4NP: does the amount of 4NP that needs to be sprayed on the plants affect the plant health and survival. Does it do anything else to the locusts than inducing phase shift? What about survival rates of 4NP- fed animals as compared to control animals.

Minor points:
The authors should discuss, why feeding on plants supplemented with Phe-d5 but not on supplemented artificial diet yielded in increased rates of 4VA-d4 (Fig. 1G vs Fig. 1I). If Phenylalanin is a necessary precursor, supplying the artificial diet with it, should be effective also.
Figure legend 1”Different letters indicate statistically significant differences between groups using one-way ANOVA (Tukey’s multiple comparisons test, $P < 0.05$) (A). P values in chemical assays were determined by a two-tailed unpaired t-test (C–I). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are presented as mean ± SEM.” I guess, the ANOVA was used for figures A and C, while the t-test was used for figures D-I
Fig. 3 The picture of the solitary locust makes sense only, if a gregarious one is shown also.
Fig.3A I guess brown (green) dots present data from gregarious (solitary) locusts? Please add information. Why did you not perform statistics with data from Fig. 3A?
Fig. 3 H-M What is the reason for the dramatic difference in the release rates of the control groups between the figures H-M and within the figures (from 24h to 72h).
“The locusts injected with dsGFP is a control for all genes.“ this refers to figure 3H-M and should better appear there (not after the text of Fig. N-O)
Fig. 6B please specify the amount of 4NP that was used for the duration experiment.
6C please specify the amount of 4NP that was sprayed on plants (this is important to judge, whether the spaying of agricultural areas to reach a similar effect is feasible)
Fig. 6 D and onwards. D is not showing VA production as mentioned in legend, but shows schematic of feeding crowded solitary locusts (which in figure legend appears only later)

“…fed with an artificial diet containing deuterated tyrosine and phenylalanine” better “fed with an artificial diet containing deuterated tyrosine or phenylalanine…”

“These imply that the locusts synthesize 4VA using building…” better “These results imply that the locusts synthesize 4VA using building…”

Deuterated phenylalanin on artificial diet no effect. Deuterated phenylalanin on host plant results in deuterated 4VA. How comes the difference?
“KEGG” Please specify what this abbreviations stands for.

“The first is phenylalanine– phenylacetaldehyde–phenethylalcohol–tyrosol–4-vinylphenol–4VA. The second route is phenylalanine–cinnamic acid (CA)–p-hydroxycinnamic acid (p-HCA)–4-vinylphenol (4VP)–4VA (Fig. 2A, Extended Data Fig. 1B).” Difficult to understand as dashes are obviously used between consecutive compounds of the pathway but of course are also part of compound names.

“Injection of deuterated cinnamic acid did not induce the production of deuterated 4VA..” should better read “Also the injection of deuterated cinnamic acid…”
“we detected the contents of these four….” better “we looked for these four….” (as “detected” already anticipates the existence of these compounds)
“Injection of deuterated p-HCA and 4VP did not induce the production of deuterated 4VA in solitary locusts…” better “Also the injection of deuterated …..”
“The step from 4VP to 4VA may contribute to the distinct emissions…” better “It rather seams to be the step from 4VP to $VA that contributes to the distinct emissions….”
“Nine genes annotated methyltransferases…” better “Nine genes annotated as methyltransferases…”
“RNAi knockdown of LOCMI16699 and LOCMI02868 significantly…” better “When next performed RNAi experiments with the identified genes, the knockdown of LOCMI16699 and LOCMI02868 significantly….
“After behavioral assays,…” better “In behavioral assays,…” Please briefly describe in text, what assays you performed.
“imcrucialor” exchange by “crucial for”
“nitropheol” exchange by “nitrophenol”
“Attraction to 4VA of locusts may arise from the attempt for more survival and reproduction resources.” Sentence unclear to me. Consider re-wording
“methyltranfereases” replace by “methyltransferases”
“Assessing the environmental safety of small molecules is a significant public concern and must be addressed before their widespread adoption.” I fully agree. It would have been nice, if e.g longevity of animals (locust and best one or two other non-related insect species) fed with the bioactive concentrations of. 4-nitrophenol would have been included here to test of the compounds toxicity

Best regards
Markus Knaden

Referee #3

(Remarks to the Author)
The report by Guo et al. addresses two main issues: 1) the biosynthesis of an aggregation factor for swarming locusts. Earlier work had established that 4-vinylanisole (4VA) was a likely aggregation pheromone, but part of being a pheromone is being biosynthesized in the same species it affects – something that had not been conclusively shown in the original report, and 2) developing an aggregation inhibitor from knowledge of the biosynthetic pathway. In my analysis, the authors did a good job on the first issue and an unsatisfactory job on the second.

It's challenging to work out biosynthetic pathways with insects, especially ones with no genetic system snd multiple possible pathways. The authors established, at least to my satisfaction, that 4VA is biosynthesized from phenylalanine derived from plants, and that a crucial step in the biosynthesis is the conversion of 4VP to 4VA – the OH group of a phenol is converted to an ether OCH3 through addition of a methyl (CH3) group. This is a well-known reaction catalyzed by methyl transferases (MT). MTs play multiple roles in biological regulation including the modulation of human neurotransmitters. Basically, the authors identified two main MTs, 4VPMT1 and 4VPMT2 from 9 possibilities. They tried to reduce two MT possibilities to one, but they gave up and concluded that either or both could carry out the crucial reaction. Crucially, there were a few remaining possibilities that were not investigated in depth, or at least those efforts are not reported.

The development of an inhibitor that could be used to limit locust aggregation and in principle decrease crop predation formed the much more significant part of the report. Two points should be noted. The first is the discussion in the opening paragraph that pesticides are polluting the environment. The second is that pheromones and pheromone mimics are used only for monitoring, not controlling populations. This choice, monitoring not controlling is the result of environmental regulations and economics in the US. The authors use structure-based drug design and synthesis to identify 4-nitrophenol (4NP) as a “behavioral regulators for environment-friendly locust control [abstract].” There are several things wrong with this statement.

1) There is no evidence that 4NA is a behavioral regulator. There is evidence that 4NA is an attractant, but there is no evidence that it’s the only attractant, and lacking that there is no essential link to behavior. There is also evidence that 4NP inhibits the production of 4NA, which creates a possible link from 4NA to behavior, but not a definitively established one. In the language of drug discovery, the target has not been derisked.
2) There is no evidence that 4VPMT1 and 4VPMT2 are the only methyltransferases that can carry out the conversion. If some of the others can carry out the transformation, even at low levels, that could be enough to cause attraction. .
3) Finally, 4NP is not environment-friendly. It causes It causes severe reactions in people and is not remotely likely to meet environmental regulations. The existing evidence is enough to get it banned for wide scale environmental release.

In summary[, there is no conclusive evidence that 4NA is a behavioral regulator of aggregation, no demonstration that it is necessary and sufficient. Likely perhaps but far from proven. And there is conclusive evidence that 4NP is environment-unfriendly.
The statement on line 393: “4-Nitrophenol is a potent behavioral regulator with great potential application in locust control.” Is wishful thinking and far from rigorously established fact.

Referee #4

(Remarks to the Author)
The frequent and destructive locust plagues pose a severe threat to agriculture, economies, and ecosystems worldwide. For nearly a century, the field of locust chemical ecology has focused on understanding the chemical signals that influence locust behavior, particularly those driving aggregation behaviors that underpin swarm formation and lead to plagues. In 2020, Guo et al. identified the aggregation pheromone, 4-vinylanisole (4VA), and elucidated the biological mechanisms of locust aggregation behavior. In the current study, Guo et al. revealed the biosynthetic pathway of 4VA, further advancing our understanding of locust aggregation. The identification and characterization of two key methyltransferases, 4VPMT1 and 4VPMT2, involved in 4VA biosynthesis offer a mechanistic understanding of how these enzymes catalyze the conversion of 4-vinylphenol (4VP) to 4VA. This work significantly enhances our knowledge of the biochemical underpinnings of locust swarm behavior at the biosynthetic level. Most notably, the elucidation of the X-ray co-crystal structure of 4VPMT in complex with 4VP and S-adenosylmethionine (SAM) is a particularly impressive feat. Based on this structure, the researchers successfully screened and identified 4-nitrophenol as a substrate surrogate that inhibits the activity of 4VPMTs, thereby blocking 4VA production and suppressing locust aggregation. This is a groundbreaking discovery in locust chemical ecology, as it presents a novel approach to targeting locust swarms by disrupting pheromone biosynthesis rather than relying on chemical pesticides. The development of 4-nitrophenol as a small molecule inhibitor is particularly noteworthy. It represents a highly targeted and environmentally friendly method of locust control, with significant potential to serve as a viable alternative to traditional pesticides. This application-driven research makes the findings not only scientifically valuable but also practically significant. Overall, the experimental design is robust, and the results are both compelling and conclusive. The identification of 4VA biosynthetic enzymes and the discovery of an effective inhibitor are landmark achievements. However, before moving forward, I have a few concerns I would like the authors to address

comments
The identification of 4-nitrophenol as a small-molecule inhibitor of 4VPMT activity is an important finding. However, there are potential confounding factors that need to be addressed. While the study tests the effect of 4-nitrophenol on locust gregarious behavior, it is also possible that this compound inhibits other enzymes unrelated to 4VA biosynthesis, which could independently disrupt locust aggregation behavior. Additionally, 4-nitrophenol might interfere with locust detection of 4VA or could itself evoke specific behavioral responses in locusts. Have you tested whether 4-nitrophenol elicits any direct behavioral effects in locusts? It would be essential to exclude the possibility that the observed behavioral changes are due to effects other than the inhibition of 4VA biosynthesis. I recommend conducting behavioral assays to determine whether 4-nitrophenol directly affects locust behavior or sensory perception of 4VA. This would strengthen the conclusion that 4-nitrophenol's impact on locust behavior is specifically due to the inhibition of 4VPMT activity.

In the abstract, the authors claim that 4-nitrophenol suppresses locust aggregation. However, the behavioral tests presented in the manuscript do not sufficiently support this claim. The current experimental design appears more focused on assessing locust phase-shift behaviors rather than directly testing aggregation. To substantiate the conclusion that 4-nitrophenol impairs locust aggregation, it is essential to verify whether it affects the attraction between individuals within a population. I recommend using a two-choice behavioral assay, as in Guo et al. (2020, Nature), to test whether 4-nitrophenol disrupts the attraction between locusts. Specifically, VOCs should be collected from both control gregarious-phase locusts and 4-nitrophenol-fed locusts, and tested to see whether other locusts show a preference between these odor sources. If the results show that locusts preferentially orient toward VOCs from control gregarious-phase locusts over those from 4-nitrophenol-fed locusts, this would provide strong evidence that 4-nitrophenol suppresses locust aggregation. Implementing this additional behavioral test would greatly strengthen the authors' claim regarding 4-nitrophenol's role in inhibiting aggregation.

While the identification of 4-nitrophenol as an inhibitor of 4VPMTs is a promising result, I have some reservations regarding the screening process. The manuscript mentions screening fewer than 20 4VP analogues as substrate competitors, which seems limited when aiming to discover an effective inhibitor to block enzyme function. Given the availability of the co-crystal structure of 4VPMT in complex with 4VP and S-adenosylmethionine (SAM), it would be highly beneficial to employ a more comprehensive approach, such as AI-assisted docking and virtual screening, to identify potential inhibitors. AI-driven docking methods could allow for the exploration of a much larger chemical space, increasing the likelihood of identifying more potent and selective inhibitors of 4VPMTs.

The identification of 4-nitrophenol as a promising chemical for inhibiting locust swarming is indeed significant. However, I would like to raise a concern regarding the cost-effectiveness of this compound for practical applications. Since the potential use of 4-nitrophenol as a behavioral regulator will involve considerations of economic viability, it would be beneficial for the authors to discuss the cost implications of this compound in the context of its application in locust control. Additionally, this study has successfully identified key biosynthetic genes involved in 4VA production. In the future, these target genes could also be utilized for developing RNAi insecticides aimed at controlling locust swarming behaviors. I suggest that the authors expand the discussion section to include these considerations, addressing both the economic aspects of 4-nitrophenol and the potential for innovative genetic approaches in pest management.

The authors report that both the emission level of 4VA and the expression level of 4VPMT genes are highly elevated in the hind leg, suggesting this tissue's pivotal role in 4VA biosynthesis. However, upon examining Figure 4A, it is evident that while 4VPMT1 is most highly expressed in the hind leg, 4VPMT2 shows its highest expression in the midgut. This discrepancy raises questions about the distinct functional roles of these genes in 4VA biosynthesis and their contributions to locust aggregation. Interestingly, previous research has shown that gregarious locust feces can also release 4VA, suggesting that the midgut expression of 4VPMT2 may be linked to microbial interactions or processes related to digestion. Could the authors elaborate on the functional differences between 4VPMT1 and 4VPMT2, particularly in the context of their distinct tissue-specific expression patterns? Additionally, how might the midgut expression of 4VPMT2, potentially

influenced by the microbiome, contribute to pheromone biosynthesis or locust behavior?

Following up on the concerns regarding the expression of 4VPMT2 in the midgut and the role of locust feces in releasing 4VA, I was curious about the method used to collect volatile compounds from locusts. When locusts are confined to a small jar or vial for odor collection, they may excrete feces during this process, and as mentioned earlier, feces can release 4VA. How did the authors control for or exclude the potential influence of feces on the volatile collection and quantification of 4VA?

The authors demonstrate that knocking down or inhibiting the activity of 4VPMTs significantly reduces the release of 4VA. However, I am curious about the impact of these manipulations on the overall profile of volatile organic compounds (VOCs) emitted from the locusts. Could the authors provide gas chromatograms showing the composition of volatiles emitted from locusts under different conditions: with and without 4VPMT expression, and with and without 4VPMT inhibition? This data would help clarify whether other VOCs are produced when 4VA is suppressed. Furthermore, if any additional compounds emerge in the VOC profile upon 4VPMT manipulation, it is essential to assess their behavioral effects on locusts. This would help exclude the possibility that these compounds influence locust behavior independently of 4VA.

In reviewing the manuscript, I noticed that the number of replicates in certain experiments appears to be quite low, which raises concerns about the robustness of the statistical analyses. For example, in Figure 1C, Figures 4E-F and Fig5, the limited number of replicates may not provide sufficient power for reliable statistical conclusions. Could the authors explain the rationale behind the choice of replication in these specific experiments?

In Figure 1C, the manuscript mentions that locusts were fed artificial food. However, it is not clear what the specific composition of this artificial feed was. Could the authors clarify the ingredients used in the artificial feed? Specifically, was the precursor compound for 4VA excluded from this feed? Given the importance of understanding the biosynthetic pathway of 4VA, it would be critical to know whether the diet provided to the locusts contained any compounds that could influence 4VA production.

I recommend that the authors consider revising the title to more accurately reflect the key findings of the study. A title that captures both the biochemical and behavioral aspects of the research would be beneficial. For reference, the study “Next Generation Neuropeptide Y Receptor Small Molecule Agonists Inhibit Mosquito Biting Behavior” provides a clear and informative structure that effectively conveys the focus of the work.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
Thius is a revised version of a manuscript previously reviewed by me. My comments on the original submission were all relatively minor and I think that the authors have responded properly and made changes where necessary. The revision is fully satisfactory to me.

Referee #2

(Remarks to the Author)
In my opinion the authors treated all suggestions from the different reviewers carefully and by performing additional experiments further increased the significance of the manuscript. I congratulate the authors to this beautiful story!
Markus Knaden
Summary of the key results
The migratory locust Locusta migratoria exists in two phases, a solitary and a gregarious one, of which the latter forms swarms and is a pest of many crops. In their manuscript “Inhibiting biosynthetic enzymes blocks the production of aggregation pheromone and behavioral swarming of locusts” Guo et al. investigate the biosynthetic pathway of the locust aggregation pheromone 4-vinylanisole (4VA), which has been shown to be relevant for phase shift and the formation of swarms in the migratory locust Locusta migratoria. By performing different feeding experiments and gene expression analyses the authors identify 4-vinylphenol as a precursor that by two methyl transferases (VPMT1 and 2) becomes transferred into 4VA. Inhibiting the transcription of those transferases results in the loss of 4VA in the locusts, which then results in gregarious locusts exhibiting a more solitary behavioral phenotype. In a next step the authors investigate the potential bounding properties of the transferases and the precursor 4VP and based on that design another molecule (4 nitrophenol, 4NP) that even binds better and blocks the methylation process. In a last experiments the authors show that feeding gregarious locusts with 4NP again results in some kind of phase shift, with the locusts after feeding exhibiting more solitary-like behaviors.In the revised version the authors now added an investigation regarding the potential risks of the application of 4VP. Despite being a rather harmful substance, they do not find any negative effects on plants, locusts, and bumble bees when applying the compound in those low concentrations that are needed to inhibit the 4VA pathway.

Originality and significance: if not novel, please include reference
The findings are novel and (due to the pest status of locusts and the potential solution to inhibit their harmful swarming

phase) of high significance.

Data & methodology: validity of approach, quality of data, quality of presentation
All data and methodology are convincing. I have only the minor suggestion to present data from the behavioural arena in a different way: instead of presenting bar scatter plots of times spent in either part of the arena (which ignores the dependence of the data as each data point has a corresponding one in the other bar), the authors should use present an attraction index (time spent with odour-time spent with control/total time) and test their results against the null hypothesis 0 (i.e. significant attraction results in values above 0, significant repulsion in values below zero)

Appropriate use of statistics and treatment of uncertainties
All looks good to me.

Conclusions: robustness, validity, reliability
All looks good to me.

Suggested improvements: experiments, data for possible revision
Nothing ore needed.

References: appropriate credit to previous work?
I am not aware of any additional study that should be cited.

Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
All sounds good to me.

Referee #3

(Remarks to the Author)
The authors have conducted an impressive number of additional studies to address the concerns raised by the referees, and in my view they have largely been successful in addressing them. There is one exception. All referees raised concerns about the possibility that 4NP might have side effects that would prohibit its use. The authors conducted studies to show, to their satisfaction, that 4NP wasn't a problem for plants, insect pests, or mammals. I do not find these studies to be convincing. 4NP has serious human health liabilities, and I have attached the MSDs (material safety data sheet) from the US EPA (Environmental Protection Agency) and the legally required MSD from Sigma-Aldrich, the main supplier of MSD for researchers in the US. The Sigma-Aldrich MSD meets EU requirements. The statement in the revised manuscript: "Although 4NP application did not affect the growth of plants and the survival of locusts, bumble bees, and mice in the present study, other potential health risks need further evaluation before large-scale application." does not come close to acknowledging the serious issues surrounding 4NP. Proving something is 'safe' is a high bar, and the authors don't come close to doing that even the large amount of published data to the contrary. The manuscript is not suitable for publication in its present form, and if it continues to advocate for the utility of 4NP, it may never be.

Referee #4

(Remarks to the Author)
After carefully reviewing the revised manuscript and considering the responses to my previous concerns, I believe the authors have addressed all issues in a satisfactory manner. The revisions improve the manuscript, and there are no further concerns on my part. I therefore recommend the paper for publication in Nature without any delay

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Point-by-point response:

Referee #1 (Remarks to the Author):

Guo et al have elucidated the biosynthetic pathway of the aggregation pheromone 4-vinylanisole (4VA) that plays a critical role in forming locust swarms and the resulting locust plagues. They identified the biosynthetic intermediates and the key enzymes controlling 4VA production. Two methyltransferases highly expressed in gregarious locusts are upregulated in response to increased population density and turn on and off 4VA synthesis. As a final twist, the authors identified 4-nitrophenol as a potent inhibitor of these methyltransferases and outline its potential use in the control of locust plagues.

This is an excellent study covering a multitude of aspects on the phenomenon and making use of appropriate and state-of-the-art techniques. The manuscript is generally well written and the presentation and reasoning is clear. I am impressed with the high quality of the work.

My few comments are all minor:

Title: “the production of aggregation pheromone and behavioral swarming of locusts” should possibly be “in locusts”?

Response: Thank you for your suggestion. We revised the title to “4-Vinylanisole biosynthetic enzyme inhibitors block the aggregation behavior in locusts”.

Line 105 does not read well to me. “wheat seedlings” refer to “host plant” whereas lignin, amino acid etc refer to compound classes. Consider rephrasing to something like “The main compound classes in the wheat seedlings used as host plants include lignin, amino acids….”

Response: Nice suggestion. We revised this sentence to “The main compound classes in the wheat seedlings used as host plants include lignin, amino acids, cellulose, and hemicellulose (Fig. 1B).” in Lines 105-106.

Line 112-113: Can you be positive about that? Is there no lignin at all present in the artificial diet used as control? Or maybe the additional lignin fed to the locusts was not available to the insects?

Response: Thank you for your question. I am sorry we did not clarify this. In fact, we placed this information in the supplemental materials of previous version. We confirm that no lignin is present in the artificial diet used as a control. Now, we continue to keep these two tables in the supplemental materials. We observed a significant increase in deuterated phenylalanine levels following locust consumption of an artificial diet with

deuterated phenylalanine, indicating that the locusts ingested and digested the lignin present in the artificial diet under identical conditions. Additionally, we added more biological replicates and verified that locusts fed on lignin with an artificial diet did not show increased levels of 4VA, compared to the control group. These results are listed below.

Table S2. Components of locust artificial diet

Ingredient	Dosage
Ultrapure water	400 mL
Powdered agar	10.0 g
Casein	8.0 g
Glucose	14.0 g
Sucrose	10.0 g
Starch	28.0 g
Cellulose	24.0 g
Methylparaben	1.0 g
Pentadiene carboxylic acid	0.5 g
Wesson salt	1.5 g
Vitamin C	2.0 g
Vitamin B1	0.3 g
Glycine	0.3 g
Glusate	0.2 g
Lysine	0.2 g
Total	500 g

Table S3. Components of Wesson salt

Ingredient	V/V%
$CaCO_3$	21.0
$FeSO_4.7H_2O$	1.56
$MgSO_4$	9.0
KCl	12.0
KH_2PO_4	31.0
NaCl	10.5
$Ca_3(PO4)_2$	14.9
$CuSO_4.5H_2O$	0.039

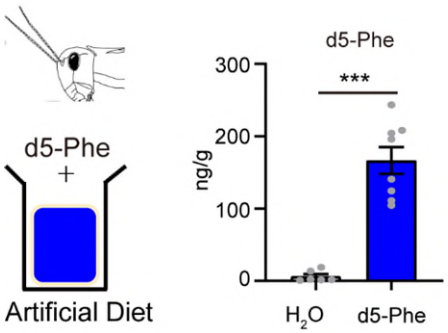
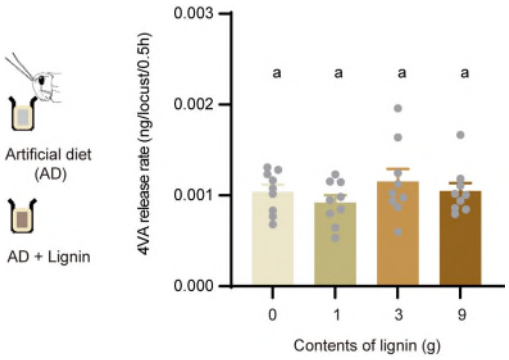


Figure R1. The contents of d5-phenylalanine in the locust gut significantly increased after the locusts were fed on an artificial diet with d5-phenylalanine.



Revised Figure 1C. The contents of 4VA remain unchanged after the locusts consumed an artificial diet containing lignin.

Line 119-120: "Feeding of host plants sprayed with….. ". Consider replacing with "Feeding the locusts with host plants sprayed with…"

Response: Thank you for your suggestion. We revised the sentences as "Locusts that are fed on the host plants sprayed with deuterated tyrosine did not produce deuterated 4VA (Two-tailed -unpaired *t*-test, $P > 0.05$) (Fig. 1H)." in Lines 120-122.

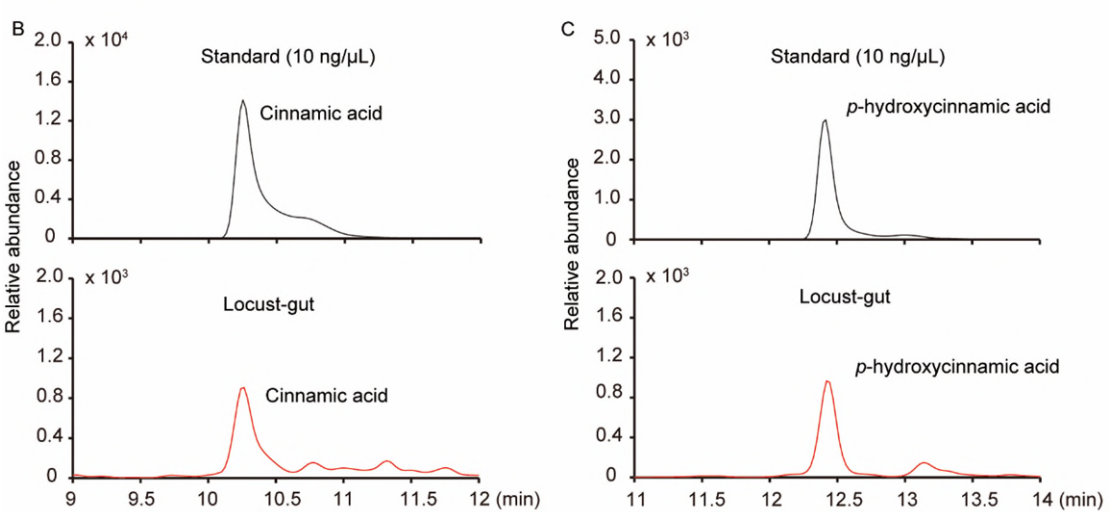
Line 128-130: Line 78: I don´t believe in the value of presenting long lists of raw data and automated identifications of various compounds (Tables S1 and S2). I would prefer a proper identification of the key compounds used for construction of the two pathways outlined in Fig 2A. The literature is full of misidentified compounds and these flaws easily live their own life when people refer to them and not confirm that the chemical identities are correctly assigned in the original study.

Response: Thank you for your comments. We are sorry we did not clarify this point. In fact, in our previous work (Wei et al., 2017), we reported the presence of two chemicals, phenylacetaldehyde and phenethylalcohol, in locusts. Table 1 in that study presented all the volatile chemicals found in the migratory locust.

Table 1 Absolute amounts of locust-associated compounds (mean ± SE), collected from the headspace volatiles of fourth-instar gregarious locusts (ng/10 locusts/h) and their feces (ng/1 g fresh weight [FW]/h).

Chemical compound	Body volatiles[§] ng/10 locusts/h	Fecal volatiles[§] ng/1 g FW/h
Aromatic compounds		
1 Phenylacetonitrile[†]	26.3 ± 1.8	1.81 ± 0.7
2 Benzaldehyde[†]	4.28 ± 1.0	5.89 ± 1.5
3 Phenol [†]	3.62 ± 0.5	11.61 ± 2.4
4 Guaiacol[†]	1.33 ± 0.3	4.47 ± 0.8
5 Veratrole[†]	0.87 ± 0.1	0.44 ± 0.2
6 Benzeneacetaldehyde[†]	0.56 ± 0.2	0.85 ± 0.1
7 4-Vinylanisole[†]	0.52 ± 0.1	0.58 ± 0.1
8 Benzoic acid[†]	0.5 ± 0.2	1.34 ± 0.5
9 Anisole[†]	0.42 ± 0.3	0.12 ± 0.1
10 Phenethyl alcohol[†]	0.15 ± 0.1	2.5 ± 0.7
11 Indole[†]	ND[¶]	1.75 ± 0.4
Aliphatic compounds		
12 Acetic acid[†]	51.4 ± 14.3	26.6 ± 5.3
13 Hexanoic acid[†]	1.27 ± 0.1	8.30 ± 1.4
14 3-Methyl, butanoic acid[‡]	0.66 ± 0.2	6.1 ± 1.3
15 Decanoic acid[†]	0.42 ± 0.1	0.16 ± 0.1
16 Pentanoic acid[†]	0.34 ± 0.1	1.66 ± 1.1
17 Butanoic acid[†]	0.31 ± 0.1	3.34 ± 1.1
18 Nonanoic acid[†]	0.26 ± 0.1	0.24 ± 0.1
19 Octanoic acid[†]	0.14 ± 0.1	0.24 ± 0.1
20 (*E*)-3-Hexenoic acid[‡]	0.09 ± 0.1	0.91 ± 0.4
21 Propanoic acid[†]	ND	1.68 ± 0.2
22 3-Hydroxy, 2-butanone[‡]	22.7 ± 2.9	5.0 ± 0.8
23 2,3-Butanediol[†]	13.11 ± 2.7	42.1 ± 8.1
24 [R-(R*,R*)], 2,3-Butanediol[†]	1.86 ± 0.3	15.1 ± 3.2
25 2,5-Dimethyl-pyrazine[†]	0.83 ± 0.1	7.75 ± 1.5
26 1-Pentanol[†]	0.63 ± 0.2	7.54 ± 1.6
27 (Z)-2-Penten-1-ol[†]	0.22 ± 0.1	8.93 ± 1.4
28 Cyclohexanol[†]	ND	5.46 ± 0.8
29 β-Ionene[†]	ND	5.40 ± 1.5
30 β-Ionone epoxide[‡]	ND	3.11 ± 0.6
31 1-Methoxy-butane[‡]	ND	2.99 ± 0.9
32 2,6-Dimethyl-pyrazine[‡]	ND	2.58 ± 0.3
33 (*E*)-2-Hexenal[†]	ND	2.43 ± 0.3
34 Methyl-pyrazine[‡]	ND	1.44 ± 0.5
35 (*Z*)-3-Hexen-1-ol[†]	ND	0.86 ± 0.2

To confirm the existence of cinnamic acid and *p*-hydroxycinnamic acid in locusts, we performed a targeted metabolic analysis. By using standard compounds as references, we were able to detect these two compounds in locust samples. We deleted these results (Tables S1 and S2) in the new version, and revised the Results section as "To explore the biosynthetic intermediates from phenylalanine to 4VA, we summarized phenylalanine metabolism annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and predicted two chemically reasonable biosynthetic routes from phenylalanine to 4VA. The first route is phenylalanine → phenylacetaldehyde → phenethylalcohol → tyrosol→ 4-vinylphenol → 4VA. The second route is phenylalanine → cinnamic acid (CA) → p-hydroxycinnamic acid (p-HCA) → 4-vinylphenol (4VP) → 4VA (Fig. 2A, Extended Data Fig. 1A). Phenylacetaldehyde and phenethylalcohol have been identified in locusts previously[13], while CA and p-HCA can also be detected in locusts using the standard compounds as referees (Extended Data Fig. 1B and 1C)." in Lines 129-138.



Revised Extended Data Fig. 1B and 1C. Targeted detection of cinnamic acid (B) and p-hydroxycinnamic acid (C).

Line 198: It would not hurt to mention in what heterologous system the two enzymes were expressed (so that we do not have to look up Methods at this point). Please add this info!

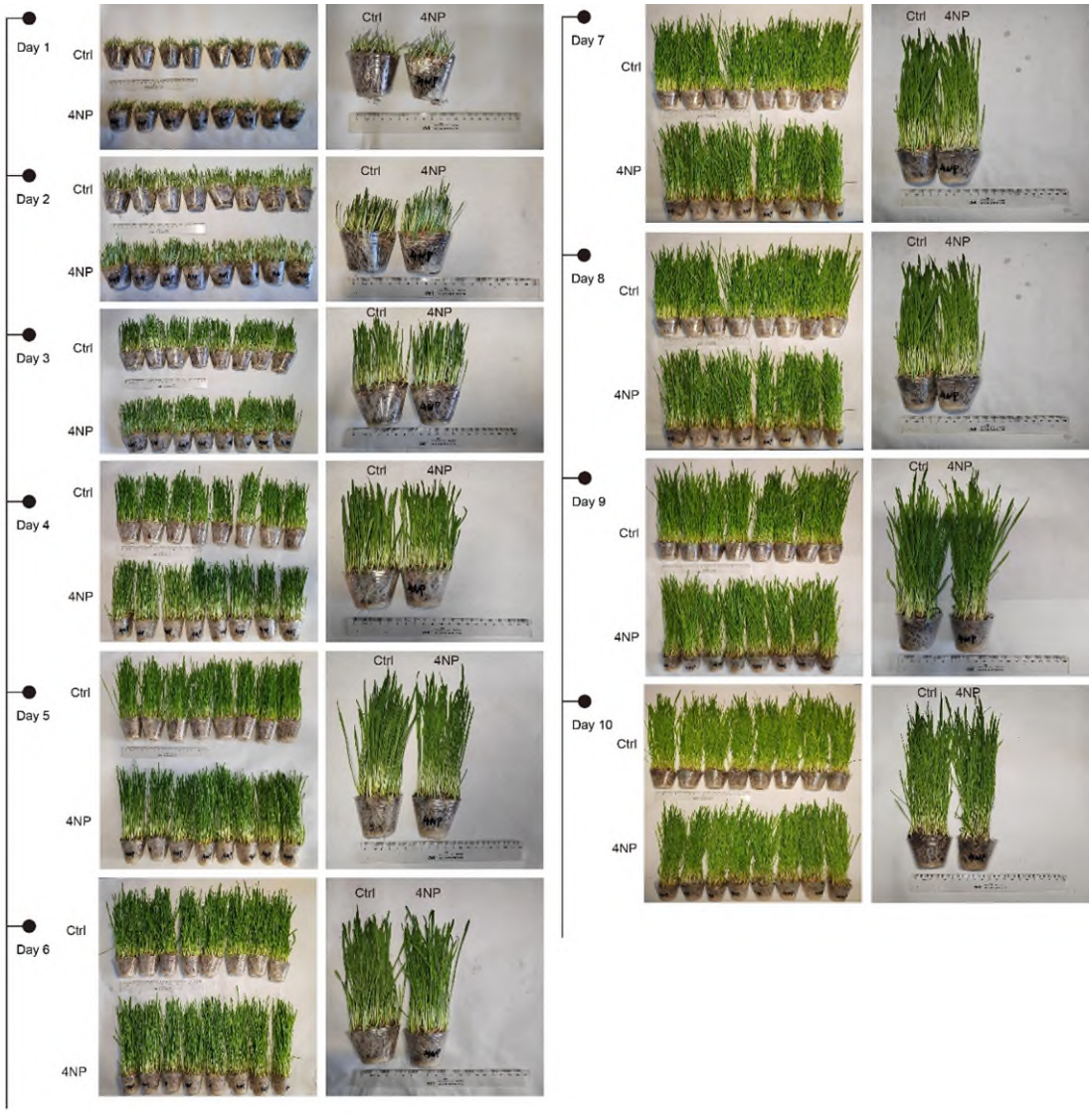
Response: Thanks for your suggestion. Based on your suggestion, we introduced the heterologous system the two enzymes were expressed as "To further verify the function of LOCMI02868 and LOCMI16699, we heterologously expressed these two proteins using a pET28a-derived vector in *Escherichia coli* BL21 (DE3) strain and tested their enzymatic activities in vitro." in Lines 201-203.

Lines 405-408: What about the potential hazards of spraying with 4-nitrophenol? 4-Nitrophenol irritates the eyes, skin, and respiratory tract. It may also cause inflammation of those parts. It has a delayed interaction with blood and forms methaemoglobin which is responsible for methemoglobinemia, potentially causing cyanosis, confusion, and unconsciousness. When ingested, it causes abdominal pain and vomiting. Prolonged contact with skin may cause allergic response. Genotoxicity and carcinogenicity of 4-nitrophenol are not known. (See for instance Wikipedia for references https://en.wikipedia.org/wiki/4-Nitrophenol). I think that it would make sense to briefly mention (with a reference) any potential health hazards associated with large-scale spraying with 4-nitrophenol as the authors refer to it as a "sustainable control strategy"./ Christer Löfstedt

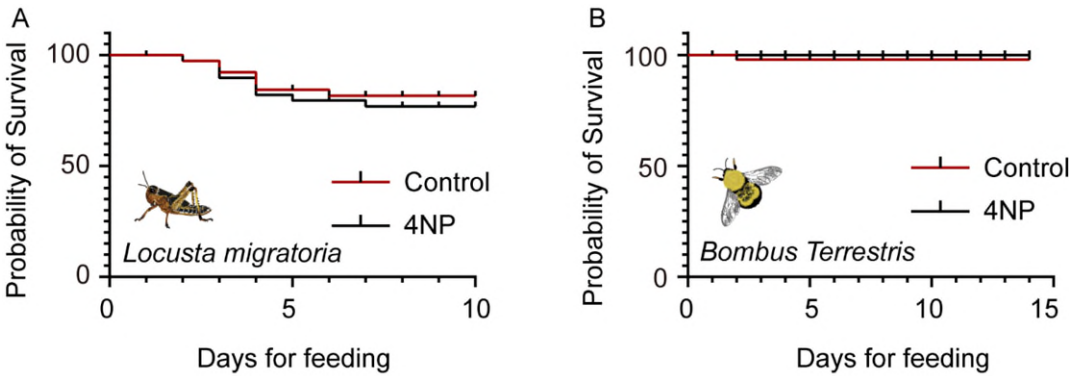
Response: Thanks for your important suggestion. We evaluated the impacts of 4NP on the plant growth, locust and bumble bee mortality, as well as mammal acute toxicity at the concentrations of 4NP employed in this study.

We found that 4NP, when applied at the bioactive dose of 2 μmol per day, did not impact

plant growth, locust and bumble bee mortality, or show noticeable acute toxicity in mice. These results indicate that 4NP has no significant effects on plants, insects, and mammals at this dosage. These results are listed below.

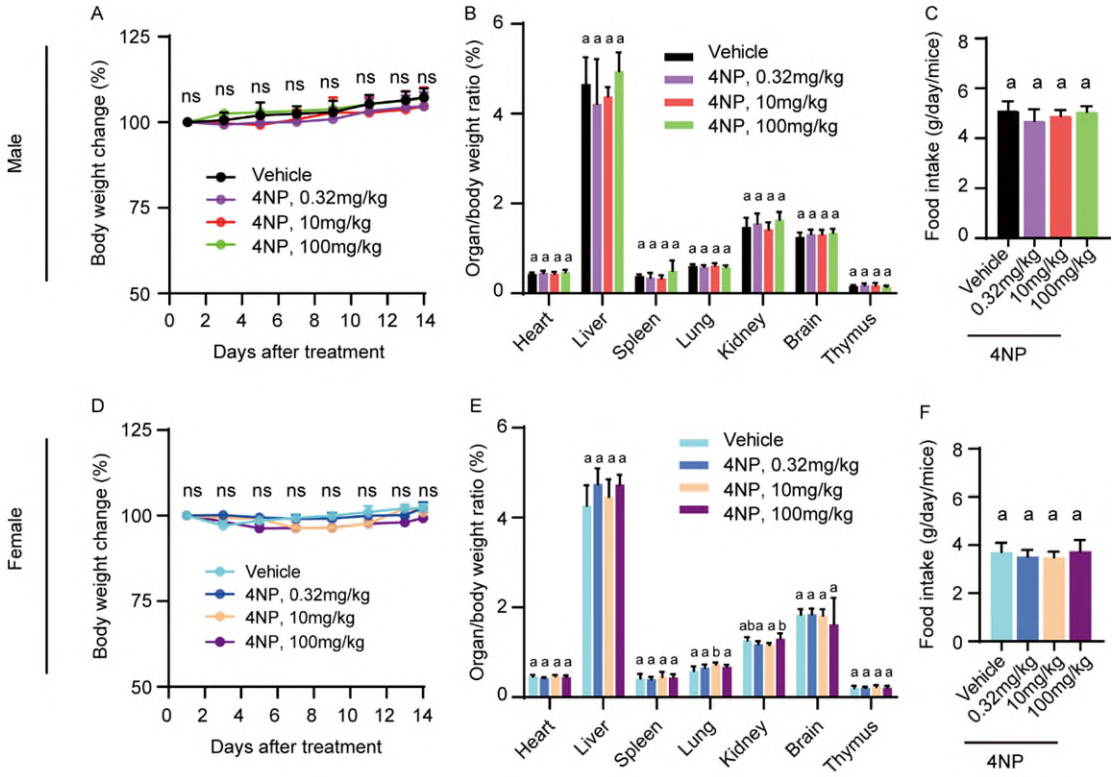


Extended Data Fig. 12. The effects of 4-nitrophenol (4NP) on the growth of wheat seedlings.



Extended Data Fig. 13. Survival curves for locusts and bumble bees following

ingestion of food containing 4-nitrophenol (4NP).



Extended Data Fig. 14. Mammal acute toxicity experiments in mice. Treatment of healthy male and female CD-1 mice with 4-nitrophenol (4NP) once daily (at doses of 0.32, 10 and 100 mg/kg, p.o.) for 14 days barely affected the body weight (A and D), organ/body weight ratios (B and E) or food intake (C and F) compared to the vehicle-treated group, suggesting that 4NP has a potentially favorable safety profile in mice. n = 8. Different letters indicate statistically significant differences between groups using one-way ANOVA (Tukey's multiple comparisons test, $P < 0.05$). ns, not significant.

Since injecting the locust (~0.5 g per one) with 1 nmol (139 ng) could significantly decrease the release amount of 4VA, the acute toxicity experiments were performed in mice under this bioactive dose (~0.32 mg/kg). Since feeding the locusts with 2 μmol (~280 μg) per day could significantly decrease the release amount of 4VA, the acute toxicity experiments were performed in mice (~28 g per one) under this bioactive dose (~10 mg/kg). Furthermore, we evaluated the acute toxicity of mice under a higher dose (~100 mg/kg).

In addition, we discussed the potential risks of spraying with 4-nitrophenol in the Discussion section "In the present study, blocking 4VA production through 4NP spraying significantly inhibits the acquisition and maintenance of gregarious behavior. Thus, 4NP is a potent regulator with great potential application in locust control. Although 4NP application did not affect the growth of plants and the survival of locusts, bumble bees, and mice in the present study, other potential health risks need further

evaluation before large-scale application. Additionally, applying newly developed adjuvants along with 4NP will facilitate controlled release and minimize environmental impact under controllable costs." in Lines 445-452.

Referee #2 (Remarks to the Author):

The migratory locust Locusta migratoria exists in two phases, a solitary and a gregarious one, of which the latter forms swarms and is a pest of many crops. In their manuscript "Inhibiting biosynthetic enzymes blocks the production of aggregation pheromone and behavioral swarming of locusts" Guo et al. investigate the biosynthetic pathway of the locust aggregation pheromone 4-vinylanisole (4VA), which has been shown to be relevant for phase shift and the formation of swarms in the migratory locust Locusta migratoria. By performing different feeding experiments and gene expression analyses the authors identify 4-vinylphenol as a precursor that by two methyl transferases (VPMT1 and 2) becomes transferred into 4VA. Inhibiting the transcription of those transferases results in the loss of 4VA in the locusts, which then results in gregarious locusts exhibiting a more solitary behavioral phenotype. In a next step the authors investigate the potential bounding properties of the transferases and the precursor 4VP and based on that design another molecule (4 nitrophenol, 4NP) that even binds better and blocks the methylation process. In a last experiments the authors show that feeding gregarious locusts with 4NP again results in some kind of phase shift, with the locusts after feeding exhibiting more solitary-like behaviors.

To my knowledge this is the first time, that a phase shift in locusts from the problematic gregarious form towards the less problematic solitary form could be artificially induced by spraying a chemical. This is of high interest, as 4NP potentially could present and alternative strategy to the use of pesticides.

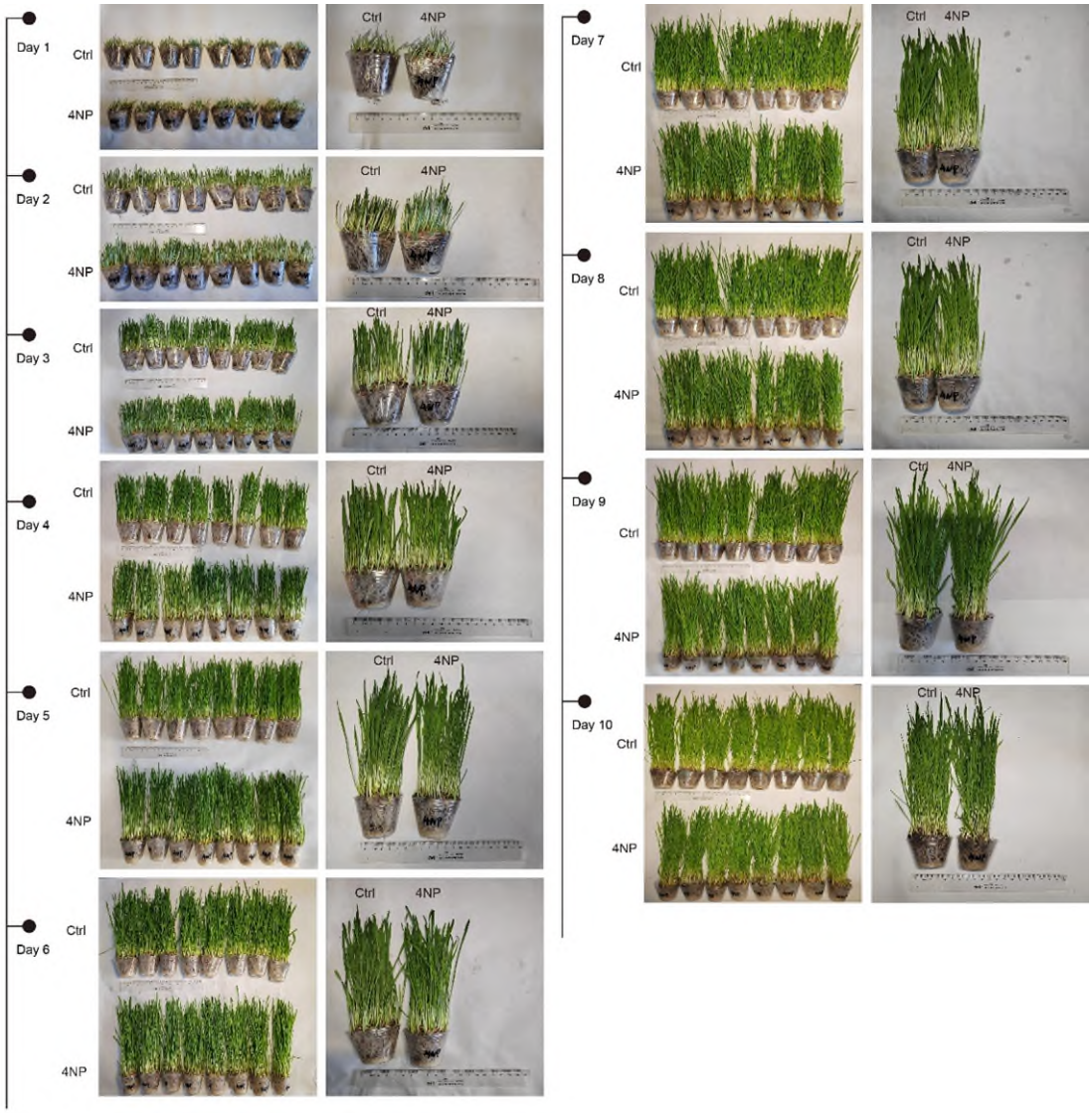
The manuscript is well written and the data, their presentation and statistical analyses are convincing (I should mention, that due to lacking expertise I cannot judge the data on binding properties presented in Fig. 5).

I feel that the conclusions are robust and reliable.

I have only one major comment: as the authors state, spraying of NP4 could due to its potential to induce phase shift become a useful alternative to the use of pesticides. However, it would be very helpful if the authors include some analyses on the potential negative side effects of 4NP:

Does the amount of 4NP that needs to be sprayed on the plants affect the plant health and survival?
Response: Thank you for your comments. It is indeed an important question. We assessed the impact of 4-nitrophenol (4NP) on the health and survival of wheat seedlings. We applied 4NP to the plants daily for a period of 10 days and monitored

their growth. Our findings revealed that the plants treated with 4NP exhibited growth patterns comparable to those of the untreated control groups. The results are listed below.



Extended Data Fig. 12. The effects of 4-nitrophenol (4NP) on the growth of wheat seedlings.

Does it do anything else to the locusts than inducing phase shift?

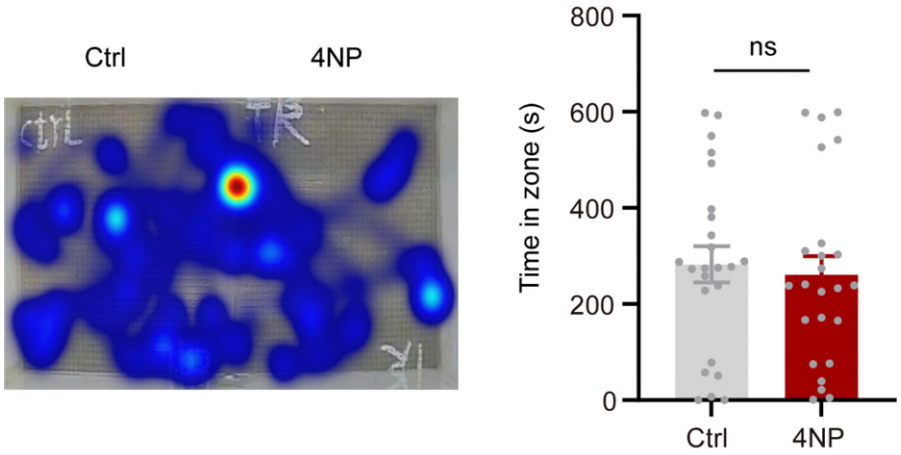
Response: Based on your comment, we first evaluated whether 4NP could directly elicits attraction or repulsion responses in locusts within a dual-choice behavioral setup. The locusts did not show any preference for 4NP. These results indicated that 4NP did not have a direct influence on the behavior of locusts.

We conducted comparative experiments using locusts fed with 4NP and control locusts as stimuli in a dual-choice behavioral system. Our findings revealed that locusts exhibited a significant preference for the area occupied by the control locust volatiles.

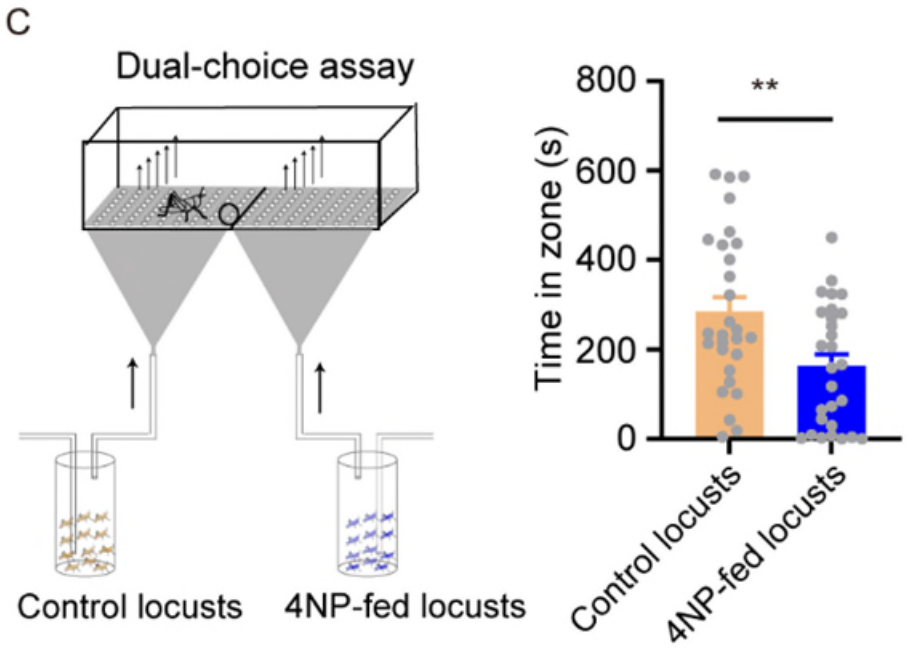
To determine whether other compounds in the volatiles of 4NP-fed locusts influenced this behavior, we quantified the VOCs present in gregarious locusts after they had consumed 4NP. We discovered that the levels of two specific compounds, m-xylene and nonane, were significantly reduced following 4NP ingestion. Subsequent behavioral tests with these compounds showed that neither could induce attraction in locusts. These findings indicate that the inhibitory effects of 4VPMTs on locust behavior are primarily due to the reduction of 4VA levels, rather than the influence of other compounds.

We conducted an evaluation to determine the impact of 4NP on the sensory perception of locusts following its application. Our findings indicated that 4NP did not influence the electrophysiological responses of the basiconic sensilla in locusts.

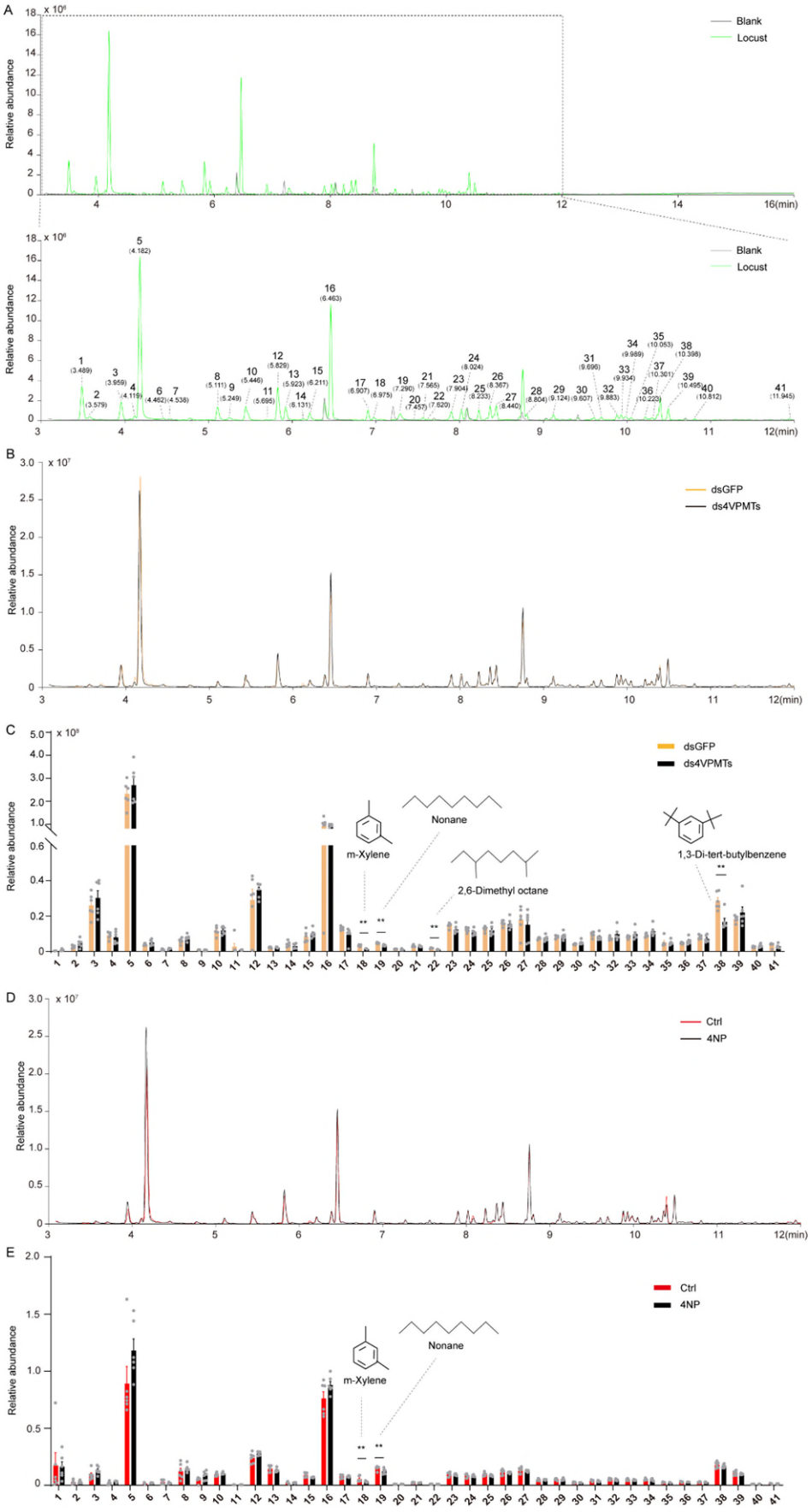
Taken together, the effects of 4NP on locust behavior is primarily attributed to its inhibitory effect on 4VPMTs and the subsequent reduction in 4VA levels. The results are listed below.



Extended Data Fig. 8. The behavioral responses of locusts to 4-vinylphenol (4NP). n = 24. *P* values were determined by the Wilcoxon signed-rank test. ns, not significant. Data are presented as mean ± SEM.

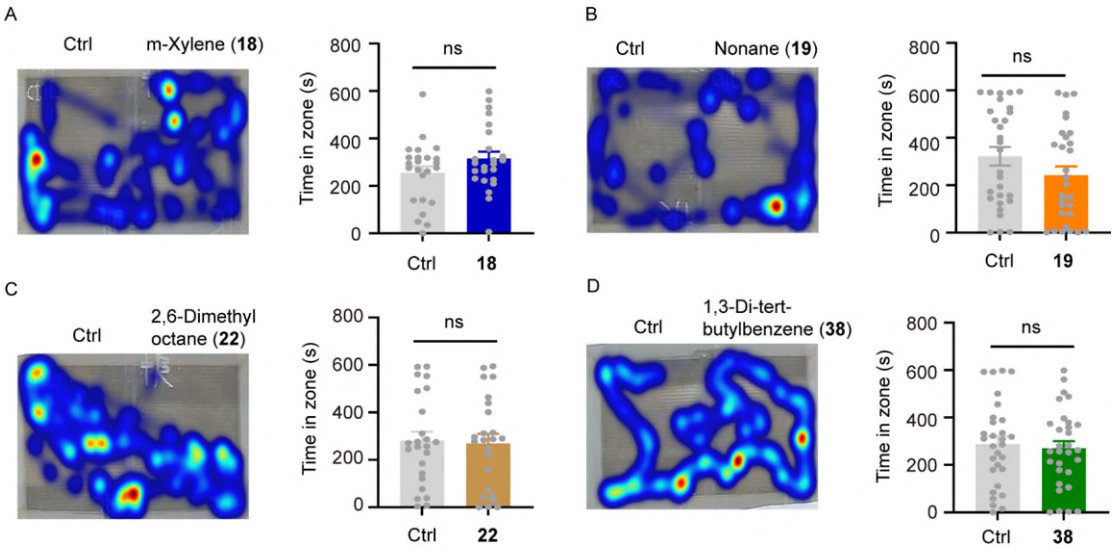


Revised Figure 6C. The preferences of locusts to the control gregarious locusts and 4NP-fed locusts.

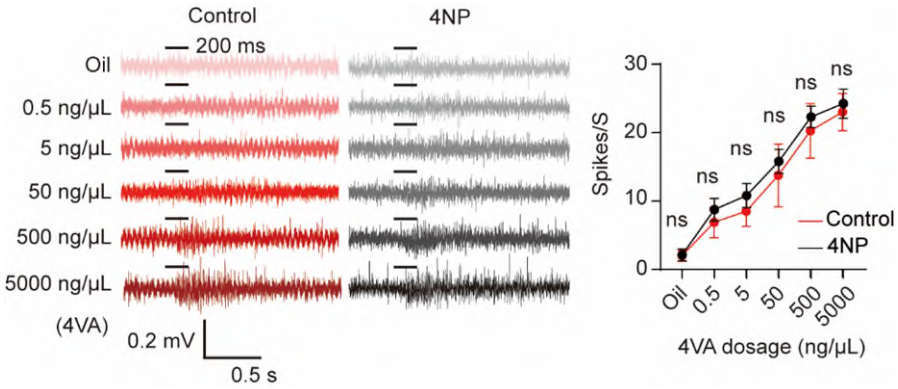


Extended Data Fig. 4. The volatile organic compounds (VOCs) of locusts after

RNAi of 4VPMTs and application of 4NP. (A) The total ion chromatograms (TICs) of locust VOCs compared to the blank. (B) The TICs of locust VOCs after RNAi of 4VPMTs. (C) The relative abundance of 41 peaks of locust VOCs after RNAi of 4VPMTs. (D) The TICs of locust VOCs after application of 4NP. (E) The relative abundance of 41 peaks of locust VOCs after application of 4NP. P values were determined by a two-tailed unpaired t-test, n = 6. *, P < 0.05; **, P < 0.01; Data are presented as mean ± SEM.



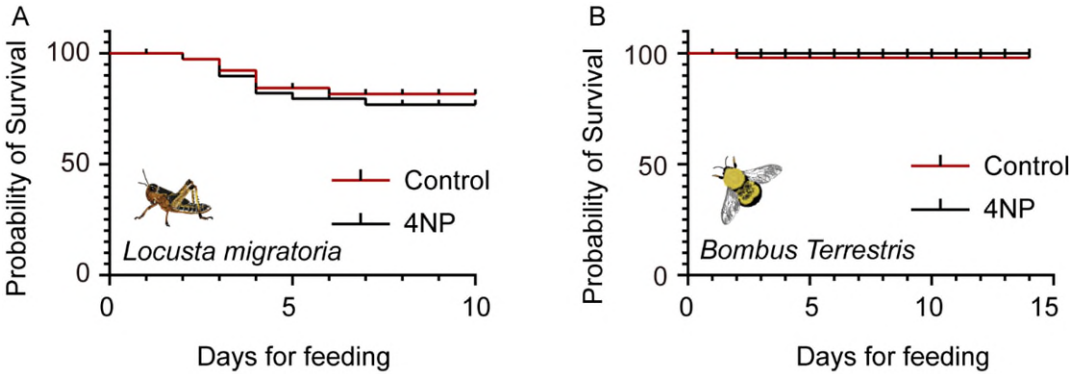
Extended Data Fig. 5. The behavioral responses of locusts to four compounds. (A-D) The behavioral responses of locusts to m-xylene (A, n = 24), nonane (B, n = 29), 2,6-dimethyl octane (C, n = 24), and 1,3-di-tert-butylbenzene (D, n = 30). P values were determined by Wilcoxon signed-rank test. ns, not significant. Data are presented as mean ± SEM.



Extended Data Fig. 11. The electrophysiological responses of basiconic sensilla to 4VA after the application of 4-vinylphenol (4NP). P values were determined by a two-tailed unpaired t-test, n = 8 for control, n = 16 for 4NP. ns, not significant.

What about survival rates of 4NP- fed animals as compared to control animals.

Response: Thank you for your question. Based on your comments, we detected the survival of locusts and bumble bees after 4NP application. We found there was no significant difference in survival rates between 4NP- fed animals and control groups.



Extended Data Fig. 13. Survival curves for locusts and bumble bees following ingestion of food containing 4-nitrophenol (4NP).

Minor points:
The authors should discuss, why feeding on plants supplemented with Phe-d5 but not on supplemented artificial diet yielded in increased rates of 4VA-d4 (Fig. 1G vs Fig. 1I). If Phenylalanine is a necessary precursor, supplying the artificial diet with it, should be effective also.

Response: Thank you for your good question. We have made great efforts to elucidate the role of phenylalanine in 4VA biosynthesis. We can detect d5-cinnamic acid (CA) after locust feeding on artificial diet with d5-phenylalanine, although we failed to detect d4-4VA simultaneously. We speculated that specific gut symbionts can convert phenylalanine to cinnamic acid, while this conversion may only contribute a small portion compared to direct ingestion of cinnamic acid from plants, considering its extremely low efficiency (~0.1%). To clarify this, we added discussion as "Gut microbiota is suggested to play a possible role in the biosynthesis of 4VA. Phenylalanine ammonia-lyase, an enzyme that catalyzes the conversion of phenylalanine to cinnamic acid, is found in plants, fungi, and bacteria 17. When locusts are fed an artificial diet enriched with deuterated phenylalanine, the presence of deuterated cinnamic acid can be detected, indicating that gut microbiota likely plays a role in the lysis of phenylalanine. However, the contribution of the gut microbiota to supplying cinnamic acid for 4VA biosynthesis may be relatively minor compared to the role of host plants." in Lines 422-429.

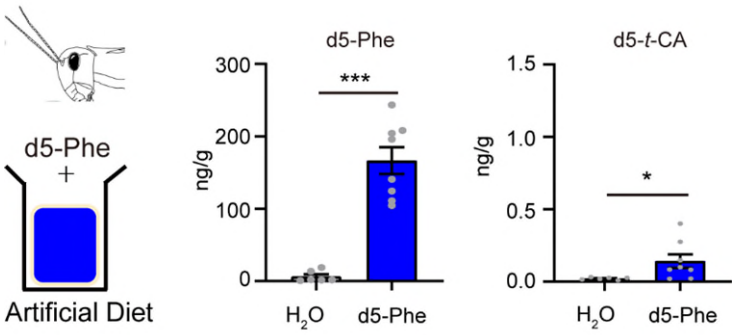


Figure R2. The contents of d5-phenylalanine and d5-cinnamic acid in locust gut significantly increased after the locusts were fed on an artificial diet with d5-phenylalanine.

Figure legend 1"Different letters indicate statistically significant differences between groups using one-way ANOVA (Tukey's multiple comparisons test, $P < 0.05$) (A). P values in chemical assays were determined by a two-tailed unpaired t-test (C–I). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are presented as mean ± SEM." I guess, the ANOVA was used for figures A and C, while the t-test was used for figures D-I

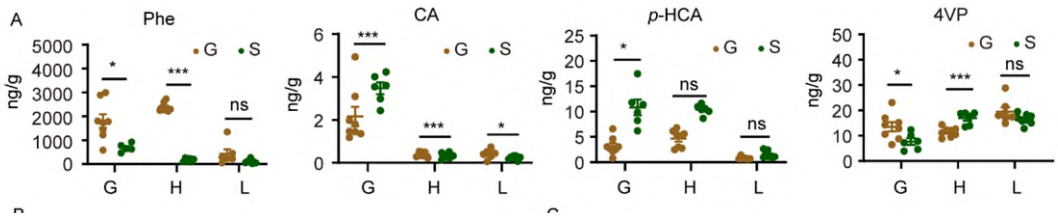
Response: Thanks for your suggestion. We revised the figure legend as the reviewer suggested.

Fig. 3 The picture of the solitary locust makes sense only, if a gregarious one is shown also.

Response: Based on your suggestion, we deleted the picture of a solitary locust.

Fig.3A I guess brown (green) dots present data from gregarious (solitary) locusts? Please add information. Why did you not perform statistics with data from Fig. 3A?

Response: Thank you for your comments. We are sorry for this fuzziness. Our results present that the four precursors can all be detected in three different tissues of both gregarious and solitary locusts, with no discernible pattern. Consequently, these four precursor chemicals do not appear to be the primary factors influencing the variation in 4VA emissions between gregarious and solitary locusts. Based on your suggestion, we added the statistical analysis with significant differences. The revised Figure 3A is listed below.

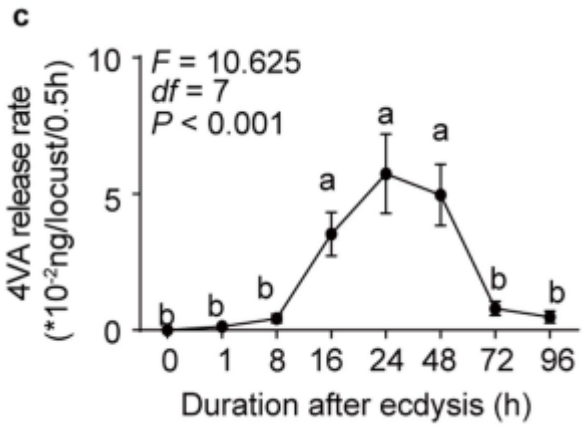


Revised Fig.3A

Fig. 3 H-M What is the reason for the dramatic difference in the release rates of the control groups between the figures H-M and within the figures (from 24h to 72h).

Response: Thanks for your question. In fact, the gene knockdown experiments spanned approximately six months, necessitating periodic adjustments to the GC-MS, including maintenance of the chromatographic column, cleaning of the ion source, and tuning of the mass spectrometer. These procedures significantly affect the sensitivity of the SPME targeted detection. Indeed, the variation in insect samples across different batches and seasons also contributes to the overall variability. Although there are variations among the release rates of the control groups, the outcomes from the pairwise comparisons between each control group and the knockdown group remain comparable across experiments. The objective of this experiment is to assess the differences between the control and knockdown treatments.

In the previous study (Guo et al., 2020), We observed that the 4VA emissions in a single instar exhibited variability due to ecdysis timing, during which 4VA release was significantly reduced. Thus, 4VA showed different release rates between different time points (from 24 h to 72 h, within the figures), even though these samples were continuously detected.



The 4VA fluctuation in gregarious locusts after ecdysis (Guo et al., 2020).

“The locusts injected with dsGFP is a control for all genes.“ this refers to figure 3H-M and should better appear there (not after the text of Fig. N-O)

Response: Thank you for your suggestion. We added this sentence after the legends of Figure 3H-M.

Fig. 6B please specify the amount of 4NP that was used for the duration experiment. 6C please specify the amount of 4NP that was sprayed on plants (this is important to judge, whether the spaying of agricultural areas to reach a similar effect is feasible)

Response: Based on your suggestion, we added the amount of 4NP in the revised Figure 6. In this study, we sprayed 2 μmol (2 mM×1mL) per day of 4NP on the plants and fed the locusts.

Fig. 6 D and onwards. D is not showing 4VA production as mentioned in legend, but shows schematic of feeding crowded solitary locusts (which in figure legend appears only later)

Response: Thanks for your suggestion. Combined with new additional experimental results, we re-organized and revised Figure 6 and the figure legends.

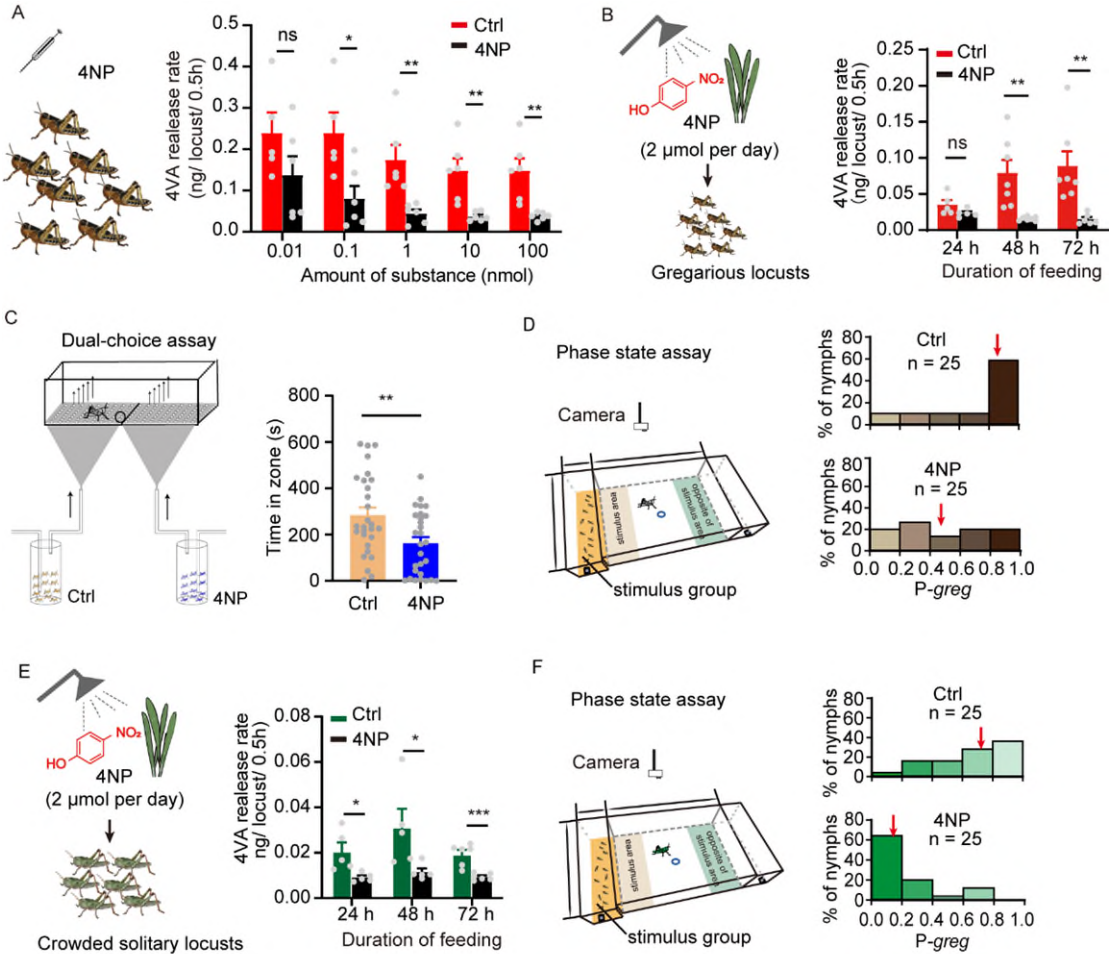


Fig.6 4-Nitrophenol effectively inhibits the gregarious behavior of locusts. (A) The effects of 4-nitrophenol (4NP) at different amounts on 4VA production in locusts, n = 6. (B) 4VA production of gregarious locusts after feeding on plants sprayed with 4NP,

n = 7. (C) The preference of locusts for volatiles emitted by control gregarious locusts compared to those fed with 4NP, n = 28. (D) The behavioral state of gregarious locusts after feeding on plants sprayed with 4NP. (E) 4VA production of crowded solitary locusts after feeding on plants sprayed with 4NP, n = 5. (F) The behavioral state of crowded solitary locusts after feeding on plants sprayed with 4NP. P values were determined by a two-tailed unpaired t-test (A, B, C, E). Comparisons of median P-greg were analyzed using the Mann–Whitney U test (D and F). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are presented as mean ± SEM. P-greg, probabilistic metric of gregariousness. Arrows indicate median P-greg values.

"…fed with an artificial diet containing deuterated tyrosine and phenylalanine" better "fed with an artificial diet containing deuterated tyrosine or phenylalanine…"

Response: Based on your suggestion, we revised this sentence as "fed with an artificial diet containing deuterated tyrosine or phenylalanine…"

"These imply that the locusts synthesize 4VA using building…" better "These results imply that the locusts synthesize 4VA using building…"

Response: We revised this sentence as the reviewer suggested.

Deuterated phenylalanine on artificial diet no effect. Deuterated phenylalanine on host plant results in deuterated 4VA. How comes the difference?

Response: Thank you for your question. We can detect d5-cinnamic acid after locust feeding on an artificial diet with d5-phenylalanine, although we failed to detect d4-4VA. A likely explanation for these differences is the varying intake of plants and artificial diets. In fact, locusts show a preference for wheat seedlings over artificial diets. Consequently, when locusts consume the artificial diet, they ingest only a small amount of d5-phenylalanine. Based on our observation, the d5-phenylalanine amount locusts take in when they feed on wheat seedlings is about 8-9 folds compared to locusts fed on artificial diet.

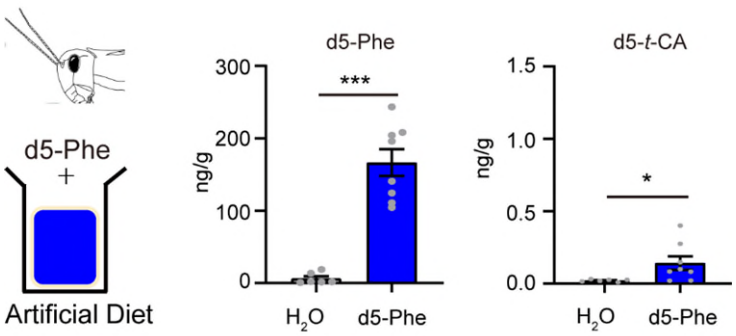


Figure R2. The contents of d5-phenylalanine and d5-cinnamic acid in the locust gut significantly increased after the locusts were fed on an artificial diet with d5-

phenylalanine.

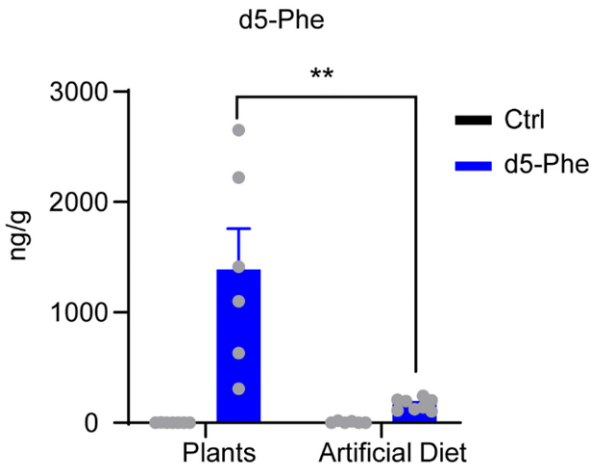


Figure R3. The contents of d5-phenylalanine (d5-Phe) in the locust gut after the locusts were fed on the plants sprayed with d5-phenylalanine or an artificial diet containing d5-phenylalanine.

"KEGG" Please specify what this abbreviation stands for.

Response: Sorry for this. We added the full name of KEGG as Kyoto Encyclopedia of Genes and Genomes.

"The first is phenylalanine– phenylacetaldehyde–phenethylalcohol–tyrosol–4-vinylphenol–4VA. The second route is phenylalanine–cinnamic acid (CA)–p-hydroxycinnamic acid (p-HCA)–4-vinylphenol (4VP)–4VA (Fig. 2A, Extended Data Fig. 1B)." Difficult to understand as dashes are obviously used between consecutive compounds of the pathway but of course are also part of compound names.

Response: Based on your suggestion, we revised these representations as "The first route is phenylalanine → phenylacetaldehyde → phenethylalcohol → tyrosol→ 4-vinylphenol → 4VA. The second route is phenylalanine → cinnamic acid (CA) → p-hydroxycinnamic acid (p-HCA) → 4-vinylphenol (4VP) → 4VA (Fig. 2A, Extended Data Fig. 1A)." in Lines 132-135.

"Injection of deuterated cinnamic acid did not induce the production of deuterated 4VA.." should better read "Also the injection of deuterated cinnamic acid…"

Response: Thanks. We revised this sentence as the reviewer suggested.

"we detected the contents of these four…." better "we looked for these four…." (as "detected" already anticipates the existence of these compounds)

Response: We revised this sentence as the reviewer suggested.

"Injection of deuterated p-HCA and 4VP did not induce the production of deuterated 4VA in solitary locusts…" better "Also the injection of deuterated ….."

Response: Thanks. Based on your suggestion, we revised this sentence to "Also the injection of deuterated..."

"The step from 4VP to 4VA may contribute to the distinct emissions…" better "It rather seems to be the step from 4VP to 4VA that contributes to the distinct emissions…."

We revised this sentence as the reviewer suggested.

"Nine genes annotated methyltransferases…" better "Nine genes annotated as methyltransferases…"

We revised this sentence as the reviewer suggested.

"RNAi knockdown of LOCMI16699 and LOCMI02868 significantly…" better "When next performed RNAi experiments with the identified genes, the knockdown of LOCMI16699 and LOCMI02868 significantly….

We revised this sentence as the reviewer suggested.

"After behavioral assays,…" better "In behavioral assays,…" Please briefly describe in text, what assays you performed.

We revised the representations as "We next evaluated the behavioral states of locusts after the knockdown of 4VPMTs, which are directly related to the aggregation state, in a well-established behavioral assay paradigm[12,15]." in Lines 241-243.

"imcrucialor" exchange by "crucial for"

We revised the sentence as the reviewer suggested.

"nitropheol" exchange by "nitrophenol"

We revised it as the reviewer suggested.

"Attraction to 4VA of locusts may arise from the attempt for more survival and reproduction resources." Sentence unclear to me. Consider re-wording

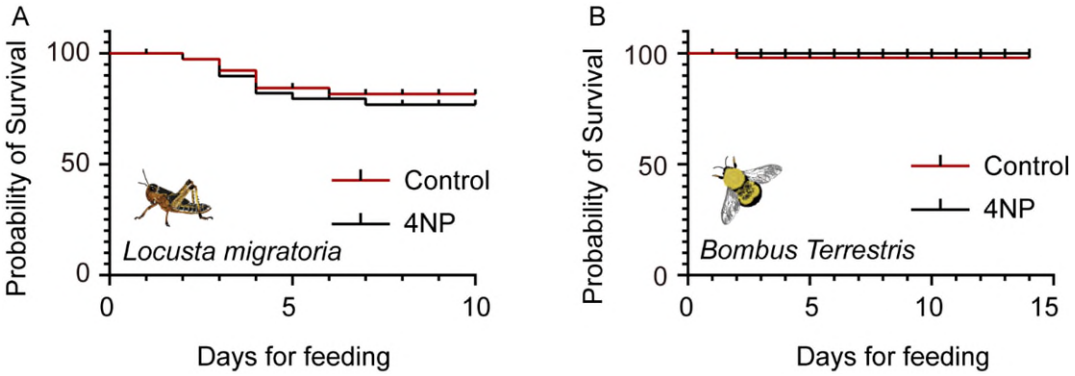
We revised the Discussion section and deleted this sentence.

“methyltranfereases” replace by “methyltransferases”

We revised it as the reviewer suggested.

“Assessing the environmental safety of small molecules is a significant public concern and must be addressed before their widespread adoption.” I fully agree. It would have been nice, if e.g longevity of animals (locust and best one or two other non-related insect species) fed with the bioactive concentrations of. 4-nitrophenol would have been included here to test of the compounds toxicity

Response: Thanks for your suggestions. We examined the survival curves for locusts and bumblebees, in which 4NP did not affect the mortality of locusts and bumble bees.



Extended Data Fig. 13. Survival curves for locusts and bumble bees following ingestion of food containing 4-nitrophenol (4NP).

Referee #3 (Remarks to the Author):

The report by Guo et al. addresses two main issues: 1) the biosynthesis of an aggregation factor for swarming locusts. Earlier work had established that 4-vinylanisole (4VA) was a likely aggregation pheromone, but part of being a pheromone is being biosynthesized in the same species it affects – something that had not been conclusively shown in the original report, and 2) developing an aggregation inhibitor from knowledge of the biosynthetic pathway. In my analysis, the authors did a good job on the first issue and an unsatisfactory job on the second.

It's challenging to work out biosynthetic pathways with insects, especially ones with no genetic system and multiple possible pathways. The authors established, at least to my satisfaction, that 4VA is biosynthesized from phenylalanine derived from plants, and that a crucial step in the biosynthesis is the conversion of 4VP to 4VA – the OH group of a phenol is converted to an ether OCH3 through addition of a methyl (CH3) group.

This is a well-known reaction catalyzed by methyl transferases (MT). MTs play multiple roles in biological regulation including the modulation of human neurotransmitters. Basically, the authors identified two main MTs, 4VPMT1 and 4VPMT2 from 9 possibilities. They tried to reduce two MT possibilities to one, but they gave up and concluded that either or both could carry out the crucial reaction. Crucially, there were a few remaining possibilities that were not investigated in depth, or at least those efforts are not reported.

The development of an inhibitor that could be used to limit locust aggregation and in principle decrease crop predation formed the much more significant part of the report. Two points should be noted. The first is the discussion in the opening paragraph that pesticides are polluting the environment. The second is that pheromones and pheromone mimics are used only for monitoring, not controlling populations. This choice, monitoring not controlling is the result of environmental regulations and economics in the US. The authors use structure-based drug design and synthesis to identify 4-nitrophenol (4NP) as a "behavioral regulators for environment-friendly locust control [abstract]." There are several things wrong with this statement.

1) There is no evidence that 4NA is a behavioral regulator. There is evidence that 4NA is an attractant, but there is no evidence that it's the only attractant, and lacking that there is no essential link to behavior. There is also evidence that 4NP inhibits the production of 4NA, which creates a possible link from 4NA to behavior, but not a definitively established one. In the language of drug discovery, the target has not been derisked.

Response: Thank you for your reminder. We probably need to clarify the research background of 4VA in our manuscript further. The 4NA mentioned by you never appears in our manuscript. We speculate that the 4NA may be a clerical error or spelling mistake of aggregation pheromone 4-vinylanisole (4VA) and is likely 4VA in your comments. To clarify this point, we attempt to introduce some background of our previous research progress about 4VA. In 2020, our group successfully identified 4VA as an aggregation pheromone of the migratory locust supported by chemical, behavior, electrophysiology, olfactory receptor identification, and field experiments (Guo et al., Nature, 2020). The role of 4VA is the biological basis of locust swarming through promoting the change from the solitary phase to the gregarious phase of the locusts (Yang et al., PNAS, 2023). Furthermore, aggregation pheromone 4VA can remarkably promote the synchrony of sexual maturation in female locusts, facilitating the formation of the high density of the offspring (Chen et al., eLife, 2022). Based on our research on 4VA, other research groups developed the peptide and nucleic acid biosensors for detecting 4VA, demonstrating its high sensitivity and specificity to its olfactory receptor (Ma et al., Sensors and Actuators B-Chemical, 2023; Hu et al., Chemical Engineering Journal, 2023). Thus, 4VA is recognized as an aggregation pheromone and crucial behavioral regulator for the migratory locust and can be used to monitor the changes in population density and attract locust aggregation (Guo et al., Nature, 2020). Importantly,

the novelty of our present manuscript lies in its focus on the 4VA biosynthesis in locusts and the search for critical small-molecule inhibitors to block the 4VA biosynthesis and release, based on the biosynthesis routes of 4VA. This work is distinct from our previous research and significantly advances our understanding of the fundamental chemistry and biology of locust aggregation mechanisms.

2) There is no evidence that 4VPMT1 and 4VPMT2 are the only methyltransferases that can carry out the conversion. If some of the others can carry out the transformation, even at low levels, that could be enough to cause attraction.

Response: Your comments are reasonable. In order to exclude other possibility as you mentioned, we used almost all methods to confirm 4VPMT1 and 4VPMT2 as the only methyltransferases. The identification of 4VPMTs involves a rigorous, step-by-step screening process that includes chemical logic reasoning, transcriptome analysis, qPCR verification, RNAi knockdown, protein expression, and enzymatic activity assays. We confirmed that 4VA levels were significantly reduced, nearly to zero, following the double knockdown of 4VPMT1 and 4VPMT2. Based on this evidence we can obtain under existing conditions, we conclude that 4VPMT1 and 4VPMT2 are the methyltransferases that carry out the conversion. We failed to find other isoenzymes to carry out the transformation.

3) Finally, 4NP is not environment-friendly. It causes It causes severe reactions in people and is not remotely likely to meet environmental regulations. The existing evidence is enough to get it banned for wide scale environmental release.

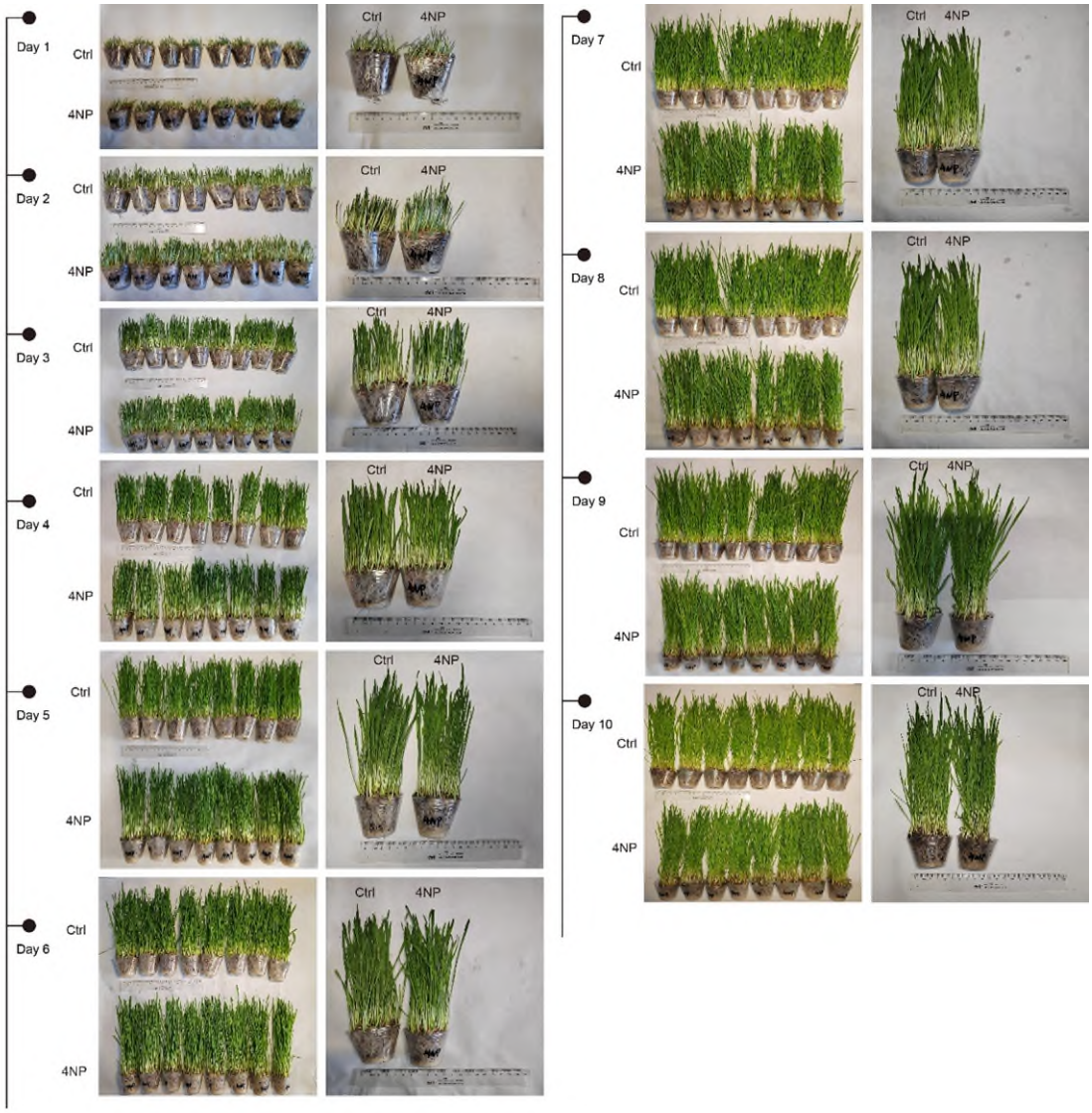
Response: Thank you for your comments. We are sorry we did not clarify our description about 4NP being environment-friendly. What we originally meant is to convey that the environment-friendliness of 4NP is comparable to that of chemical pesticides. In response to your comments, we specially designed experiments to assess the potential toxicity of 4NP. Our supplemental results demonstrate that 4NP, at the bioactive dose in this study, did not affect the growth of plants, or the survival of locusts, bumbles, and mice. Additionally, various types of adjuvants can be developed to facilitate controlled release and minimize environmental impact. In fact, the toxicity of all chemicals is indeed dose-dependent. Thus, your comments prompted us to delve deeper into the relationship between 4NP dosage and potential toxic risks. Right now, our experiments clearly indicate that 4NP is safe at current dose levels. Our present study aims to show that 4NP can significantly impede 4VA biosynthesis in the framework of basic research. When 4NP is actually used for locust control in the future, it will, of course, undergo rigorous environmental safety assessments to ensure compliance with safety standards

We evaluated the effects of 4NP on the plant health and survival. We continuously sprayed 4NP on the plants for 10 days and recorded the growth of the plants. We found that the treated plants showed a similar growth compared to the control groups. The

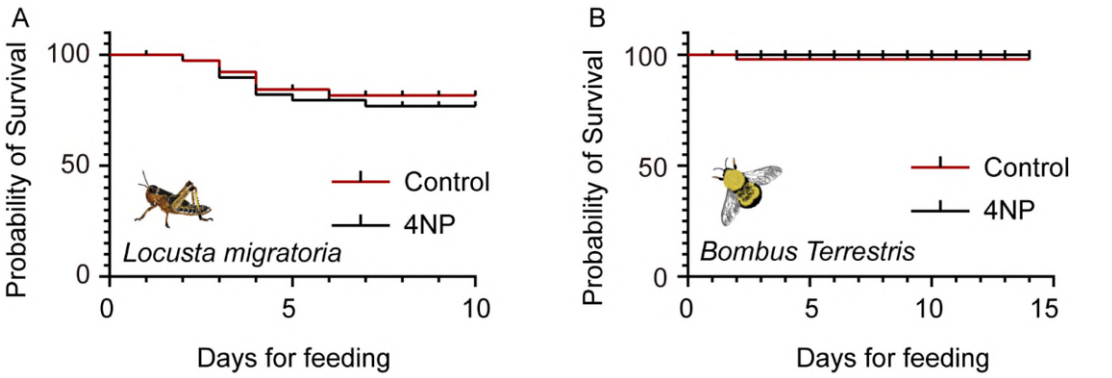
results are listed below.

We examined the survival curves for locust and bumblebee, in which 4NP did not affect the mortality of locusts and bumble bees.

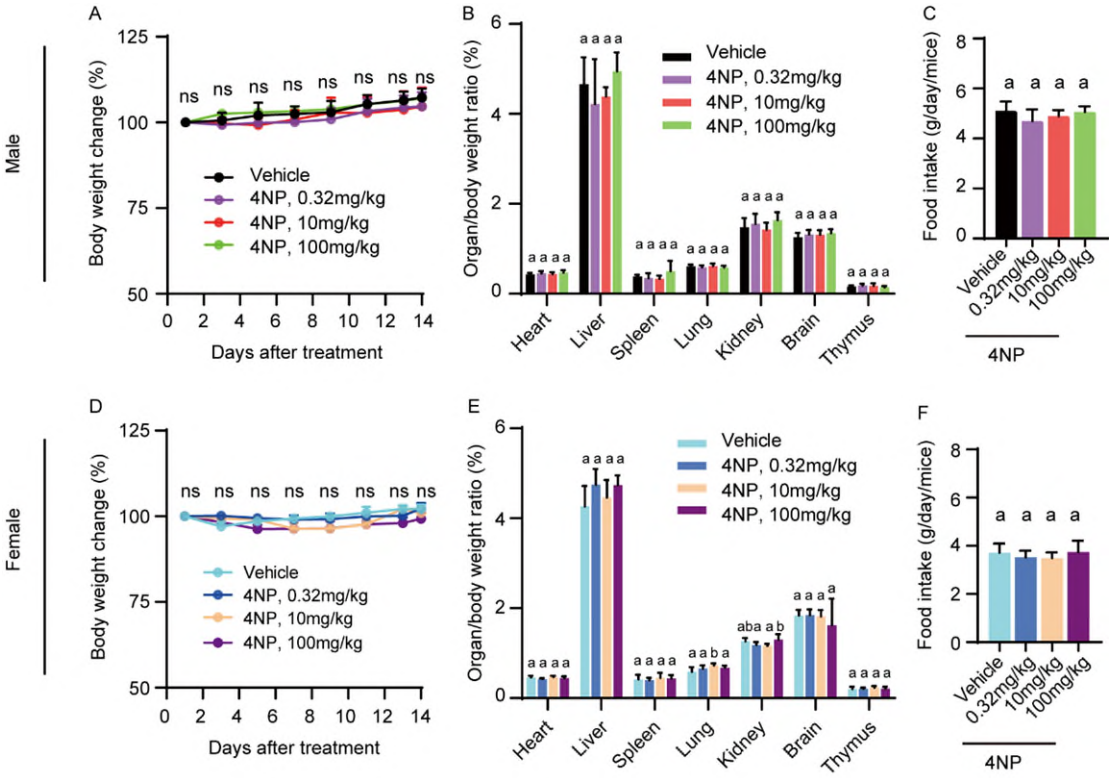
Importantly, treatment of healthy male and female CD-1 mice with 4NP once daily (at doses of 0.32, 10, and 100 mg/kg, p.o.) for 14 days shows no significant changes in body weight, organ/body weight ratios or food intake compared to the vehicle-treated group, suggesting that 4NP has a potential safety profile in mice.



Extended Data Fig. 12. The effects of 4-nitrophenol (4NP) on the growth of wheat seedlings.



Extended Data Fig. 13. Survival curves for locusts and bumble bees following ingestion of food containing 4-nitrophenol (4NP).



Extended Data Fig. 14. Mammal acute toxicity experiments in mice. Treatment of healthy male and female CD-1 mice with 4-nitrophenol (4NP) once daily (at doses of 0.32, 10 and 100 mg/kg, p.o.) for 14 days barely affected the body weight (A and D), organ/body weight ratios (B and E) or food intake (C and F) compared to the vehicle-treated group, suggesting that 4NP has a potentially favorable safety profile in mice. n = 8. Different letters indicate statistically significant differences between groups using one-way ANOVA (Tukey's multiple comparisons test, $P < 0.05$). ns, not significant.

Referee #4 (Remarks to the Author):

The frequent and destructive locust plagues pose a severe threat to agriculture, economies, and ecosystems worldwide. For nearly a century, the field of locust chemical ecology has focused on understanding the chemical signals that influence locust behavior, particularly those driving aggregation behaviors that underpin swarm

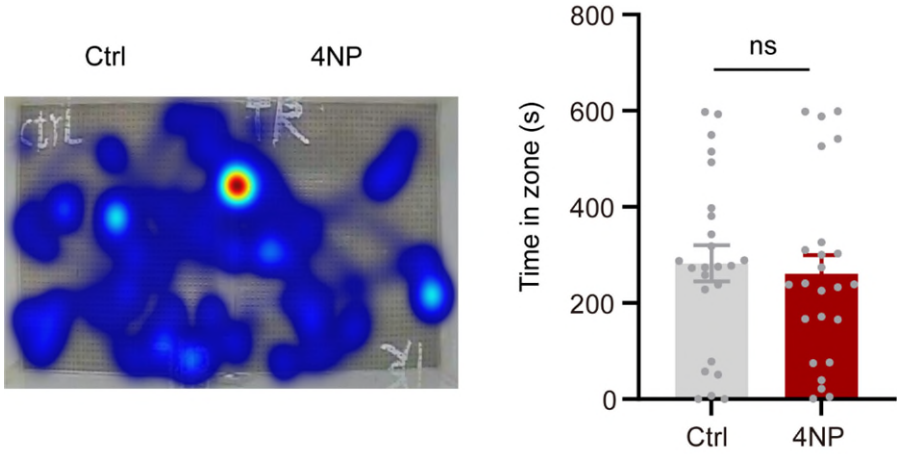
formation and lead to plagues. In 2020, Guo et al. identified the aggregation pheromone, 4-vinylanisole (4VA), and elucidated the biological mechanisms of locust aggregation behavior. In the current study, Guo et al. revealed the biosynthetic pathway of 4VA, further advancing our understanding of locust aggregation. The identification and characterization of two key methyltransferases, 4VPMT1 and 4VPMT2, involved in 4VA biosynthesis offer a mechanistic understanding of how these enzymes catalyze the conversion of 4-vinylphenol (4VP) to 4VA. This work significantly enhances our knowledge of the biochemical underpinnings of locust swarm behavior at the biosynthetic level. Most notably, the elucidation of the X-ray co-crystal structure of 4VPMT in complex with 4VP and S-adenosylmethionine (SAM) is a particularly impressive feat. Based on this structure, the researchers successfully screened and identified 4-nitrophenol as a substrate surrogate that inhibits the activity of 4VPMTs, thereby blocking 4VA production and suppressing locust aggregation. This is a groundbreaking discovery in locust chemical ecology, as it presents a novel approach to targeting locust swarms by disrupting pheromone biosynthesis rather than relying on chemical pesticides. The development of 4-nitrophenol as a small molecule inhibitor is particularly noteworthy. It represents a highly targeted and environmentally friendly method of locust control, with significant potential to serve as a viable alternative to traditional pesticides. This application-driven research makes the findings not only scientifically valuable but also practically significant. Overall, the experimental design is robust, and the results are both compelling and conclusive. The identification of 4VA biosynthetic enzymes and the discovery of an effective inhibitor are landmark achievements. However, before moving forward, I have a few concerns I would like the authors to address

comments

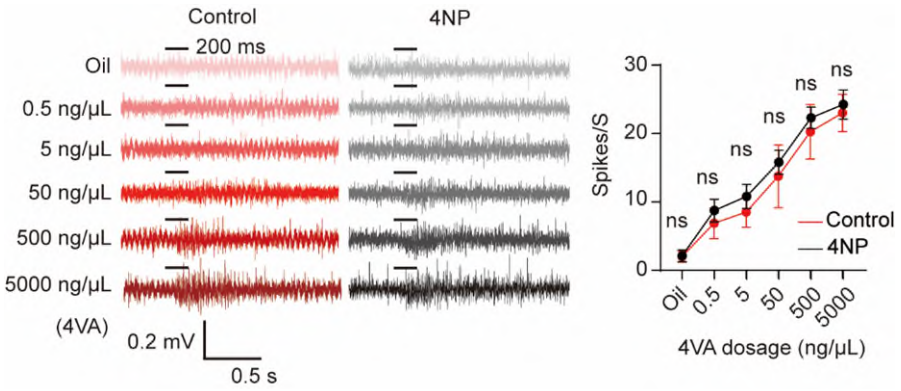
The identification of 4-nitrophenol as a small-molecule inhibitor of 4VPMT activity is an important finding. However, there are potential confounding factors that need to be addressed. While the study tests the effect of 4-nitrophenol on locust gregarious behavior, it is also possible that this compound inhibits other enzymes unrelated to 4VA biosynthesis, which could independently disrupt locust aggregation behavior. Additionally, 4-nitrophenol might interfere with locust detection of 4VA or could itself evoke specific behavioral responses in locusts. Have you tested whether 4-nitrophenol elicits any direct behavioral effects in locusts? It would be essential to exclude the possibility that the observed behavioral changes are due to effects other than the inhibition of 4VA biosynthesis. I recommend conducting behavioral assays to determine whether 4-nitrophenol directly affects locust behavior or sensory perception of 4VA. This would strengthen the conclusion that 4-nitrophenol's impact on locust behavior is specifically due to the inhibition of 4VPMT activity.

Response: Thank you for your valuable suggestions. We first assessed the behavioral responses of locusts to 4NP in the dual-choice behavioral system. We found that 4NP did not elicit any direct attract or repulsion responses. We then conducted an evaluation to determine the impact of 4NP on the sensory perception of locusts following its

application. The results showed that 4NP did not affect the electrophysiological responses of basiconic sensilla, which are the primary sensilla responsible for detecting 4VA. These findings indicate that the impact of 4NP on locust behavior is primarily due to the inhibition of 4VPMTs and the consequent reduction in 4VA levels. The results are listed below.



Extended Data Fig. 10. The behavioral responses of locusts to 4-vinylphenol (4NP). n = 24. *P* values were determined by the Wilcoxon signed-rank test. ns, not significant. Data are presented as mean ± SEM.

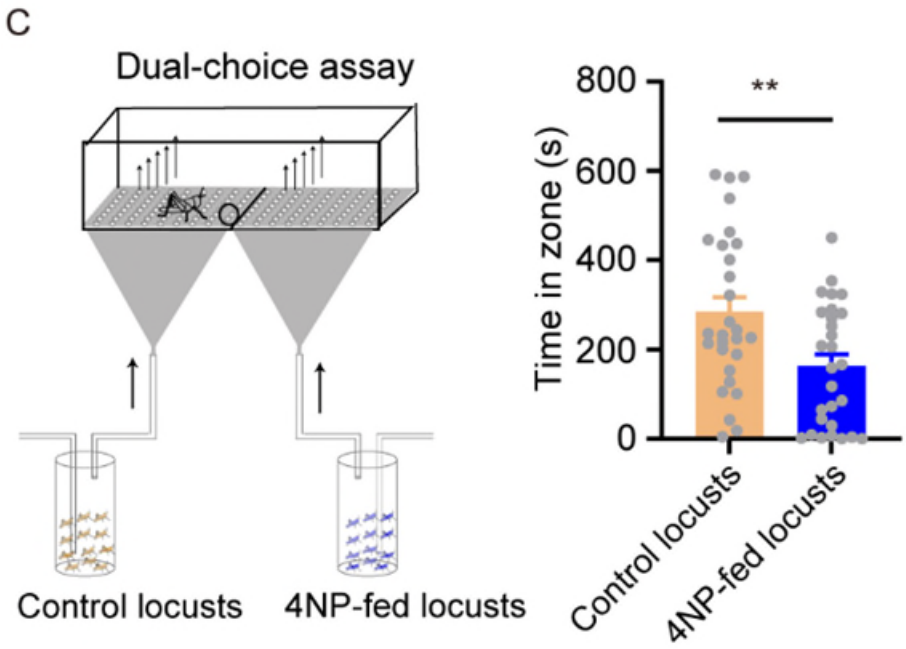


Extended Data Fig. 11. The electrophysiological responses of basiconic sensilla to 4VA after application of 4NP. *P* values were determined by a two-tailed unpaired *t*-test, n = 8 for control, n = 16 for 4NP. ns, not significant.

In the abstract, the authors claim that 4-nitrophenol suppresses locust aggregation. However, the behavioral tests presented in the manuscript do not sufficiently support this claim. The current experimental design appears more focused on assessing locust phase-shift behaviors rather than directly testing aggregation. To substantiate the conclusion that 4-nitrophenol impairs locust aggregation, it is essential to verify whether it affects the attraction between individuals within a population. I recommend using a two-choice behavioral assay, as in Guo et al. (2020, Nature), to test whether 4-nitrophenol disrupts the attraction between locusts. Specifically, VOCs should be

collected from both control gregarious-phase locusts and 4-nitrophenol-fed locusts, and tested to see whether other locusts show a preference between these odor sources. If the results show that locusts preferentially orient toward VOCs from control gregarious-phase locusts over those from 4-nitrophenol-fed locusts, this would provide strong evidence that 4-nitrophenol suppresses locust aggregation. Implementing this additional behavioral test would greatly strengthen the authors' claim regarding 4-nitrophenol's role in inhibiting aggregation.

Response: Thank you for your suggestions. Based on your suggestion to use gregarious locusts as controls and 4NP-fed locusts as experimental subjects, we conducted tests in a dual-choice behavioral system. The results indicated a significant preference for the control zone among the tested locusts, suggesting that the 4NP-fed locusts were unable to release 4VA. The results are listed below.



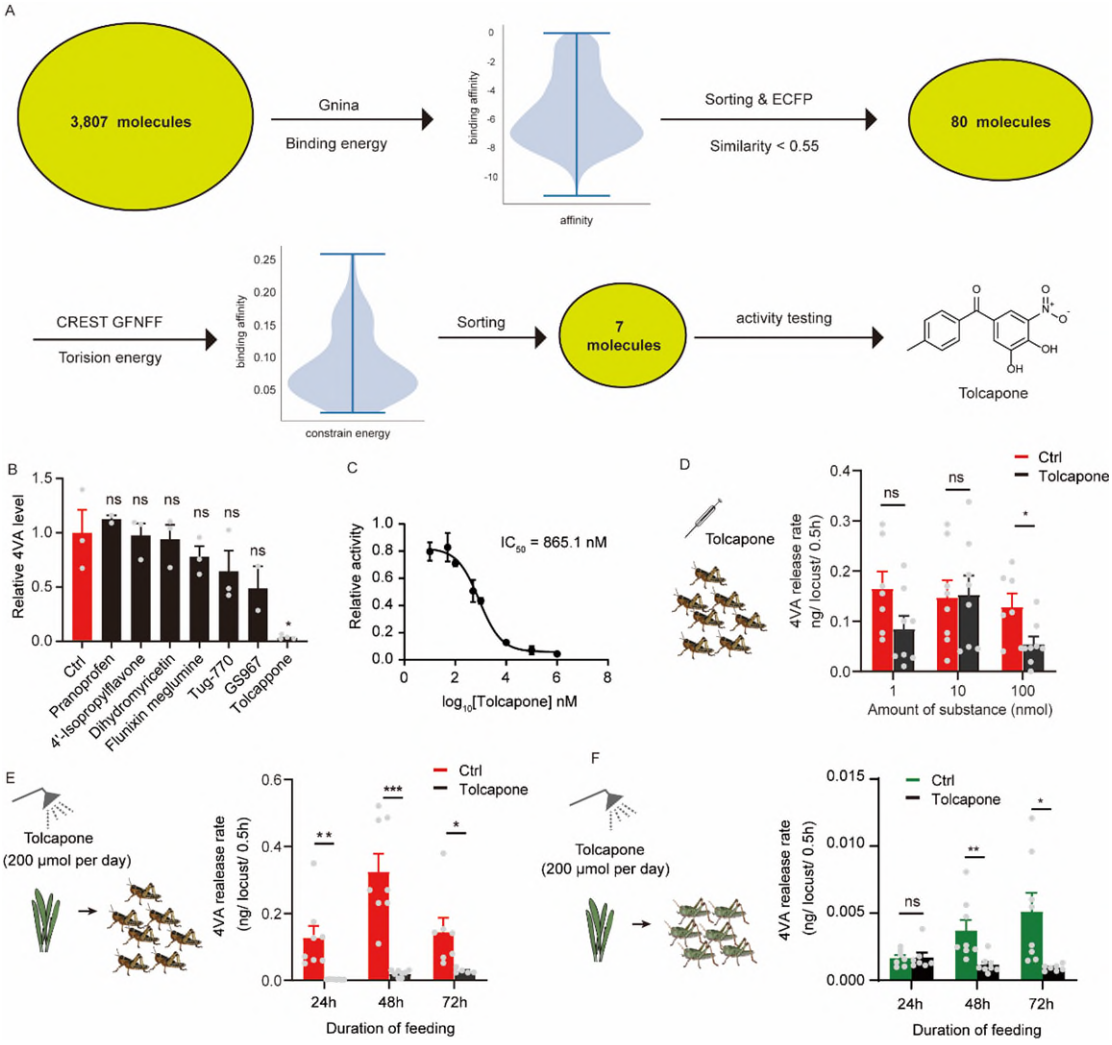
Revised Figure 6C. The preference of locusts for volatiles emitted by control gregarious locusts compared to those fed with 4NP.

While the identification of 4-nitrophenol as an inhibitor of 4VPMTs is a promising result, I have some reservations regarding the screening process. The manuscript mentions screening fewer than 20 4VP analogues as substrate competitors, which seems limited when aiming to discover an effective inhibitor to block enzyme function. Given the availability of the co-crystal structure of 4VPMT in complex with 4VP and S-adenosylmethionine (SAM), it would be highly beneficial to employ a more comprehensive approach, such as AI-assisted docking and virtual screening, to identify potential inhibitors. AI-driven docking methods could allow for the exploration of a much larger chemical space, increasing the likelihood of identifying more potent and

selective inhibitors of 4VPMTs.

Response: Excellent suggestion! According to your suggestions, we identified as much as possible inhibitors of 4VPMTs through virtual screening with higher potency and biosafety from the library of preclinical and clinical drugs in our lab. We conducted a virtual screening on a library of 3,807 active small molecules using the GNINA software to identify potential target compounds. During the screening process, we first ranked all the small molecules based on their scores from low to high. To enhance the diversity of the screening results, we analyzed the structural similarity of the top-scoring small molecules and excluded compounds that are similar to the previously selected compounds. Ultimately, 80 candidate molecules with structural diversity were chosen for the next round of screening. In the second round of screening, we used CREST to determine the optimal conformation of each molecule and to compare these optimal conformations with their bound conformations. The purpose of this step was to evaluate the conformational stability of the binding models of candidate molecules in the pocket, in particular, to exclude compounds with excessive torsional strain that might render them unstable. After rigorous screening, seven small molecules emerged as the most promising candidates and were selected for subsequent experimental validation.

Among the 7 molecules, one molecule exhibited a noticeable inhibiting effect (~85% inhibition) on the enzymatic activity of 4VPMT1. It turned out to be tolcapone, a clinically used drug to treat symptoms of Parkinson's disease. Despite lower inhibiting efficiency (IC_{50} = 865.1 nM) than 4NP, feeding the locusts with tolcapone can significantly inhibit the production of 4VA *in vivo*, demonstrating its potential application in locust control. Since tolcapone is a clinically used drug, its safety profiles have been well established.



Extended Data Fig.15. Tolcapone is a small-molecule inhibitor for 4VA production. (A) Virtual screening was performed to identify potential inhibitors of 4VPMTs with higher potency and biosafety from the library of preclinical and clinical drugs. (B) The relative enzymatic activities of 4VPMT1 after adding candidate small molecules. n = 3. (C) IC_{50} plots from *in vitro* enzymatic assay of 4VPMT1 against tolcapone. (D) The effects of tolcapone at different amounts on 4VA production in locusts, n = 7. (E and F) 4VA production of gregarious locusts (E) and crowded solitary locusts (F) after feeding of plants sprayed with tolcapone, n = 8. P values were determined by a two-tailed unpaired t-test (B, D, E, F). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Data are presented as mean ± SEM.

The identification of 4-nitrophenol as a promising chemical for inhibiting locust swarming is indeed significant. However, I would like to raise a concern regarding the cost-effectiveness of this compound for practical applications. Since the potential use of 4-nitrophenol as a behavioral regulator will involve considerations of economic viability, it would be beneficial for the authors to discuss the cost implications of this

compound in the context of its application in locust control. Additionally, this study has successfully identified key biosynthetic genes involved in 4VA production. In the future, these target genes could also be utilized for developing RNAi insecticides aimed at controlling locust swarming behaviors. I suggest that the authors expand the discussion section to include these considerations, addressing both the economic aspects of 4-nitrophenol and the potential for innovative genetic approaches in pest management.

Response: Your comments are reasonable. Based on your suggestions, we expanded the discussion section as "4-Nitrophenol (4NP) inhibits 4VA biosynthesis via competitively binding to 4VPMTs. As a substrate analog, the nucleophilicity of the phenol group in 4NP is dramatically reduced due to the existence of the electron-withdrawing nitro group. Thus, 4NP is a less reactive substrate, showing lower reactivity for 4VPMTs. Moreover, 4NP shows higher binding affinity to 4VPMTs than 4VP, therefore competitively binding with the enzymes against 4VP. 4VA can attract individuals into groups[6] and affect the social interactions between members and the acquisition of gregarious behaviors after they join the groups[12]. In the present study, blocking 4VA production through 4NP spraying significantly inhibits the acquisition and maintenance of gregarious behavior. Thus, 4NP is a potent regulator with great potential application in locust control. Although 4NP application did not affect the growth of plants and the survival of locusts, bumble bees, and mice in the present study, other potential health risks need further evaluation before large-scale application. Additionally, applying newly developed adjuvants along with 4NP will facilitate controlled release and minimize environmental impact under controllable costs. On the other hand, virtual screening was performed to identify a new candidate inhibitor, tolcapone, which is a clinically used drug. This small molecule can also significantly inhibit 4VPMTs *in vitro* and *in vivo* and consequently block the production of 4VA. Thus, efficient, low cost, and safe inhibitors can be identified and optimized for future applications. Besides applying small-molecule inhibitors, RNAi insecticides targeting 4VPMTs can also be developed to control locust swarming behaviors. Taken together, manipulation of 4VA biosynthesis is an effective and sustainable control strategy for future locust control." in Lines 436-457.

The authors report that both the emission level of 4VA and the expression level of 4VPMT genes are highly elevated in the hind leg, suggesting this tissue's pivotal role in 4VA biosynthesis. However, upon examining Figure 4A, it is evident that while 4VPMT1 is most highly expressed in the hind leg, 4VPMT2 shows its highest expression in the midgut. This discrepancy raises questions about the distinct functional roles of these genes in 4VA biosynthesis and their contributions to locust aggregation. Interestingly, previous research has shown that gregarious locust feces can also release 4VA, suggesting that the midgut expression of 4VPMT2 may be linked to microbial interactions or processes related to digestion. Could the authors elaborate on the functional differences between 4VPMT1 and 4VPMT2, particularly in the context of their distinct tissue-specific expression patterns? Additionally, how might the midgut expression of 4VPMT2, potentially influenced by the microbiome, contribute to

pheromone biosynthesis or locust behavior?
Following up on the concerns regarding the expression of 4VPMT2 in the midgut and the role of locust feces in releasing 4VA, I was curious about the method used to collect volatile compounds from locusts. When locusts are confined to a small jar or vial for odor collection, they may excrete feces during this process, and as mentioned earlier, feces can release 4VA. How did the authors control for or exclude the potential influence of feces on the volatile collection and quantification of 4VA?

Response: Good questions. Since 4VA has been detected in both locust bodies and feces, albeit at lower concentrations in feces (Guo et al., Nature, 2020), we hypothesize that the elevated expression of 4VPMT2 in the gut is a response to the 4VP provided by gut symbionts. The 4VA produced from 4VP by 4VPMT2 in the gut may be excreted with feces. Consequently, we did not exclude the emissions of 4VA from feces, as it is likely a product of the locust's 4VPMT2. In the Discussion section, we will discuss the interplay between 4VPMT2, gut microbiota, 4VA, and behavior. Additionally, our research on 4VA and symbionts will be expanded into a separate study focusing on locust gut microbiota.

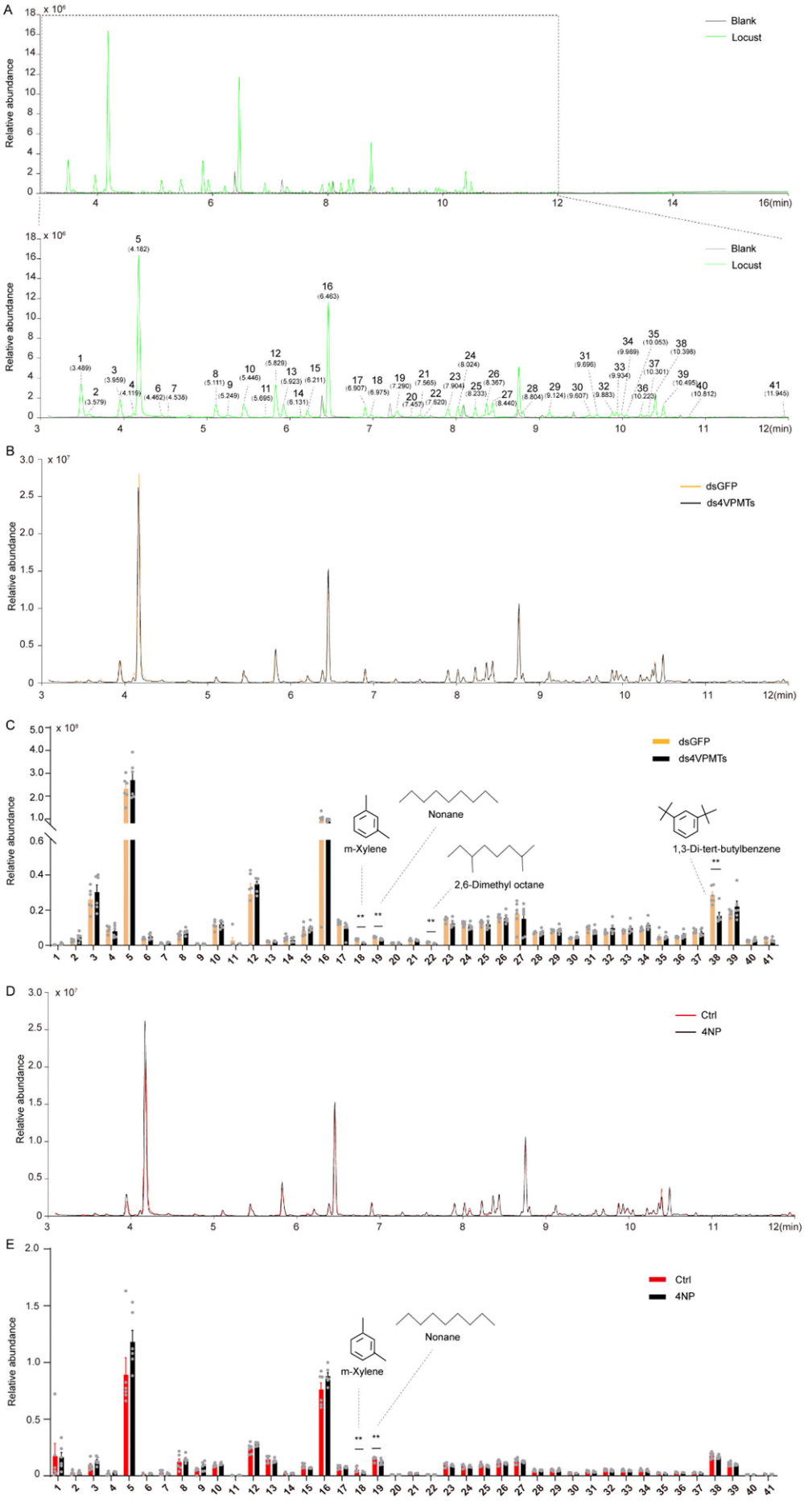
We added some discussion about this point in the revised Discussion as "Gut microbiota is suggested to play a possible role in the biosynthesis of 4VA. Phenylalanine ammonia-lyase, an enzyme that catalyzes the conversion of phenylalanine to cinnamic acid, is found in plants, fungi, and bacteria [17]. When locusts are fed an artificial diet enriched with deuterated phenylalanine, the presence of deuterated cinnamic acid can be detected, indicating that gut microbiota likely plays a role in the lysis of phenylalanine. However, the contribution of the gut microbiota to supplying cinnamic acid for 4VA biosynthesis may be relatively minor compared to the role of host plants. Our previous study indicated that feces can release 4VA[6]. Furthermore, our current findings show that 4VPMT2 is highly expressed in the gut compared to other tissues. Additionally, 4-vinylphenol can be detected in various tissues, including the gut of locusts. These results imply that gut microbiota may serve as a substrate supplier for 4VPMT2 to produce 4VA in the feces of locusts. Further investigation is needed to understand the gut microbiota and the complex interactions between plants, gut microbiota, and locusts." in Lines 422-435.

The authors demonstrate that knocking down or inhibiting the activity of 4VPMTs significantly reduces the release of 4VA. However, I am curious about the impact of these manipulations on the overall profile of volatile organic compounds (VOCs) emitted from the locusts. Could the authors provide gas chromatograms showing the composition of volatiles emitted from locusts under different conditions: with and without 4VPMT expression, and with and without 4VPMT inhibition? This data would help clarify whether other VOCs are produced when 4VA is suppressed. Furthermore, if any additional compounds emerge in the VOC profile upon 4VPMT manipulation, it is essential to assess their behavioral effects on locusts. This would help exclude the

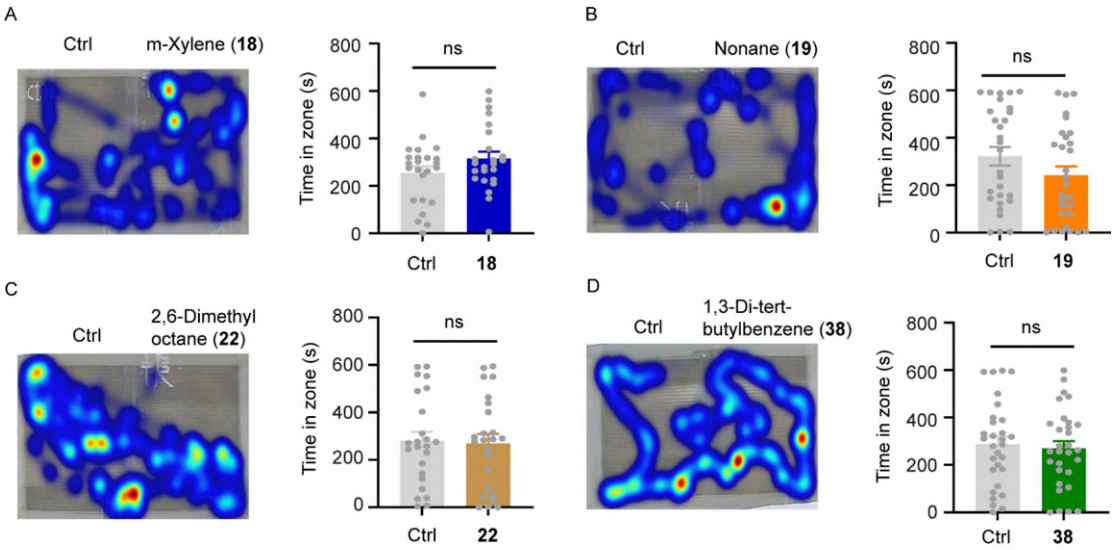
possibility that these compounds influence locust behavior independently of 4VA.

Response: Thanks for your suggestion. Based on your comments, we compared the VOCs between gregarious locusts after the knockdown of 4VPMTs and dsGFP groups. When quantifying the peaks between dsGFP groups and ds4VPMTs groups, we found that the most significant changes of four compound peaks, m-xylene (**18**), nonane (**19**), 2,6-dimethyl octane (**22**), and 1,3-di-tert-butylbenzene (**38**), showed a significant decrease after the knockdown of 4VPMTs. Then evaluating the VOCs of gregarious locusts after feeding of 4NP, we found that two compounds, m-xylene (**18**) and nonane (**19**), showed significant decreases after feeding of 4NP. Thus, new VOCs are not produced when 4VA is suppressed.

Upon testing the behavioral responses of locusts to these four compounds, we found that none of them were able to elicit either attractive or repellent responses in the locusts. These results indicated that the impact of inhibiting 4VPMTs on locust behavior is primarily due to the reduction of 4VA levels, rather than changes in other compounds or the abundance of specific substances. These results are listed below.


A
x 10^6
Relative abundance
Blank
Locust
16(min)
B
x 10^7
dsGFP
ds4VPMTs
12(min)
C
x 10^6
Nonane
m-Xylene
2,6-Dimethyl octane
1,3-Di-tert-butylbenzene
D
x 10^7
Ctrl
4NP
12(min)
E
Ctrl
4NP
Nonane
m-Xylene

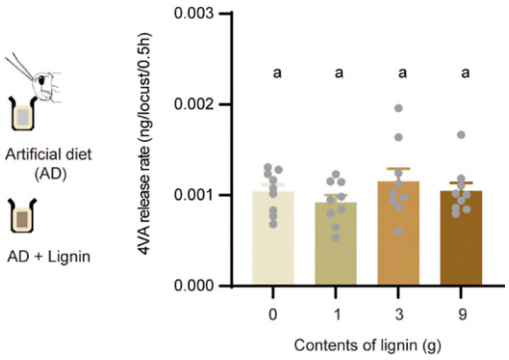
Extended Data Fig. 4. The volatile organic compounds (VOCs) of locusts after RNAi of 4VPMTs and application of 4NP. (A) The total ion chromatograms (TICs) of locust VOCs compared to the blank. (B) The TICs of locust VOCs after RNAi of 4VPMTs. (C) The relative abundance of 41 peaks of locust VOCs after RNAi of 4VPMTs. (D) The TICs of locust VOCs after application of 4NP. (E) The relative abundance of 41 peaks of locust VOCs after application of 4NP. P values were determined by a two-tailed unpaired t-test, n = 6. *, P < 0.05; **, P < 0.01; Data are presented as mean ± SEM.



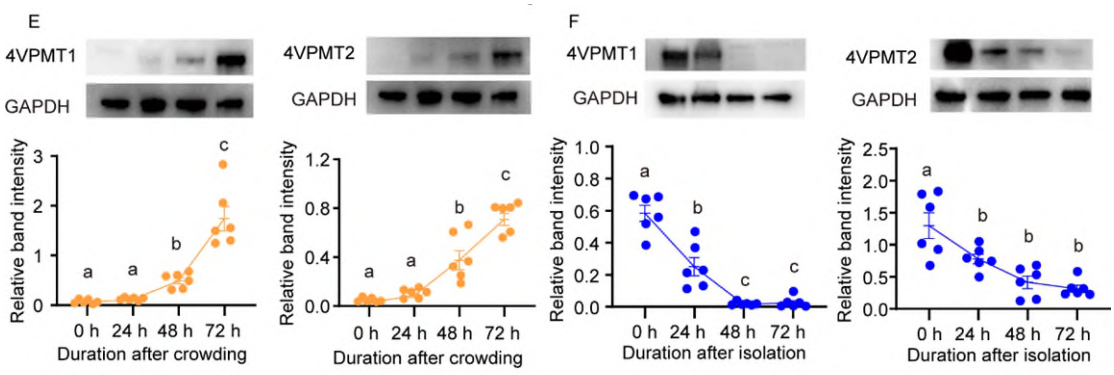
Extended Data Fig. 5. The behavioral responses of locusts to four compounds. (A-D) The behavioral responses of locusts to m-xylene (A, n = 24), nonane (B, n = 29), 2,6-dimethyl octane (C, n = 24), and 1,3-di-tert-butylbenzene (D, n = 30). P values were determined by Wilcoxon signed-rank test. ns, not significant. Data are presented as mean ± SEM.

In reviewing the manuscript, I noticed that the number of replicates in certain experiments appears to be quite low, which raises concerns about the robustness of the statistical analyses. For example, in Figure 1C, Figures 4E-F and Fig5, the limited number of replicates may not provide sufficient power for reliable statistical conclusions. Could the authors explain the rationale behind the choice of replication in these specific experiments?

Response: Based on your comments, we added more experiments for biological replicates in Figure 1C and Figures 4E-F. More biological replications still support the original conclusions. The results are listed below.



Revised Figure 1C.



Revised Figure 4E-F.

For the *in vitro* screen of small molecular inhibitors, we used three biological replicates referred to the previous studies (Hong et al., 2022, Nature; Gao et al., 2020, Nature Chemistry; Guo et al., 2021, J. Biol. Chem.).

In Figure 1C, the manuscript mentions that locusts were fed artificial food. However, it is not clear what the specific composition of this artificial feed was. Could the authors clarify the ingredients used in the artificial feed? Specifically, was the precursor compound for 4VA excluded from this feed? Given the importance of understanding the biosynthetic pathway of 4VA, it would be critical to know whether the diet provided to the locusts contained any compounds that could influence 4VA production.

Response: Thank you for your suggestion. We provided the detailed components of artificial diet in the supplementary materials.

Table S2. Components of locust artificial diet

Ingredient	Dosage
Ultrapure water	400 mL
Powdered agar	10.0 g
Casein	8.0 g
Glucose	14.0 g
Sucrose	10.0 g
Starch	28.0 g

Cellulose	24.0 g
Methylparaben	1.0 g
Pentadiene carboxylic acid	0.5 g
Wesson salt	1.5 g
Vitamin C	2.0 g
Vitamin B1	0.3 g
Glycine	0.3 g
Glusate	0.2 g
Lysine	0.2 g
Total	500 g

Table S3. Components of Wesson salt

Ingredient	V/V%
$CaCO_3$	21.0
$FeSO_4.7H_2O$	1.56
$MgSO_4$	9.0
KCl	12.0
KH_2PO_4	31.0
NaCl	10.5
$Ca_3(PO4)_2$	14.9
$CuSO_4.5H_2O$	0.039

I recommend that the authors consider revising the title to more accurately reflect the key findings of the study. A title that captures both the biochemical and behavioral aspects of the research would be beneficial. For reference, the study "Next Generation Neuropeptide Y Receptor Small Molecule Agonists Inhibit Mosquito Biting Behavior" provides a clear and informative structure that effectively conveys the focus of the work.

Response: Excellent suggestion. Based on your suggestions, we revised the manuscript title to "4-Vinylanisole biosynthetic enzyme inhibitors block the aggregation behavior in locusts".

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Comments of Referee #3 (Remarks to the Author):

The authors have conducted an impressive number of additional studies to address the concerns raised by the referees, and in my view they have largely been successful in addressing them. There is one exception. All referees raised concerns about the possibility that 4NP might have side effects that would prohibit its use. The authors conducted studies to show, to their satisfaction, that 4NP wasn't a problem for plants, insect pests, or mammals. I do not find these studies to be convincing. 4NP has serious human health liabilities, and I have attached the MSDs (material safety data sheet) from the US EPA (Environmental Protection Agency) and the legally required MSD from Sigma-Aldrich, the main supplier of MSD for researchers in the US. The Sigma-Aldrich MSD meets EU requirements. The statement in the revised manuscript: "Although 4NP application did not affect the growth of plants and the survival of locusts, bumble bees, and mice in the present study, other potential health risks need further evaluation before large-scale application." does not come close to acknowledging the serious issues surrounding 4NP. Proving something is 'safe' is a high bar, and the authors don't come close to doing that even the large amount of published data to the contrary. The manuscript is not suitable for publication in its present form, and if it continues to advocate for the utility of 4NP, it may never be.

Response: To address the reviewer's concerns regarding the safety of 4NP in its application, we removed all sections pertaining to the perspectives and discussions on the potential application of 4NP in the revised manuscript. The identification and functional validation of 4VPMTs have pinpointed key targets for controlling locust aggregation and thus enable us to develop small molecules (*e.g.* 4NP) to inhibit locust aggregation, providing a new strategy for locus swarm management.

Since 4NP was only used as a chemical tool to reveal the pivotal role of 4VPMTs in locust aggregation, we did not focus on the toxicity issue of this compound. To avoid potential misunderstandings about the utility of 4NP, we have removed all the statements on the application of 4NP in the Abstract and Discussion sections.