Hearing, echolocation, and beam steering from day 0 in tongue-clicking bats

Grace C. Smarsh, Yifat Tarnovsky and Yossi Yovel

**Article citation details**
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**Review timeline**
- Original submission: 27 November 2020
- 1st revised submission: 25 August 2021
- 2nd revised submission: 5 October 2021
- Final acceptance: 6 October 2021

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

**Review History**
RSPB-2020-2971.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?**
Acceptable

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

**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.
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
The study tests the ontogeny of echolocation in Rousettus bats by measuring hearing and quantifying echolocation click structure (temporal and spectral) and beam steering. While I do believe that the overall findings are correct, I have issues with two of the methods described:

1) The hearing test is poorly justified, and not described in full detail in the methods. It is not until the results section you state that you use isolation call responses as an indication of hearing. How do you justify this method? From your description the pups will spontaneously produce the isolation calls, so a “response” cannot unequivocally be deemed an indication of hearing. Why not use a conventional method such as ABR?

I would also argue that to show a fully functional echolocation system, you need to test echolocation clicks and you need to measure the audiogram. Arguably, your response proxy could be an indication of hearing, but it is completely unquantifiable, the pup hearing could have an entirely different audiogram than the adult and still respond to the sounds you produce. Based on this, your conclusion about fully developed echolocation system at day 0 is not justified.

2) The beam steering test and comparison is too inaccurate to quantify the difference in aim between adults and pups - you use two different recordings setups (hand-held pups and perching adults), and your recoding setup consists of only two microphones. Given that the animals can steer the beam and readily modify the separation between click-pairs, this method is much to imprecise to quantify this.

Line 22-23. This statement needs references.

Line 32-33. Your argument indicates that this study will reveal general information about click-based echolocation systems. Given the difference between the click-echolocation systems you list later (birds, humans and whales), if find this claim highly speculative. I would suggest rewording to indicate that this sentence only concerns Rousettus click-echolocation or to justify its general applicability.

Line 45-49. None of the references listed (11,25,26) are for the features of Rousettus echolocation you describe…

Line 82. You state that “Noise and call files were calibrated to a peak amplitude of 78-80 dB SPL”. This is very ambiguous. Is this measured at the pup’s location? If so, write it explicitly. Is the measurement performed in the box? What is the size of the box? The box will have a large impact
on the sound field experienced by the pub, as such the measurement should be performed in the box and it should be stated very clearly if you did so. Otherwise your claimed exposure level is not trustworthy. Also, did you account for the frequency response of the speaker in this calibration?

Line 75-96. How do you measure the hearing with this approach? I can see that you quantify responses from Line 200-201, but that should be stated here, otherwise the reader is left guessing at the method. How do you justify this method, is there a study that does so?

Line 100. Gras should be GRAS and you should state size (1/4", 1/8"?) and model.

Line 122. Did you not calibrate the Knowles microphones? Given you method, I appreciate that you do not need an absolute calibration of the entire frequency response, but a simple relative calibration should be performed (exposing both microphones to the same sound) to test how similar they are.

Line 121-134. I find this method highly inaccurate and artificial. You are holding the animal in your hand, why not place it on a platform or hang it from a mesh?

Line 171-173. I do not understand this sentence.

Line 180-182. You mention an “automated measurement feature” – you should either explain this in detail or post a reference that does so.

Line 187-193. You compare pup beam-alternations to adult beam-alternations using an intensity ratio. Given the difference in measurement scenario and low control you have over aim (hand-held pups compared to perching adults and only two microphones for animals that can vary the beam aim/beam separation) this method seems highly inaccurate. I agree that you can test if the left-left – right-right pattern is present, but comparing angles is in my opinion too uncertain with this method.

Line 196-206. This method is not justified in your description. Interpreting calling response as validation of hearing is not entirely unjustified, but it does not guarantee it either. It is also very questionable to test the ontogeny of echolocation without exposing the animals to echolocation sounds. While you may be covering the frequency range, the call duration is dramatically different. Again, an appropriate method would be ABR instead.

Line 246-257. It would be very informative to see a spectrum of the signals. While your argument concerning fft-points from line 156-157 is somewhat valid, there is a vast selection of toothed-whale literature on this that provides justification and the means to do so.

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?
Good

General interest: Is the paper of sufficient general interest?
Excellent
Quality of the paper: Is the overall quality of the paper suitable?
Excellent

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
The ms by Smarsh and Yovel describes ontogenetic development of auditory responsiveness and of echolocation calls in the bat Rousettus. In most laryngeal bats, call production can be delayed after birth and the emission of adult-like calls often is coupled to the onset of flight. Rousettus, however, uses a simpler lingual echolocation production system that is based on tongue clicking. The authors show that right after birth, Rousettus echolocates with clicks and the clicks are quite adult like in terms of their pairwise production. In addition, the neonate bats emit left/right click amplitude patterns that are similar to beam steering of adults. In the next weeks of life, the interpair interervals grow sucessively shorter and the call duration increases while the instantaneous frequency is reduced somewhat. Those changes, however, are small, compared to more massive changes during the ontogeny of laryngeal bats. In addition, the authors showed that the young bats increase the number of their own emitted isolation calls when louder sounds like echolocation clicks are presented. This of course is not a demonstration of intact hearing, but at least shows that the bats are reactive to some sounds right after birth.

The short ms generally is written well and the methods are sound. The data are important when assessing evolution of echolocation and comparing laryngeal versus lingual sound production and therfore should be published in Proc B. Lingual sound production and a concomitant perception of the clicks seems to be an innate feature and is less shaped by experience, inner ear maturation, and sensitive periods, as it is the case in laryngeal sound production.

Specific comments
Title:
your study does not demonstrate intact adult-like hearing, you did not show hearing threshold curves. But you showed that neonates did respond to echolocation signals, please change the title accordingly.
Introduction (and discussion):
I am a little bit surprised that you are not aware of a series of relevant literature with ample data on postnatal maturation of echolocation calls and corresponding behavioural adaptations in laryngeal bats (Vater M Kössl M Foeller E Coro F Mora E Russell I (2003) Development of Echolocation Calls in the Mustached Bat, Pteronotus parnellii. J Neurophysiol 90: 2274–2290) as well as their corresponding cochlear (Kössl M Foeller E Drexl M Vater M Mora E Coro E Russell IJ (2003). Postnatal development of cochlear function in the mustached bat, Pteronotus parnellii. J Neurophysiol 90:2261-2273) and auditory system maturation (Vater M Foeller E Mora EC Coro F Russell IJ Kössl M (2010) Postnatal Maturation of Primary Auditory Cortex in the Mustached Bat, Pteronotus parnellii J Neurophysiol 103: 2339–2354). Those data very nicely show that the mustached bat, as laryngeal bat, takes long time for maturation and would strongly emphasize the point that you make in your ms. Please cite and discuss those data.

Experimental protocol:

Hearing test: To use echolocation signals for your auditory responsiveness test makes sense, but what was the rationale of using 5-110 kHz noise which probably extends beyond the auditory range of those bats. Are there hearing threshold curves obtained with pure tones available for young Rousettus (maybe from another publication)?

Give a little more information about the behaviour of the young bats, did they usually hang on their mothers or stayed very close? In which natural situation do they emit echolocation calls?

Echolocation signal collection: what was the sound pressure level of the echolocation calls of the young bats? Since their tongues still undergo anatomical maturation, were the signal levels lower than those from adults?

Hearing assessment: When you acoustically stimulated the bats and they produced more isolation calls, they also emitted echolocation calls in this situation. Was their number also increased?

Results
L199: detachment of pubs from mothers also happens naturally? Stay the pubs at the roost while the mothers fly out to feed?

Discussion
1.278 I am not quite sure how to interpret your statement about cochlear growth rate. Do you want to say that cochlear development in lingual bats is finished earlier than cochlear development in laryngeal bats (which would support your suggestion that hearing also matures faster)? I would not rely too much on macroscopic cochlea growth data since there is an extended period of maturation of cochlear function after the cochlea has reached its final size. In this respect, microstructural changes of basilar membrane and tectorial membrane stiffness and coupling between hair cells and those membranes are important and will increase cochlear sensitivity in a frequency specific way in young bats. Could you give more information what you are referring to here?

Decision letter (RSPB-2020-2971.R0)
12-Jan-2021
Dear Dr Smarsh:
I am writing to inform you that your manuscript RSPB-2020-2971 entitled "Intact hearing, echolocation, and beam steering from Day 0 in tongue-clicking bats" 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.
4) Data - please see our policies on data sharing to ensure that you are complying (https://royalsociety.org/journals/authors/author-guidelines/#data).

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Sincerely,
Dr Maurine Neiman
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Thank you for submitting your manuscript to Proceedings B. We have now received two reviews of your manuscript. One of the reviewers agrees that this study supports most of your conclusions and is an important contribution to understanding the evolution and development of diverse types of echolocation. However, both reviewers express concerns about the methods used to assess hearing in the pups, and both object to the conclusion that pups have "intact" hearing as stated in the title. The second reviewer is also concerned that the method used to assess beam steering was not accurate enough to compare with data from adults, and therefore, combined with the concerns about hearing, the conclusion that the pups of this species are capable of echolocation from day zero has not been adequately demonstrated. Both reviewers provide valuable comments and suggestions that will improve a future version of the manuscript.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
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Author's Response to Decision Letter for (RSPB-2020-2971.R0)

See Appendix A.

RSPB-2021-1714.R0

Review form: Reviewer 1

Recommendation
Accept as is

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?
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
The authors have addressed all my concerns in a very nice way and I have no further comments. I am happy to recommend the study for publication

Decision letter (RSPB-2021-1714.R0)

28-Sep-2021

Dear Dr Smarsh

I am pleased to inform you that your manuscript RSPB-2021-1714 entitled "Hearing, echolocation, and beam steering from Day 0 in tongue-clicking bats" has been accepted for publication in Proceedings B.

The referee(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the referee(s)’ comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.
To revise your manuscript, log into https://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. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original 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.

Before uploading your revised files please make sure that you have:

1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".

2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. 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.

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].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

5) Data accessibility section and data citation
It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository (https://royalsociety.org/journals/authors/author-guidelines/#data).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a ‘data accessibility’ section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:

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- Phylogenetic data: TreeBASE accession number S9123
- Final DNA sequence assembly uploaded as online supplemental material
- Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311

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as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

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. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

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Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Dr Maurine Neiman
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member
Comments to Author:
Thank you for your careful revisions of this manuscript.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s).
The authors have addressed all my concerns in a very nice way and I have no further comments. I am happy to recommend the study for publication.

Author's Response to Decision Letter for (RSPB-2021-1714.R0)
See Appendix B.

Decision letter (RSPB-2021-1714.R1)
06-Oct-2021
Dear Dr Smarsh

I am pleased to inform you that your manuscript entitled "Hearing, echolocation, and beam steering from Day 0 in tongue-clicking bats" 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.
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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.

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

Dr Maurine Neiman
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Thank you for submitting your manuscript to Proceedings B. We have now received two reviews of your manuscript. One of the reviewers agrees that this study supports most of your conclusions and is an important contribution to understanding the evolution and development of diverse types of echolocation. However, both reviewers express concerns about the methods used to assess hearing in the pups, and both object to the conclusion that pups have “intact” hearing as stated in the title. The second reviewer is also concerned that the method used to assess beam steering was not accurate enough to compare with data from adults, and therefore, combined with the concerns about hearing, the conclusion that the pups of this species are capable of echolocation from day zero has not been adequately demonstrated. Both reviewers provide valuable comments and suggestions that will improve a future version of the manuscript.

Thank you. We have addressed the concerns regarding hearing in detail, and have collected ABRs in response to pure tones and a click to better address the concerns regarding hearing abilities in newborn pups. Hearing is not as sensitive as adults on Day 0 but the hearing range covers the main range for Rousettus. While not fully intact, the results point to another difference in ontogeny between laryngeal and lingual echolocating bats, in which laryngeal bats generally do not hear the main hearing range at this age and have an upward shift in frequency during development.

Regarding the behavioral tests on Day 0, we have added an additional analysis and figure of the click response to stimuli on Day 0, to provide greater information on echolocation use in newborns, as requested by Reviewer 2.

We have revisited the beam steering concerns as well, and agree with the reviewer’s argument that we cannot compare beam angle at this time. We have removed this, but have kept the assessment of directional changes, with which neither reviewers had faults.

We have added additional figures of the power spectra of the clicks, and added supplemental figures and a video to help illustrate the behavior and vocal abilities of young pups.

Below we have sought to fully address each reviewers’ individual comments and adjust the manuscript accordingly.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
The study tests the ontogeny of echolocation in Rousettus bats by measuring hearing and quantifying echolocation click structure (temporal and spectral) and beam steering. While I do believe that the overall findings are correct, I have issues with two of the methods described:
1) The hearing test is poorly justified, and not described in full detail in the methods. It is not until the results section you state that you use isolation call responses as an indication of hearing. How do you justify this method? From your description the pubs
will spontaneously produce the isolation calls, so a “response” cannot unequivocally be
deemed an indication of hearing. Why not use a conventional method such as ABR?
I would also argue that to show a fully functional echolocation system, you need to test
echolocation clicks and you need to measure the audiogram. Arguably, your response
proxy could be an indication of hearing, but it is completely unquantifiable, the pup
hearing could have an entirely different audiogram than the adult and still respond to the
sounds you produce. Based on this, your conclusion about fully developed echolocation
system at day 0 is not justified.

Thank you for your careful revision of our manuscript and detailed comments. We have
sought to address your concerns in detail below and adjust accordingly. First, here we
will address in detail the concerns about testing hearing, as this came up several times
in your comments:

We see and agree with your point that our conclusion on fully intact hearing could not be
justified as we did not show the sensitivity of hearing to different frequencies. It is
important to note that behavior has been and is still used to assess hearing abilities.
Behavioral audiograms can also be considered a “conventional” method as they are
widely used across taxa and have been conducted successfully in bats for decades (e.g.
Dalland 1965, Long & Schnitzler 1975, Suthers & Summers 1980, Schmidt et al 1984,
Esser & Schmidt 1990) and are argued to demonstrate greater sensitivity than ABRs
(e.g. Wenstrup 1984, Obrist & Wenstrup 1998, Koay et al. 1998, Lattenkamp et al 2020).

Our initial interest was presence of hearing. This can be assessed behaviorally by a
significant response to acoustic stimuli. The design is similar to numerous other acoustic
playback studies in bats, birds, and other taxa which establish recognition,
discrimination, or detection of sounds with a significant behavioral response. We have
reviewed and added/adjusted the methods and results regarding this test, and
additionally analyzed the bats’ response to sound using echolocation, which is also
significant (L 80-104, 166-172, 227-241, Fig 1, Supplementary Fig 1). To further illustrate
the responsiveness of these robust animals, I have included an example video clip from
one of our trials, as well as spectrograms in the supplements.

Moreover, in order to address the reviewer’s concerns regarding frequency sensitivity we
recorded ABRs for four pups Day 0 to Day 2 in in response to tone pips (6, 12, 18, 24,
30, 35 KHz) and a 0.1 ms click (0 to 50 kHz, most energy 0 to 10 kHz). These
frequencies encompass the most sensitive hearing range of *R. aegyptiacus* (8 to 25 kHz,
Koay et al. 1998). This part of the project was conducted with Yifat Tarnovsky, a PhD
candidate in the Yovel lab, whose has training and experience in recording ABRs in *R.
aegyptiacus*. Additionally, we included adult thresholds acquired from ABRs that
Tarnovsky previously recorded in the same setup (L 115-128, 173-181, 242-250, Fig 1).

2) The beam steering test and comparison is too inaccurate to quantify the difference in
aim between adults and pups – you use two different recordings setups (hand-held pups
and perching adults), and your recording setup consists of only two microphones. Given
that the animals can steer the beam and readily modify the separation between click-
pairs, this method is much too imprecise to quantify this.

We accept this comment and removed this assessment of aim comparison.
We have added references

We provide context regarding click echolocation systems as it is not specific to bats and there is much to learn about click-based sensory systems (L 30, L 32-33). On L 31 we specifically state that we investigate the Rousettus click sensory system. On lines 34-35 we now state that the mechanism of click based echolocation differ, and then go on to discuss lingual echolocation systems specifically. We hope this is clear now

Line 45-49. None of the references listed (11,25,26) are for the features of Rousettus echolocation you describe… Thank you for this catch. We have corrected this reference library issue and checked the rest of the document to ensure there were no others.

Line 82. You state that “Noise and call files were calibrated to a peak amplitude of 78-80 dB SPL”. This is very ambiguous. Is this measured at the pup’s location? If so, write it explicitly. Is the measurement performed in the box? What is the size of the box? The box will have a large impact on the sound field experienced by the pub, as such the measurement should be performed in the box and it should be stated very clearly if you did so. Otherwise your claimed exposure level is not trustworthy. Also, did you account for the frequency response of the speaker in this calibration?

Yes, this is important to include. We did calibrate the stimuli by holding the calibrated GRAS microphone from within the box at the location of the pup’s head when the pup is perching there. We have added this point here L 88-L 90.

We have added the details that the box was lined with foam to reduce echoes, and its dimensions on L 93-94.

We considered the frequency response of the speaker before using it for the experiment. It covers 1-120 kHz, with variability of up to 6 db between 1 and 60 kHz. Since the response of the microphone we used to calibrate the system is flat and calibrated, it accounts for the entire frequency response of the system including the speaker. We have added this point here L 90-92.

Line 75-96. How do you measure the hearing with this approach? I can see that you quantify responses from Line 200-201, but that should be stated here, otherwise the reader is left guessing at the method. How do you justify this method, is there a study
that does so.
We have clarified the methods of testing (L 78-104) and analysis L 166-172
We have addressed concerns about behavioral testing above. L 75-78 we add in
information about behavioral audiograms.

Line 100. Gras should be GRAS and you should state size (1/4”, 1/8”?) and model.
Yes, I have added this information on L89.

Line 122. Did you not calibrate the Knowles microphones? Given you method, I
appreciate that you do not need an absolute calibration of the entire frequency response,
but a simple relative calibration should be performed (exposing both microphones to the
same sound) to test how similar they are.
Yes, we compared the signals of the microphone in the setup in response to artificial
clicks. The microphones were placed in the same position each time. The gain
difference between them was 0.17 V and I have added this information on L 152. We
have adjusted the amplitude measurements from the weaker channel accordingly and
recalculated the transitions, which did not affect the overall result (L 218-219).

Line 121-134. I find this method highly inaccurate and artificial. You are holding the
animal in your hand, why not place it on a platform or hang it from a mesh?
It was important in this study to get very clear recordings of such hyper-short clicks, and
enough of them. In the hand, we could gently stimulate the pup and get good recordings
in the direction of the microphone in a reasonable amount of time (which is important in
a species in which the newborn is always attached to the mother). While artificial, this
method has been used previously in other studies (e.g. Sterbing 2002).

Line 171-173. I do not understand this sentence.
This sentence was an error, thank you for noting. We have corrected and rewritten it L
208-209.

Line 180-182. You mention an “automated measurement feature” – you should either
explain this in detail or post a reference that does so.
We have added in additional information on Batalef L215-217.

Line 187-193. You compare pup beam-alternations to adult beam-alternations using an
intensity ratio. Given the difference in measurement scenario and low control you have
over aim (hand-held pups compared to perching adults and only two microphones for
animals that can vary the beam aim/beam separation) this method seems highly
inaccurate. I agree that you can test if the left-left – right-right pattern is present, but
comparing angles is in my opinion too uncertain with this method.
We see your concerns and removed this part.

Line 196-206. This method is not justified in your description. Interpreting calling
response as validation of hearing is not entirely unjustified, but it does not guarantee it
either. It is also very questionable to test the ontogeny of echolocation without exposing
the animals to echolocation sounds. While you may be covering the frequency range,
the call duration is dramatically different. Again, an appropriate method would be ABR instead. We have addressed the concerns about hearing tests from a behavioral standpoint and from ABRs above.

Following the reviewer’s concerns, we used ABR’s in addition to the behavioral tests. The standard ABR setup does not allow playing back an exact Rousettus click, so we have played back a click similar in duration (L 115). We also tested the response to pure tones, so altogether now demonstrate sensitivity to both echolocation click frequencies and duration. It will be an exciting advancement of this research to play back various types of stimuli (communication, echolocation, echolocation from different angles, etc) with a specialized setup in the future.

To add more information regarding the abilities of the sensory system at Day 0, we added the assessment of use of echolocation in response to sound stimuli on Day 0, showing that they emit echolocation spontaneously and at greater rates in response to a stimulus. This tells us that there is a functional basis of circuitry between sound reception with echolocation production. We also played back echolocation clicks in preliminary test to a week old pup and found she responded to the passes with isolation calls and clicks (Supplementary Fig. 3).

Line 246-257. It would be very informative to see a spectrum of the signals. While your argument concerning fft-points from line 156-157 is somewhat valid, there is a vast selection of toothed-whale literature on this that provides justification and the means to do so. We have included spectra examples of Day 0 and Day 35 pups, and an adult (Fig. 2). Yes the toothed-whale literature does provide justification, however, this approach is a very straightforward and accurate method to acquire the peak frequency. We have referenced work by Boonman et al 2020, in which the authors compared the instantaneous and FFT frequency measurements and confirmed the correlation between these two approaches. I have added this on Line 193-195.

Referee: 2

Comments to the Author(s)
The ms by Smarsh and Yovel describes ontogenetic development of auditory responsiveness and of echolocation calls in the bat Rousettus. In most laryngeal bats, call production can be delayed after birth and the emission of adult-like calls often is coupled to the onset of flight. Rousettus, however, uses a simpler lingual echolocation production system that is based on tongue clicking. The authors show that right after
birth, Rousettus echolocates with clicks and the clicks are quite adult like in terms of their pairwise production. In addition, the neonate bats emit left/right click amplitude patterns that are similar to beam steering of adults. In the next weeks of life, the interpairo interervalls grow successivly shorter and the call duration increases while the instantaneous frequency is reduced somewhat. Those changes, however, are small, compared to more massive changes during the ontogeny of laryngeal bats. In addition, the authors showed that the young bats increase the number of their own emitted isolation calls when louder sounds like echolocation clicks are presented. This of course is not a demonstration of intact hearing, but at least shows that the bats are reactive to some sounds right after birth.

The short ms generally is written well and the methods are sound. The data are important when assessing evolution of echolocation and comparing laryngeal versus lingual sound production and therfore should be published in Proc B. Lingual sound production and a concomitant perception of the clicks seems to be an innate feature and is less shaped by experience, inner ear maturation, and sensitive periods, as it is the case in laryngeal sound production.

Thank you for the careful consideration of our manuscript and comments. We have sought to address them thoroughly below.

Specific comments
Title:
your study does not demonstrate intact adult-like hearing, you did not show hearing threshold curves. But you showed that neonates did respond to echolocation signals, please change the title accordingly.

True, we have adjusted the title, and we have recorded ABRs for several day 0 – day 1 pups to add more information on the sensitivity to frequencies 6, 12, 18, 24, 30, and 35 kHz, which encompasses the most sensitive region of the R. aegyptiacus audiogram (Koay et al 1998). (L 115-128, 173-181, 242-250, Fig 1)

Introduction (and discussion):
I am a little bit surprised that you are not aware of a series of relevant literature with ample data on postnatal maturation of echolocation calls and corresponding behavioural adaptations in laryngeal bats (Vater M Kössl M Foeller E Coro F Mora E Russell I (2003) Development of Echolocation Calls in the Mustached Bat, Pteronotus parnellii. J Neurophysiol 90: 2274–2290) as well as their corresponding cochlear (Kössl M Foeller E Drexl M Vater M Mora E Coro E Russell IJ (2003). Postnatal development of cochlear function in the mustached bat, Pteronotus parnellii. J Neurophysiol 90:2261-2273) and auditory system maturation (Vater M Foeller E Mora EC Coro F Russell IJ Kössl M (2010) Postnatal Maturation of Primary Auditory Cortex in the Mustached Bat, Pteronotus parnellii J Neurophysiol 103: 2339–2354). Those data very nicely show that the mustached bat, as laryngeal bat, takes long time for maturation and would strongly emphasize the point that you make in your ms. Please cite and discuss those data.

There is an extensive amount of echolocation ontogeny literature to consider. Thank you for the suggestion, as the research on the development of the auditory system in
*Pteronotus* is very interesting and informative. I have referred to it in the introduction (L24, 28) and the discussion (L 306, 308-310, 320-321).

Experimental protocol:

Hearing test: To use echolocation signals for your auditory responsiveness test makes sense, but what was the rationale of using 5-110 kHz noise which probably extends beyond the auditory range of those bats. Are there hearing threshold curves obtained with pure tones available for young *Rousettus* (maybe from another publication)?

The purpose of using broad band noise was as a control. We didn’t know what frequencies such a young animal would be sensitive to so we chose a broad frequency sound that would encompass the adult sensitivity range and also all of the harmonics of the communication calls we played back. We presumed they wouldn’t respond to the higher frequencies of noise but also assumed it wouldn’t hurt to do so.

There are no threshold curves in the literature for young *Rousettus*, so we have subsequently recorded ABR for several pups age Day 0 to 1. We reported the thresholds for the pups as well as the average for 5 adults whose ABRs were recorded in the same setup. The frequencies we used were in the main frequency sensitivity window for adult *Rousettus* (8 to 30 kHz, Koay et al 1998) (see line numbers listed in above response).

Give a little more information about the behaviour of the young bats, did they usually hang on their mothers or stayed very close? In which natural situation do they emit echolocation calls?

Thank you for noting this gap in information. We have added more information on L 70-75. Pups are always on their mothers for the first 3 weeks of age. As for echolocation, we did not have expectations or prior knowledge of echolocation use at such young ages. We observed the echolocation emission when we first started playing back adult social calls and noise at the start of the project. We have gone back through the data and observed that pups untouched in the playback experiment will emit echolocation click pairs on their own in between isolation calls, but the acoustic stimuli significantly increase emission. We have added a video of playback to a Day 0 pup and example spectrograms of the response in the supplemental to illustrate this (Supplementary Media, Supplemental Fig 2). We have analyzed the echolocation response and included it in the methods (L 80-104, L166-172), and results (L234-241), and in the figures (Fig. 1). This provides greater input in hypotheses regarding the development of echolocation vs communication from a neural standpoint. We have only found one study (Esser & Daucher 1996 Hearing in the FM-bat *Phyllostomus discolor*: a behavioral audiogram) in the literature in which interactive playbacks of pups were conducted in neonates. The response to playbacks of stimuli in our study illustrates that there is a functional basis of the auditory and vocal motor circuitry at this age, and also leads to further research questions regarding differentiation of sensorimotor circuitry at this age for laryngeal sounds vs tongue clicks.

Echolocation signal collection: what was the sound pressure level of the echolocation calls of the young bats? Since their tongues still undergo anatomical maturation, were
the signal levels lower than those from adults? We have gone back and calibrated the recording the settings and estimated the source levels (range 94.8 to 107 db SPL 10 cm from the mouth). We have added it here, L 286-287. We couldn’t compare these values to our adult recordings, but the pup values were not as high as the adult intensities reported in the literature: 75-125 db SPL or 105-115 db SPL at 10 cm from the mouth measured in the lab (reviewed in Yovel et al 2011).

Hearing assessment: When you acoustically stimulated the bats and they produced more isolation calls, they also emitted echolocation calls in this situation. Was their number also increased?

Interesting question. We went back and analyzed the trials for the same individuals from 2018, and included the data as stated above. They did respond to stimuli with more echolocation (L234-241, Fig. 1). We also included spectrograms showing vocal responses to echolocation passes in a young pup in the Supplemental material (Supp. Fig. 3).

Results
L199: detachment of pubs from mothers also happens naturally? Stay the pubs at the roost while the mothers fly out to feed?
They are on the mother continuously for the first 3 weeks, then the mothers start leaving the pups behind while foraging. We have added this information L 72-73.

Discussion
L.278 I am not quite sure how to interpret your statement about cochlear growth rate. Do you want to say that cochlear development in lingual bats is finished earlier than cochlear development in laryngeal bats (which would support your suggestion that hearing also matures faster)? I would not rely too much on macroscopic cochlea growth data since there is an extended period of maturation of cochlear function after the cochlea has reached its final size. In this respect, microstructural changes of basilar membrane and tectorial membrane stiffness and coupling between hair cells and those membranes are important and will increase cochlear sensitivity in a frequency specific way in young bats. Could you give more information what you are referring to here?

We originally stated this as evidence strengthening the hypothesis that laryngeal and lingual echolocators had a common echolocating ancestor, but following the reviewer’s comment we have reviewed and reformulated this section in the discussion. In lingual bats the majority of external cochlear development occurs prenatally, followed by a slow trajectory postnatally, rather than what may be continual rapid changes in laryngeal bats. This points to different ontogenetic and perhaps evolutionary patterns. Furthermore, we may hypothesize that multiple aspects of the sensory system are in place at birth in lingual bats, with additional, yet minimal, refinements occurring slowly compared to laryngeal bats (which have dramatic shifts in frequency corresponding to many changes in the hair cells, cochlear neurons, and auditory cortex). Flight occurs much later in Rousettus as well. We have rewritten this section and our emphasis L 307-319, 320-324. We welcome your input on these ideas.

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Journal Name: Proceedings of the Royal Society B
Abstract: Bats of the genus Rousettus are the only animals known to naturally produce click echolocation from the tongue. There have been many studies examining the dramatic changes of laryngeal echolocation in pups across bat families, but little is known about the ontogeny of lingual echolocation. Here, we examined the echolocation development of *Rousettus aegyptiacus*, the Egyptian fruit bat, which uses rapid tongue movements to produce hyper-short clicks and steer the direction of the beam. We recorded echolocation once a week from Day 0 through Day 35 postbirth and examined temporal emission patterns, signal frequency, and signal duration. We assessed the age of hearing and beam-steering abilities. On Day 0 postbirth *R. aegyptiacus* pups hear and produce hyper-short clicks in a paired pattern, the same as adults. Remarkably, newborn pups were able to use their tongues to steer the sonar beam, showing that Rousettus pups are born with highly developed tongue control. As they aged, pups produced click pairs faster, converging with adult intervals by age of first flights (7-8 weeks). In contrast to laryngeal bats, *Rousettus* echolocation frequency and duration is stable through Day 35, but shift by the time pups begin to fly, possibly due to tongue-diet maturation effects. Furthermore, frequency and duration shift in the opposite direction of mammalian laryngeal vocalizations. *Rousettus* lingual echolocation thus appears to be a highly functional, intact sensory system from birth. This nearly-innate sensing might come at the cost of behavioral flexibility that allows laryngeal bats to adapt their calls to different environments.
Appendix B

Author response to second round of reviews for “Hearing, echolocation, and beam steering from Day 0 in tongue-clicking bats:"

Associate Editor
Board Member
Comments to Author:
Thank you for your careful revisions of this manuscript.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
The authors have addressed all my concerns in a very nice way and I have no further comments. I am happy to recommend the study for publication

Dear Reviewer(s),

We were happy to see that our efforts to respond thoroughly to the Reviewers and the Assoc. Editor sufficiently addressed the concerns on our manuscript.

Thank you for the careful reviews and comments throughout this process. We think that the manuscript and overall story has been greatly improved.

Kind regards,

Authors