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# Octopamine and dopamine mediate waggle dance following and information use in honeybees

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**Article citation details**
*Proc. R. Soc. B* **287**: 20201950.
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**Review timeline**

| | |
|---|---|
| Original submission: | 26 April 2020 |
| 1st revised submission: | 11 August 2020 |
| 2nd revised submission: | 15 September 2020 |
| Final acceptance: | 18 September 2020 |

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

# Review History

## RSPB-2020-0947.R0 (Original submission)

### Review form: Reviewer 1

**Recommendation**
Major revision is needed (please make suggestions in comments)

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Good

**General interest: Is the paper of sufficient general interest?**
Acceptable

**Quality of the paper: Is the overall quality of the paper suitable?**
Acceptable

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
No

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
Yes

**Is it clear?**
Yes

**Is it adequate?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
This is an interesting study. I have some comments the authors might consider.

It's potentially problematic that there is no validation of the pharmacological octopamine and dopamine treatments reaching the brain. As a pharmacologist this is something I would have liked to see, and I would argue it would be a standard control for a study like this. I am sympathetic to the counterarguments, however. It's not clear how much an elevation of OA or DA in the brain would be needed for the subtle behavioural effects reported in this study. The precise measurement of OA and DA at brain or brain subregion level are not easy and require very specialised equipment. The authors might argue that there have been plenty of other studies showing oral treatments are effective in raising brain levels of biogenic amines. My cautions here is that the evidence of OA is much better than for DA. DA is a much more fragile molecule that OA in aqueous solution, even if an antioxidant is present. Further, if I understood the methods correctly individual bees were exposed to the compounds at a feeder on a treatment day before the testing day, and I don't think they were exposed to OA or DA during the testing day. I don't think it has been established how long an oral treatment might persist, but certainly these are molecules that are rapidly metabolised and cleared in neural tissue.

Do the authors have any data they could add to the study to validate their treatment method?

The introduction sets up an expectation of OA and DA having opposing effects on dance behaviour. I'm not sure this is right. Missing from the article is a discussion of Burke et al 2012 Nature 492 433-437, one of Scott Waddell's papers arguing that specific dopaminergic neurons in flies mediate both reward and punishment. A comparison of possible reward circuits in bees and flies was described by Sovik et al 2015 Adv Ins Phys 48 189-226. I think the discussion of possible brain roles of dopamine needs to be enhanced. It's not clear a-priori what this treatment would do to a bee's involvement in dance behaviour. The slightly ambiguous results for dopamine might be reflective of the complexity of the biology. At the moment this is discussed only in terms of dopaminergic memory circuits. It is as plausible that the authors applied two treatments that affected reward processes in different ways: one response and perception of sugar sweetness and one satiation and consumption of caloric reward.

I would like to see consideration of the possible rewarding roles of dopamine in insects included in the paper.

I also noticed that Felsenberg et al 2017 is still cited as in press, so this reference needs to be updated.

The analyses seem fine, but quite complex GLMM and LME models are used. The structure of the models are well described in the stats sections. As a supplement might it be possible to include summary tables of the models? I find that helpful for considering the model structure and how the deviance partitions among the variables and their interactions.

## Review form: Reviewer 2

**Recommendation**
Major revision is needed (please make suggestions in comments)

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Excellent

**General interest: Is the paper of sufficient general interest?**
Excellent

**Quality of the paper: Is the overall quality of the paper suitable?**
Good

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
No

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
Yes

**Is it clear?**
Yes

**Is it adequate?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
Linn and colleagues investigated whether octopamine and dopamine (previously shown to be involved in learning and reward signalling) mediated the use of private and social information in honeybees by tracking bees' persistence at a known resource and dance-following behaviour respectively. Oral administration of octopamine increased persistence at a familiar food source

and inhibited dance-following behaviour, suggesting that these bees valued private information more strongly and/or were less motivated to seek out social information. Conversely, dopamine treatment led to increased interest in following dances, though this did not translate into an increased likelihood of recruitment.

The neurophysiological underpinnings of different information use strategies is an important topic. I especially like that this study was able to investigate it in a very naturalistic context—deciding whether to continue revisiting a known resource or seek out a novel one is a choice honeybee foragers face every day. I also thought that the experiment was cleverly designed, with the use of paired training feeders providing essentially equivalent private information to control bees and those administered either OA or DA. That said, I have some concerns about the specificity and effective duration of the oral administration approach used here that I would like the authors to address.

Based on previous work that administered biogenic amines orally to honeybees, foragers were provided OA/DA at the feeders themselves. However, in most of these previous studies, treated food was provided to individuals or entire colonies that had no other food option (sometimes over multiple days). In the present study, much of the food collected by the OA/DA foragers is subsequently regurgitated and distributed throughout the colony (e.g. Grüter et al. (2006) Behav. Ecol. Sociobiol. 60). Is simply retaining treated sucrose in the crop (rather than digesting it) sufficient for OA/DA to reach and affect the brain? In addition, could the effects of OA/DA be stronger for individuals that visited the TF more often during the treatment (having presumably received a higher dose)? As the authors have this information (L125-126), could it be included in the statistical analysis?

I also wondered if administering OA/DA in this way means that control and DF foragers also received treated sucrose in the hive through trophallactic exchanges (either from TF foragers themselves or through second-order contacts). If so, could this have potentially impacted the outcomes of the experiment? For example, if OA leads to a feeder being perceived as more rewarding and DF foragers were exposed the previous day to OA in the hive, could DF foragers in these trials have danced more vigorously for their feeder than their counterparts in DA trials?

Finally, is there any information on how long the effects of orally administered OA/DA are expected to last, given that ~24 hours elapsed between treatment and testing?

Line-by-line comments:

Abstract: I think it would be best to avoid abbreviations in the abstract.

L36: “increase in”

L55-63: These sentences felt like a bit of a tangent to me. Perhaps they might be better placed in the Discussion (somewhere around L255)? That way the first paragraph just introduces social vs. personal information use, which leads in nicely to discussing what is known about the molecular mechanisms underpinning these strategies. Not a big issue- just something to consider.

L155-156: Include citations for these packages.

L157-160: Is it appropriate to treat hive as a random effect with only 3 hives in the study? When there are fewer than ~5 levels of a grouping factor, variance estimates for random effects are often not robust (see Harrison et al. (2018) PeerJ 6, e4794; Bolker et al. (2009) TREE 24, 127). Include hive as a fixed effect instead?

L168: Were these visits during the test day? The OA/DA treatment day?

Table 1: Is it the case that OA trials always preceded DA trials as this table seems to suggest? If so, could this have impacted the outcomes of the DA trials?

Figure 2: It would be nice to see the data points overlaid on the boxplots.

Thank you for a fascinating read.

## Review form: Reviewer 3

**Recommendation**
Major revision is needed (please make suggestions in comments)

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Good

**General interest: Is the paper of sufficient general interest?**
Good

**Quality of the paper: Is the overall quality of the paper suitable?**
Marginal

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
Yes

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
Yes

**Is it clear?**
Yes

**Is it adequate?**
Yes

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
In this manuscript, the authors test the hypothesis that biogenic amines (Dopamine and Octopamine) influence the use of personal and social information in honeybees.

Overall, I found that the manuscript was generally well written and presented an interesting and novel question that has not received much research attention.

My main issue with the manuscript was that I found the method section quite vague. There is some key information missing which is necessary to understand if the hypotheses have been well tested. I explain in more detail below:

Methods:

The authors mention that each colony underwent two trials (one with DA and the other with OA) however there is little information about when these trials took place. What was the time interval between trials? Isn't it likely that trial 1 interfered with trial 2, especially if the same scent was used per colony?

It's not clear how long the test period was? In the manuscript (L134-136) it says "foragers were allowed to collect 1.8 M sucrose solution for 60 to 180 min (approx. 12.00-15.00 h) at the DF, whereas both TFs remained empty". If this was the test period, why does it vary? The time should have been standardized across all trials.

Why do the authors use "number of waggle dances followed" and then "total number of waggle runs followed"? What was the response for the number of waggle dances followed? Not total? For someone that is perhaps not so familiar with honeybee dance behavior it would also be useful to have some clarification why you have distinguished between dances followed and waggle runs followed. The authors mention earlier on in the manuscript that the number of runs followed positively correlates to waggle dances followed, is it therefore informative to have both these measures?

There's no mention if TF forager choices were recorded more than once. Did the authors just record the first foraging choice the TF foragers made? If not then they should account for TF forager identity in their models otherwise there is a pseudoreplication problem. This should be clarified.

For the statistical analysis, including random effects that have less that 5 levels can result in imprecise variance estimates (Harrison et al 2018, Peer J). Here the authors include colony (3 levels) and trial (2 levels) as random effects in their models. They should redo their statistical analyses with this in mind.

General points:

Maybe a better control would have been to use an octopamine antagonist to see if the effect then subsides. The current setup shows that DA treated bees prefer the TF feeder (and follow less dances) but there could be other reasons for this preference other than biogenic amine signalling effects (e.g. maybe OA sugar tastes better and therefore there's stronger fidelity). An antagonist would directly confirm whether change in personal and social information use is affected by biogenic amine signalling.

Discussion

L255-256: I find that the manuscript is missing some biological relevance to make this statement. Were the levels of biogenic amines found in the TF honeybees comparable to natural variation levels?

# Decision letter (RSPB-2020-0947.R0)

15-May-2020

Dear Mrs Linn:

I am writing to inform you that your manuscript RSPB-2020-0947 entitled "Octopamine and dopamine mediate waggle dance following and information-use in honeybees" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

To upload a resubmitted manuscript, log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,
Dr Sasha Dall
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Your manuscript has received 3 detailed reviews from experts in the field. I have gone through these reviews, and mapped them against your manuscript. While all reviewers found aspects of the study interesting, and possibly appropriate for this journal, you will see that the reviewers also raise a range of issues, with these being largely focused around key aspects of the methodology of your study. Any revision of your manuscript would need to address these in a convincing manner, both in a response letter and in the manuscript itself. In addition, one reviewer raises issues with the contextualisation of the study in the Introduction (and, as a corollary, the Discussion). Again, this point would need to be addressed comprehensively in any revision.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
This is an interesting study. I have some comments the authors might consider.

It's potentially problematic that there is no validation of the pharmacological octopamine and dopamine treatments reaching the brain. As a pharmacologist this is something I would have liked to see, and I would argue it would be a standard control for a study like this. I am sympathetic to the counterarguments, however. It's not clear how much an elevation of OA or DA in the brain would be needed for the subtle behavioural effects reported in this study. The precise measurement of OA and DA at brain or brain subregion level are not easy and require very specialised equipment. The authors might argue that there have been plenty of other studies showing oral treatments are effective in raising brain levels of biogenic amines. My cautions here is that the evidence of OA is much better than for DA. DA is a much more fragile molecule that OA in aqueous solution, even if an antioxidant is present. Further, if I understood the methods correctly individual bees were exposed to the compounds at a feeder on a treatment day before the testing day, and I don't think they were exposed to OA or DA during the testing day. I don't think it has been established how long an oral treatment might persist, but certainly these are molecules that are rapidly metabolised and cleared in neural tissue.

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L155-156: Include citations for these packages.

L157-160: Is it appropriate to treat hive as a random effect with only 3 hives in the study? When there are fewer than ~5 levels of a grouping factor, variance estimates for random effects are often not robust (see Harrison et al. (2018) PeerJ 6, e4794; Bolker et al. (2009) TREE 24, 127). Include hive as a fixed effect instead?

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Figure 2: It would be nice to see the data points overlaid on the boxplots.

Thank you for a fascinating read.

Referee: 3

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General points:

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Discussion

L255-256: I find that the manuscript is missing some biological relevance to make this statement. Were the levels of biogenic amines found in the TF honeybees comparable to natural variation levels?

## Author's Response to Decision Letter for (RSPB-2020-0947.R0)

See Appendix A.

# RSPB-2020-1950.R0

## Review form: Reviewer 2

**Recommendation**
Accept with minor revision (please list in comments)

**Scientific importance: Is the manuscript an original and important contribution to its field?**
Excellent

**General interest: Is the paper of sufficient general interest?**
Good

**Quality of the paper: Is the overall quality of the paper suitable?**
Good

**Is the length of the paper justified?**
Yes

**Should the paper be seen by a specialist statistical reviewer?**
No

**Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.**
No

**It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.**

**Is it accessible?**
N/A

**Is it clear?**
N/A

**Is it adequate?**
N/A

**Do you have any ethical concerns with this paper?**
No

**Comments to the Author**
In their response letter, the authors do a nice job of clarifying and addressing the reviewers' comments and questions. However, there are a few instances where I would like to see more of this information in the manuscript itself. Many readers may have these same questions and shouldn't need to dig through the reviewer reports to find the answer.

In particular, I believe more information should be included in the manuscript about the efficacy of the oral treatment method used here. For example, regarding how long the effects of OA and DA are expected to last, the authors write in response to referee 1: "there is evidence that experimentally administered biogenic amines do not last very long in the brain, probably not much more than a couple of hours (e.g. Barron et al. 2007 J. Insect. Physiol.). Our thinking was that biogenic amines would affect the perception of, and learning about, the food source during the treatment time. The bee would then remember, for example, that she visited a particularly rewarding food source (with OA), which would affect her behaviour on the testing day even though the OA had already been cleared in brain tissues". It would be great to have this reasoning spelled out more explicitly in the paper itself, perhaps after L126-132 or somewhere near L92.

Similarly, the authors discuss in the response why they believe sucrose collected at the feeder and held in the crop is likely sufficient for OA/DA to reach the brain, even though much of it is subsequently regurgitated to nectar receivers in the hive (e.g. nectar in the crop is used to power the flight home; rapid increases in OA titres have been observed following ingestion). Again, I would prefer to see a note to this effect included in the text itself.

Could it also be noted (e.g. at L105-106) that the order of OA and DA trials was randomised within colonies?

Finally, at L181, I believe it would be helpful to specify that the numbers in parentheses refer to waggle runs per dance.

Thanks again for an interesting read. I look forward to seeing it published.

## Decision letter (RSPB-2020-1950.R0)

01-Sep-2020

Dear Mrs Linn:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article (https://royalsociety.org/journals/authors/author-guidelines/#data). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online

figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Dr Sasha Dall
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Thank you for your positive response to the reviews. As you can see, the reviewer is looking forward to seeing your manuscript published. However, they also suggest that various points addressed in your response should be in the manuscript itself. As a general editorial rule, if something has to be explained to a reviewer, then it should be explained in the manuscript itself, as future readers are likely to have similar questions. So, I would be grateful if you could revise your manuscript following the instructions given by the reviewer.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
In their response letter, the authors do a nice job of clarifying and addressing the reviewers' comments and questions. However, there are a few instances where I would like to see more of this information in the manuscript itself. Many readers may have these same questions and shouldn't need to dig through the reviewer reports to find the answer.

In particular, I believe more information should be included in the manuscript about the efficacy of the oral treatment method used here. For example, regarding how long the effects of OA and DA are expected to last, the authors write in response to referee 1: "there is evidence that experimentally administered biogenic amines do not last very long in the brain, probably not much more than a couple of hours (e.g. Barron et al. 2007 J. Insect. Physiol.). Our thinking was that biogenic amines would affect the perception of, and learning about, the food source during the treatment time. The bee would then remember, for example, that she visited a particularly rewarding food source (with OA), which would affect her behaviour on the testing day even though the OA had already been cleared in brain tissues". It would be great to have this reasoning spelled out more explicitly in the paper itself, perhaps after L126-132 or somewhere near L92.

Similarly, the authors discuss in the response why they believe sucrose collected at the feeder and held in the crop is likely sufficient for OA/DA to reach the brain, even though much of it is subsequently regurgitated to nectar receivers in the hive (e.g. nectar in the crop is used to power

the flight home; rapid increases in OA titres have been observed following ingestion). Again, I would prefer to see a note to this effect included in the text itself.

Could it also be noted (e.g. at L105-106) that the order of OA and DA trials was randomised within colonies?

Finally, at L181, I believe it would be helpful to specify that the numbers in parentheses refer to waggle runs per dance.

Thanks again for an interesting read. I look forward to seeing it published.

## Author's Response to Decision Letter for (RSPB-2020-1950.R0)

See Appendix B.

## Decision letter (RSPB-2020-1950.R1)

18-Sep-2020

Dear Mrs Linn

I am pleased to inform you that your manuscript entitled "Octopamine and dopamine mediate waggle dance following and information-use in honeybees" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

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Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Dr Sasha Dall
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

# Appendix A

Dear Editor,

Thank you for your correspondence and for the reviewer comments concerning our manuscript "Octopamine and dopamine mediate waggle dance following and information-use in honeybees" (RSPB-2020-0947). The comments were very helpful for revising and improving our manuscript. The line numbers in this cover letter refer to the new version of the manuscript with highlighted changes.

We hope that you will find our revision satisfactory, and look forward to hearing from you.

Best regards,

Melissa Linn

**Reply to comments made by referee #1**

**Referee**: It's potentially problematic that there is no validation of the pharmacological octopamine and dopamine treatments reaching the brain. As a pharmacologist this is something I would have liked to see, and I would argue it would be a standard control for a study like this. I am sympathetic to the counterarguments, however. It's not clear how much an elevation of OA or DA in the brain would be needed for the subtle behavioural effects reported in this study. The precise measurement of OA and DA at brain or brain subregion level are not easy and require very specialised equipment. The authors might argue that there have been plenty of other studies showing oral treatments are effective in raising brain levels of biogenic amines. My cautions here is that the evidence of OA is much better than for DA. DA is a much more fragile molecule that OA in aqueous solution, even if an antioxidant is present. Further, if I understood the methods correctly individual bees were exposed to the compounds at a feeder on a treatment day before the testing day, and I don't think they were exposed to OA or DA during the testing day. I don't think it has been established how long an oral treatment might persist, but certainly these are molecules that are rapidly metabolised and cleared in neural tissue. Do the authors have any data they could add to the study to validate their treatment method?

**Our reply:** We agree with the referee that it would have been desirable to quantify how our treatment changes OA and DA levels in the brain. We have attempted to establish these methods in our laboratory, using GC-MS, but have not yet been able to make it work. However, our main

reason for not attempting these measurements in the first place was that we felt that there is convincing evidence, especially with OA, that feeding of biogenic amines with this concentration (2mg/ml) leads to a significant increase in brain titres (Schulz & Robinson 2001). Barron et al. (2007) also provide evidence, even though they used very small quantities (10 µl). Our bees visited the feeders on average about ~5 times during the 60-min treatment phase and probably drank about 30-50 µl per visit and, thus, consumed about 150-250 µl OA- and DA-solution on average. We therefore had no reason to believe that, using the same concentration and allowing bees to drink from the feeder repeatedly, our treatment would not lead to an increased brain titre. As mentioned by the referee, the evidence for DA is less clear (Scheiner et al. 2002), but there have been more recent studies showing that feeding of DA affects learning in a way that strongly suggest that DA present in food reaches the brain (Agarwal et al. 2011). It would be very difficult to explain the results of Agarwal et al. (2011) without DA reaching the brain. Following these effects and in the absence of more detailed studies, we decided to use the same concentration of DA as for OA. But we agree with the referee that more detailed pharmacological analyses should be performed in the future to better understand to what extent biogenic amines pass the brain-haemolymph barrier and reach different parts of the brain. Given our findings, however, we are very confident that our treatment worked.

The temporal dynamics of the treatment effects are an interesting issue. As mentioned by the referee, there is evidence that experimentally administered biogenic amines do not last very long in the brain, probably not much more than a couple of hours (e.g. Barron et al. 2007 J. Insect. Physiol.). Our thinking was that biogenic amines would affect the perception of, and learning about, the food source during the treatment time. The bee would then remember, for example, that she visited a particularly rewarding food source (with OA), which would affect her behaviour on the testing day even though the OA had already been cleared in brain tissues. This would be similar to us remembering a tasty or bad meal for a long time.

**Referee:** The introduction sets up an expectation of OA and DA having opposing effects on dance behaviour. I'm not sure this is right. Missing from the article is a discussion of Burke et al 2012 Nature 492 433-437, one of Scott Waddell's papers arguing that specific dopaminergic neurons in flies mediate both reward and punishment. A comparison of possible reward circuits in bees and flies was described by Sovik et al 2015 Adv Ins Phys 48 189-226. I think the discussion of possible brain roles of dopamine needs to be enhanced. It's not clear a-priori what this treatment would do to a bee's involvement in dance behaviour. The slightly ambiguous results for dopamine might be reflective of the complexity of the biology. At the

moment this is discussed only in terms of dopaminergic memory circuits. It is as plausible that the authors applied two treatments that affected reward processes in different ways: one response and perception of sugar sweetness and one satiation and consumption of caloric reward. I would like to see consideration of the possible rewarding roles of dopamine in insects included in the paper.

**Our reply:** This is an interesting suggestion and we have modified our introduction and discussion to mention these studies and the hypotheses highlighted by the referee (L84-86 and L93-94 and L282-285). The effects of DA indeed seem to be more complex, which could explain our results. The reason why we expected DA to have opposing effects to OA was that DA treatment caused a reduction in sucrose responsiveness in bees, whereas OA caused an increased responsiveness (Scheiner et al. 2002). Our expectation seemed plausible if dance interest is indeed mediated by reward perception. But we agree that this view may be too simplistic.

**Referee:** I also noticed that Felsenberg et al 2017 is still cited as in press, so this reference needs to be updated.

**Our reply:** Felsenberg et al 2017 has been updated.

**Referee:** The analyses seem fine, but quite complex GLMM and LME models are used. The structure of the models are well described in the stats sections. As a supplement might it be possible to include summary tables of the models? I find that helpful for considering the model structure and how the deviance partitions among the variables and their interactions.

**Our reply:** We have added this information in a supplementary file and refer to this file in the Methods section (L165).

**Reply to comments made by referee #2**

**Referee:** Based on previous work that administered biogenic amines orally to honeybees, foragers were provided OA/DA at the feeders themselves. However, in most of these previous studies, treated food was provided to individuals or entire colonies that had no other food option (sometimes over multiple days). In the present study, much of the food collected by the OA/DA foragers is subsequently regurgitated and distributed throughout the colony (e.g. Grüter et al. (2006) Behav. Ecol. Sociobiol. 60). Is simply retaining treated sucrose in the crop (rather than

digesting it) sufficient for OA/DA to reach and affect the brain? In addition, could the effects of OA/DA be stronger for individuals that visited the TF more often during the treatment (having presumably received a higher dose)? As the authors have this information (L125-126), could it be included in the statistical analysis?

**Our reply**: The referee raises an interesting question. It seems that the transfer of biogenic amines from ingested food to the brain happens quite quickly: even if only a small quantity of OA solution was fed to harnessed bees (10 µl of OA solution), this increased OA titres in different body parts, including the head capsule, often within minutes (Barron et al. 2007). When bees are flying, they use energy stored in their crop, most likely also nectar they collect. Gmeinbauer & Crailsheim (1993), for example, found that glucose solution that was consumed by a bee just after flight very quickly appeared in the haemolymph. Even though we do not know the exact route of biogenic amines through the body in our experimental conditions, evidence suggests that biogenic amines present in food samples can reach the brain fast.

We thank the referee for suggesting to include the number of treatment visits in the model to test if this impacts the effects of OA and DA. We now did this by testing the interaction between treatment and the number of visits during treatment (e.g. Dances followed ~ Treatment*Visits). While the main effect "treatment" remained significant, we also found a significant interaction. The follow-up analysis showed that treatment visit number had a positive effect on dance interest in OA- treated bees, but not in the other two groups. We have added this to the results section (L202-213). This suggests that OA has less of an (inhibitory) effect on dance following in bees that visited the food source more often. We can think of two explanations for this. First, it is possible that the most motivated bees, i.e. those that visited the food source most often, are less affected by OA. Second, larger doses of OA might lead to a lower inhibition of dance interest. Both could be the result of molecular mechanisms that attenuate OA signalling if OA brain titres are high (e.g. Böhm et al. 1997, Biochem. J. 322:1). We briefly mention this in the discussion of the revised version (L260-265).

**Referee:** I also wondered if administering OA/DA in this way means that control and DF foragers also received treated sucrose in the hive through trophallactic exchanges (either from TF foragers themselves or through second-order contacts). If so, could this have potentially impacted the outcomes of the experiment? For example, if OA leads to a feeder being perceived as more rewarding and DF foragers were exposed the previous day to OA in the hive, could DF foragers in these trials have danced more vigorously for their feeder than their counterparts in DA trials?

**Our reply**: It is indeed possible that bees from the control group might have received small samples of food containing OA or DA inside the hive. If true, we would expect that the quantity experienced by control bees was much lower than in OA- and DA-treated bees. This could mean that we underestimate the effects of the OA and DA treatment. We are therefore confident that our conclusions are conservative and warranted.

The suggestion that bees might dance more or more vigorously the day after an OA treatment than after a DA treatment is interesting. We had another look at the dance data (see Table 1), but there doesn't seem to be a clear pattern. For example, in 2 of 3 colonies, we recorded more dances for the DF after the DA treatment and in 1 of 3 colonies more waggle runs were performed after the DA treatment. On the other hand, we tend to see strong day-to-day variation in dance intensity, which are most likely driven by weather effects or the availability of alternative food sources (see also Seeley 1995). It is also important to note that in our statistical analysis, we compared treatment bees to control bees, rather than DA vs. OA-treated bees and that we used "trial" (= day) as a random effect to "inform" the model about day effects.

**Referee**: Finally, is there any information on how long the effects of orally administered OA/DA are expected to last, given that ~24 hours elapsed between treatment and testing?
**Our reply**: As far as we know, it is not yet well known for how long OA and DA treatment can affect behaviour. Experimentally administered OA and DA seems to be cleared quite quickly from the brain, probably within a couple of hours (see e.g. Barron et al. 2007 J. Insect Physiol.). However, if OA and DA affect the acquisition of learned information (Agarwal et al. 2011; Behrends & Scheiner 2012), then it could potentially affect long-term memory. Our results indeed support this possibility.

**Referee:** Abstract: I think it would be best to avoid abbreviations in the abstract.
**Our reply:** We have changed this accordingly.

**Referee:** L36: "increase in".
**Our reply**: L36: "increase" has been changed into "increase in".

**Referee:** L55-63. These sentences felt like a bit of a tangent to me. Perhaps they might be better placed in the Discussion (somewhere around L255)? That way the first paragraph just introduces social vs. personal information use, which leads in nicely to discussing what is

known about the molecular mechanisms underpinning these strategies. Not a big issue- just something to consider.

**Our reply:** For brevity reasons, we have condensed this part (L57-63).

**Referee**: L155-156: Include citations for these packages.

**Our reply**: We have added this information.

**Referee:** L157-160. Is it appropriate to treat hive as a random effect with only 3 hives in the study? When there are fewer than ~5 levels of a grouping factor, variance estimates for random effects are often not robust (see Harrison et al. (2018) PeerJ 6, e4794; Bolker et al. (2009) TREE 24, 127). Include hive as a fixed effect instead?

**Our reply**: We are aware of the discussion regarding rules of thumb about when to use a grouping factor as a fixed- or a random effect. This seems particularly important if one is interested in the estimates of the random effects themselves, but might be less relevant in a study such as our since we were not interested in "hive" effects per se, but wanted to account for the fact that bees from the same colony are not biologically independent samples. We suspect that including "hive" as a fixed-effect would violate the assumption of independence, an important assumption for models that only use fixed-effects (Sokal & Rohlf 1995).

For our analysis, we tested whether having "hive" in the models improved the AIC compared to models without "hive". This was not usually the case, whereas "trial" turned out to be a very important random effect. Put differently, the p-values for the fixed-effects were almost identical irrespective of whether we included "hive" or not. Our final models, therefore, usually only contained "trial" (with 6 levels) as a random effect, thus fulfilling the criteria of having at least 5 levels. We provide details about our models in a new supplementary file. We prefer to present our statistics in this way, but can add "hive" as a fixed-effect if the referees and editor prefer this.

**Referee:** L168. Were these visits during the test day? The OA/DA treatment day?

**Our reply**: Yes, this refers to the visits of the feeder during the treatment time, we clarified this in the new version.

**Referee:** Table 1: Is it the case that OA trials always preceded DA trials as this table seems to suggest? If so, could this have impacted the outcomes of the DA trials?

**Our reply**: No, the order was random. We presented it in the table like this for a better overview.

**Referee:** Figure 2: It would be nice to see the data points overlaid on the boxplots.
**Our reply**: We have changed the figure as suggested.

**Reply to comments made by referee #3**

**Referee:** The authors mention that each colony underwent two trials (one with DA and the other with OA) however there is little information about when these trials took place. What was the time interval between trials? Isn't it likely that trial 1 interfered with trial 2, especially if the same scent was used per colony?
**Our reply:** We thank the referee for bringing this omission to our attention. There were 6-14 days between the two trials performed with the same colony. We added this information in the revised version (L111). It is important to note that we never used the same bees for two trials, but always trained and marked new cohorts. Additionally, trial order was assigned randomly. The turnover among the foragers is considerable, about 10% of foragers die each day (Seeley 1995). Thus, we deem it unlikely that one trial affected the next one in any significant and systematically biased way.

**Referee:** It's not clear how long the test period was? In the manuscript (L134-136) it says "foragers were allowed to collect 1.8 M sucrose solution for 60 to 180 min (approx. 12.00-15.00 h) at the DF, whereas both TFs remained empty". If this was the test period, why does it vary? The time should have been standardized across all trials.
**Our reply**: We agree with the referee that in an ideal world we would have used a standardized duration. The main problem with this kind of experiment is that there is considerable daily variation in activity at the DF, e.g. depending on the temperature, sunshine, alternative foraging options or simply coincidence. It can happen that only 1 or 2 bees are collecting at the DF for the first hour, which do not dance. Sometimes more than an hour passes before an efficient group (>5) of DF foragers collects at the DF and performs waggle dances. This is why we usually try to remain flexible to some extent, while standardizing the factors we can control.

**Referee:** Why do the authors use "number of waggle dances followed" and then "total number of waggle runs followed"? What was the response for the number of waggle dances followed? Not total? For someone that is perhaps not so familiar with honeybee dance behavior it would also be useful to have some clarification why you have distinguished between dances followed and waggle runs followed. The authors mention earlier on in the manuscript that the number of runs followed positively correlates to waggle dances followed, is it therefore informative to have both these measures?

**Our reply**: We agree that we should have explained this better. The two measurements indicate two slightly different aspects of dance following. Even bees that do not decode the dance often follow dances (e.g. Biesmeijer & Seeley 2005; Grüter et al. 2008). So, it was plausible that TF foragers treated with OA follow an equal number of dances, but still show an increased tendency for using private information. We were interested in whether this was the case. The number of waggle runs followed, on the other hand, provides a more immediate insight into whether bees are interested in using the social dance information. Bees that are decoding the waggle dance follow more waggle runs (e.g. Biesmeijer & Seeley 2005; Grüter & Ratnieks 2011). Hence, we considered this to be an interesting measure as well. Overall, they are good complementary measures to assess dance following. In the revised version, we added information about the two measurements and hope it makes more sense (L150-152).

**Referee:** There's no mention if TF forager choices were recorded more than once. Did the authors just record the first foraging choice the TF foragers made? If not then they should account for TF forager identity in their models otherwise there is a pseudoreplication problem. This should be clarified.

**Our reply:** This is indeed an important point. Each bee provided only one value for a statistical test to avoid pseudoreplication. We now also show the individual data points representing each bee in the revised Figure 2. In Fig. 3, we show whether bees visited the dance feeder at least once or not. Thus, each bee enters either with a yes or a no. Likewise, in the survival analysis we record the time lapse before a TF bee lands at the TF for the first time.

**Referee:** For the statistical analysis, including random effects that have less that 5 levels can result in imprecise variance estimates (Harrison et al 2018, Peer J). Here the authors include colony (3 levels) and trial (2 levels) as random effects in their models. They should redo their statistical analyses with this in mind.

**Our reply:** We are aware of the discussion regarding rules of thumb about when to use a grouping factor as a fixed- or a random effect. This seems particularly important if one is interested in the variance estimates of the random effects themselves, but might be less relevant in a study such as our since we were not interested in "hive" effects per se, but wanted to account for the fact that bees from the same colony are not biologically independent samples. We suspect that including "hive" as a fixed-effect would violate the assumption of independence, an important assumption for models that only use fixed-effects (Sokal & Rohlf 1995).

For our analysis, we tested whether having "hive" in the models improved the AIC compared to models without "hive". This was not usually the case, whereas "trial" turned out to be a very important random effect. Put differently, the p-values for the fixed-effects were almost identical irrespective of whether we included "hive" or not. Our final models, therefore, usually only contained "trial" (with 6 levels) as a random effect, thus fulfilling the criteria of having at least 5 levels. We provide details about our models in a new supplementary file. We prefer to present our statistics in this way, but can add "hive" as a fixed-effect if the referees and editor prefer this.

**Referee:** Maybe a better control would have been to use an octopamine antagonist to see if the effect then subsides. The current setup shows that DA treated bees prefer the TF feeder (and follow less dances) but there could be other reasons for this preference other than biogenic amine signalling effects (e.g. maybe OA sugar tastes better and therefore there's stronger fidelity). An antagonist would directly confirm whether change in personal and social information use is affected by biogenic amine signalling.

**Our reply:** This is an interesting suggestion. We had another look at our data to check whether bees visited feeders offering OA and DA solution more or less often than the control solution during the treatment time. We found that the number of visits was very similar and did not depend on the solution offered at a feeder (visits to the OA-feeder vs. control-feeder: $6.3 \pm 3.8$ vs. $5.9 \pm 4.1$, Poisson GLMM, $p = 0.36$; visits to the DA-feeder vs. control-feeder: $4.7 \pm 3.65$ vs. $5.2 \pm 3.35$, $p = 0.13$). Thus, there does not seem to be a preference for a particular type of solution.

We also would like to mention that we have indeed done similar experiments using antagonists in the past (mianserin, SCH-23390). Interestingly, the bees did not like solution containing either of these two antagonists and we have abandoned these plans for the very reason mentioned by the referee.

**Referee:** L255-256: I find that the manuscript is missing some biological relevance to make this statement. Were the levels of biogenic amines found in the TF honeybees comparable to natural variation levels?

**Our reply**: We agree that this was a bit too speculative and have changed this sentence (L290-292). Unfortunately, we do not have information about how high natural levels of OA and DA were in bees in our colonies or our treated bees.

# Appendix B

Dear Editor,

Thank you for your correspondence and comments concerning our manuscript "Octopamine and dopamine mediate waggle dance following and information-use in honeybees". We have added the information as suggested by the associate editor and the referee. The line numbers in this cover letter refer to the new version of the manuscript with highlighted changes.

We hope that you will find our revision satisfactory, and look forward to hearing from you.

Best regards,

Melissa Linn

**Reply to comments made by referee #2**

**Referee**: In their response letter, the authors do a nice job of clarifying and addressing the reviewers' comments and questions. However, there are a few instances where I would like to see more of this information in the manuscript itself. Many readers may have these same questions and shouldn't need to dig through the reviewer reports to find the answer.
In particular, I believe more information should be included in the manuscript about the efficacy of the oral treatment method used here. For example, regarding how long the effects of OA and DA are expected to last, the authors write in response to referee 1: "there is evidence that experimentally administered biogenic amines do not last very long in the brain, probably not much more than a couple of hours (e.g. Barron et al. 2007 J. Insect. Physiol.). Our thinking was that biogenic amines would affect the perception of, and learning about, the food source during the treatment time. The bee would then remember, for example, that she visited a particularly rewarding food source (with OA), which would affect her behaviour on the testing day even though the OA had already been cleared in brain tissues". It would be great to have this reasoning spelled out more explicitly in the paper itself, perhaps after L126-132 or somewhere near L92.

**Our reply:** We agree and have made the changes as suggested (L138-142). Our addition reads: While experimentally administered biogenic amines are metabolised and cleared relatively quickly from the brain, probably within a couple of hours [29,51], we expected that our treatment would affect the perception of, and learning about food sources during treatment

[29,31], which is likely to have long-term effects. Long-term memory can affect honeybee foraging decisions for several days [48].

**Referee:** Similarly, the authors discuss in the response why they believe sucrose collected at the feeder and held in the crop is likely sufficient for OA/DA to reach the brain, even though much of it is subsequently regurgitated to nectar receivers in the hive (e.g. nectar in the crop is used to power the flight home; rapid increases in OA titres have been observed following ingestion). Again, I would prefer to see a note to this effect included in the text itself.
**Our reply**: We have added this (L132-137) and wrote: The exact routes of biogenic amines from the crop to the brain remain to be investigated. Gmeinbauer & Crailsheim [51], for example, found that glucose solution consumed by bees after flight quickly appeared in the haemolymph, suggesting a rapid transfer from the crop to the open circulatory system. This would explain why the feeding of biogenic amines leads to rapid changes in biogenic amine titres in the head [52] and in reward perception [40].

**Referee:** Could it also be noted (e.g. at L105-106) that the order of OA and DA trials was randomised within colonies?
**Reply**: Done.

**Referee:** Finally, at L181, I believe it would be helpful to specify that the numbers in parentheses refer to waggle runs per dance.
**Reply**: Done