THE ROYAL SOCIETY PUBLISHING

PROCEEDINGS B

Influenza A viruses remain infectious for more than seven months in northern wetlands of North America

Andrew M. Ramey, Andrew B. Reeves, Judith Z. Drexler, Joshua T. Ackerman, Susan De La Cruz, Andrew Lang, Christina Leyson, Paul Link, Diann J. Prosser, Gregory J. Robertson, Jordan Wight, Sungsu Youk, Erica Spackman, Mary Pantin-Jackwood, Rebecca L. Poulson and David E. Stallknecht

Article citation details
Proc. R. Soc. B **287**: 20201680.
http://dx.doi.org/10.1098/rspb.2020.1680

Review timeline

Original submission:	4 May 2020
1st revised submission:	13 July 2020
2nd revised submission:	10 August 2020
Final acceptance:	11 August 2020

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

Review History

RSPB-2020-0959.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?
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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Ramey et al address the interesting question of viability of influenza viruses within the environment. To this extent, they perform field and laboratory experiments to determine that these viruses can remain infectious for up to 7 months, suggesting the importance of environmental transmission for avian influenza viruses.

I think this is an important study, combining both field and experimental study, which deserve to be published. Nevertheless, I have a couple of recommendations before.

First, I clearly do appreciate the combination of field and experimental works. However, they feel quite disconnected in some way. The samples from the field have been used to do experimental work, but we do not know from the field study if the viruses found in water at the beginning of the season are the same than at the end of the season. Obviously, regarding the huge diversity of AIV, it is not possible to determine if they are the same virus. But I think it could be extremely interesting to see if the decay in number of subtype isolates decreases through time at a speed compatible with the environmental viability measured in the lab. The subtype with a short experimental viability in the lab should disapear first from the field data. This nevertheless require to have subtype identification for all field isolates through time.

I think also the consequences of this study are not elaborated enough. Regarding cross-immunity processes and evolutionary dynamics, it is non obvious that a longer environmental persistence would yield a larger diversity of strains within avian populations, neither in human populations

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?
No

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript describes a field test of influenza A virus persistence for viruses collected from wild waterfowl. The experiments and data are very interesting and present a rigorous test of viral persistence in a field setting which is matched to a laboratory assessment and animal challenge. The finding that nearly 20% of isolated viruses are still viable by VI approximately 7 months after field exposure to ambient temperatures is an important demonstration that these viruses can overwinter in the environment. The test period encompassed fall-spring migration so the results have significance for our understanding of the natural ecology of these viruses. The experimental design, methods, and interpretation are sound and I do not have any major concerns. The primary suggestion I have for improving the manuscript is to edit for conciseness and clarity (primarily in the Results section).

ABSTRACT
Line 34: Not sure why there is a hyphen in far-less.
Line 38 "replicate samples of duck swabs in surface water" isn't immediately clear; might be clearer to say duck swabs stored in surface water or duck swabs inoculated into surface water?
Line 40: I initially read this to mean that IAVs were molecularly detected directly from collected surface water samples (not samples spiked with swabs). It should be clarified that these are the same samples as described above; i.e., surface water inoculated with duck swabs.

KEYWORDS
Reservoir might be useful as well.

INTRODUCTION

Line 73: Potential reference: Hénaux V, Samuel MD, Dusek RJ, Fleskes JP, Ip HS (2012) Presence of Avian Influenza Viruses in Waterfowl and Wetlands during Summer 2010 in California: Are Resident Birds a Potential Reservoir? PLoS ONE 7(2): e31471. https://doi.org/10.1371/journal.pone.0031471

METHODS

Line 109: How many vials were inoculated per site? Exact numbers can be reported in Results, but sample size is an important aspect of experimental design and should be reported in Methods.

Line 111: Paired swabs in 40mLs seems like a significant dilution. How many replicates were aliquoted per 40mL vial? This was confusing to me upon first read (reading the abstract led me to think you might have pulled samples periodically rather than only once), but upon a full read it's clear there were 2 replicates. It might be clearer to indicate duplicates or paired/matched replicates or refer to the samples as replicate 1 and replicate 2 as you do in Fig. 1.

Line 114: The text implies that the samples were tested prior to submerging the paired sample. If so, why submerge the negatives since only the positives were tested post recovery?

Line 117: Please specify what you mean by "appropriate control."

Line 134: Was the same vial used for all monthly sampling or multiple vials aliquoted at the outset? Standardize to either ml or mL throughout text/figs.

Line 142: Consider specifying what the positive control was (i.e., surface water spiked with H3N8).

Line 143: What was the dose of the inoculum? If you didn't titer the samples, you can at least specify VI sensitivity and/or the Ct values. Because the swabs were diluted into 40mL of surface water it seems that the inoculum may have been at a relatively low dose for at least some of the inoculums which is important for interpreting the challenge results. Maybe add Cts/dose information to Figure 5. This information seems particularly important given that most mallards shed viral RNA, but did not seroconvert. For low doses, the ducks have been able to clear the infection with only a cellular immune response before mounting an antibody response.

Line 145: Does paired OP/CL swabs mean both were collected or samples were placed in a single vial?

Line 146: Were paired swabs collected from each individual in a group?

Line 149: Please be more specific about which samples were selected for testing. The lowest Ct per individual/sample type/group?

Line 158: A single reference to the Supp Info at the outset of the methods would improve readability.

RESULTS

I found some of the results difficult to follow due to repetition of methods and distracting details. The comments below are potential instances where the results could be improved. These suggestions don't need to be addressed individually, but as a guide for potential improvements for readability/reducing redundancy.

Line 168: As all vials tested are replicate vials, calling them replicate vials doesn't add clarity without further characterization (e.g. Rep1 or Rep 2 or Initial vs Recovered/Submerged).

Line 180: Excessive detail in this sentence obscures the main result. "Using samples from our field experiment" could be deleted as these results are in the "Field Experiment" Section. Experimental/Sample details are well documented in the Methods/Supp Info/Table so don't need to be repeated; e.g., We obtained genomic information for all 51 viruses isolated in the initial screen of inoculated surface water samples and for 10 viruses isolated from the matched replicate samples that we recovered approximately 7 months later.

Line 191: Redundant details could be removed/generalized by describing these samples as virus isolates from submerged samples; e.g., There were no predominate combined HA-NA subtypes among isolates recovered from submerged samples at either location (Figure 2).

Line 195: Recommend limiting details; e.g., All isolates recovered from submerged samples shared >99.6% nucleotide identity at each viral gene segment with the virus isolated from the corresponding replicate sample.

Line 199: Details could be generalized for ease of reading; e.g. Fall for August or September 2018 Spring for April 2019. Same for Lines 205-206. Or timing detail could be generalized to the paired samples; i.e., as initial and recovered/submerged.
Line 216: Minimize reiteration of methods. That the samples were held between Sept-April isn't important since the samples were held at 4C.
Line 219: Don't need to repeat 40 samples as it was specified in Line 216.
Line 220: Rather than April 2019, the important result is the samples were still infectious by VI after appx. 7 months.
Line 224: Probably more straight-forward to say corresponding submerged samples rather than repeating that the samples are from MN and were recovered in April 2019.
Line 233: Redundant to restate that the viruses were recovered between 209-229 days.
Line 235: Redundant to restate the inoculum which was just identified in Line 233.
Line 238: Do not need a 3rd reiteration of the inoculum.
Line 239: This sentence is repetitive; all the specifics other than 99.8% nucleotide identity (which could be added parenthetically to the previous sentence) are in the Table/Figure.
Line 247: Details are all available in the Table/Figure; consider limiting reporting here to seroconversion was observed in the positive control and 3 of the 9 experimental groups.
Line 250: The main point is somewhat lost in details; e.g., Based on positive rRRT-PCR, VI, seroconversion, and > 99% nucleotide identity with the IAV isolated in the inoculum, one sample each from Alaska and Minnesota was infectious in a mallard model after being held at ambient temperature in the field for seven months (Figure 5).

RESULTS Other Comments
Line 164: Not sure you need to point out that you are presenting the results in descending order. Consider providing total sample numbers (e.g., positive samples in Minnesota (n = 65/208).
Line 168: Line 151 indicates an individual swab sample was tested but the earlier reference to then as paired might be interpreted as the pair was placed in a single tube.
Line 183: The sentence about GenBank submission could be moved to the end of the paragraph as it is more of a housekeeping detail and detracts from the results.
Line 217: The word monotonic might be clearer in place of successive.
Line 228: The information on this bird needs to be reported but might be better mentioned a bit later in the paragraph so as to not detract from the main results birds.
Line 236: Consider replacing the "or" with an "and."
Line 246: Missing the word "on" before 15 dpi?
Line 294: Including PCR result details might be informative with respect to the discrepancy between PCR results and seroconversion: were any/most of the birds PCR positive on more than 1 day post inoculation? Were the Cts relatively low/high? The inoculum dose might also be relevant if birds were able to clear/prevent infection for low exposure doses.

DISCUSSION
Line 272: Wouldn't 0.2micron filtration remove bacteria?
Line 273: The important discussion here should be whether the pH change was similar for submerged samples compared to the surrounding surface water and whether a difference would impact inference from results to natural systems.

TABLES & FIGURES
Table 1: The "Samples Collected" column heading should be renamed to "Paired swabs" or "Samples inoculated" since only water samples were collected. If the goal of the experiment was to test viral persistence, then the sample size is 51 (# of samples with infectious virus). If the goal is to test different water conditions, then the relevant sample size is 6. I appreciate the work that went into the collection and testing of 686 samples, but that number deflates the assessment of viral persistence. A major result of the work is that nearly 20% of infectious samples tested in the fall were still infectious by VI 6-7 months later under field conditions, but that result gets lost in this table since the number of positives looks so low. Consider adding percentages to the table. Also, consider moving the deployment date column after the PCR and VI columns since the samples were tested and then deployed. For consistency and to be more specific, consider

referring to the samples as submerged rather than deployed. Adding a column summarizing the number of days samples were submerged might be useful.
Figure 1: Maybe reword or switch 3 & 4 – it seems like samples would be collected before they were characterized. Clarify how 9 is different from 3? Laboratory Experiments – the methods indicate that the samples were maintained in their original tubes and not aliquoted. Challenge Study – "virus isolation positive contents" might be clearer to just say virus isolate from…
Figure 2: The figure is not a histogram; it is a bar chart. See: https://www.forbes.com/sites/naomirobbins/2012/01/04/a-histogram-is-not-a-bar-chart/#33ada0256d77 Consider replacing "environmental conditions" with ambient temperature. Or, it might be more informative to refer to them as submerged samples since maintaining them at environmental conditions could be done in the lab and you might want to emphasize that it was done in the field.
Figure 3: Define MN and AK in the figure legend as they are abbreviated on the Figure.
Figure 5 Legend: Consider specifying that the inoculum was contaminated surface water so readers don't assume it was propagated. The legend might be improved by emphasizing the primary goal of the figure and de-emphasizing details (e.g., 209-229 d could be appx. 7 months, the study sites could just be MN) in the first sentence. Consider replacing "environmental conditions" with ambient temperature.

Decision letter (RSPB-2020-0959.R0)

22-Jun-2020

Dear Dr Ramey:

I am writing to inform you that your manuscript RSPB-2020-0959 entitled "Influenza A viruses remain infectious for more than seven months in northern wetlands of North America" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken after considering the advice of referees, who have recommended that revisions are necessary. The reviewers are positive overall though, as am I. 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,
Professor Hans Heesterbeek,
mailto: proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
Ramey et al address the interesting question of viability of influenza viruses within the environment. To this extent, they perform field and laboratory experiments to determine that these viruses can remain infectious for up to 7 months, suggesting the importance of environmental transmission for avian influenza viruses.

I think this is an important study, combining both field and experimental study, which deserve to be published. Nevertheless, I have a couple of recommendations before.

First, I clearly do appreciate the combination of field and experimental works. However, they feel quite disconnected in some way. The samples from the field have been used to do experimental work, but we do not know from the field study if the viruses found in water at the beginning of the season are the same than at the end of the season. Obviously, regarding the huge diversity of AIV, it is not possible to determine if they are the same virus. But I think it could be extremely interesting to see if the decay in number of subtype isolates decreases through time at a speed compatible with the environmental viability measured in the lab. The subtype with a short experimental viability in the lab should disapear first from the field data. This nevertheless require to have subtype identification for all field isolates through time.

I think also the consequences of this study are not elaborated enough. Regarding cross-immunity processes and evolutionary dynamics, it is non obvious that a longer environmental persistence would yield a larger diversity of strains within avian populations, neither in human populations

Referee: 2

Comments to the Author(s)
This manuscript describes a field test of influenza A virus persistence for viruses collected from wild waterfowl. The experiments and data are very interesting and present a rigorous test of viral persistence in a field setting which is matched to a laboratory assessment and animal challenge. The finding that nearly 20% of isolated viruses are still viable by VI approximately 7 months after field exposure to ambient temperatures is an important demonstration that these viruses can overwinter in the environment. The test period encompassed fall-spring migration so the results have significance for our understanding of the natural ecology of these viruses. The experimental design, methods, and interpretation are sound and I do not have any major concerns. The primary suggestion I have for improving the manuscript is to edit for conciseness and clarity (primarily in the Results section).

ABSTRACT
Line 34: Not sure why there is a hyphen in far-less.
Line 38 “replicate samples of duck swabs in surface water” isn’t immediately clear; might be clearer to say duck swabs stored in surface water or duck swabs inoculated into surface water?
Line 40: I initially read this to mean that IAVs were molecularly detected directly from collected surface water samples (not samples spiked with swabs). It should be clarified that these are the same samples as described above; i.e., surface water inoculated with duck swabs.

KEYWORDS
Reservoir might be useful as well.

INTRODUCTION
Line 73: Potential reference: Hénaux V, Samuel MD, Dusek RJ, Fleskes JP, Ip HS (2012) Presence of Avian Influenza Viruses in Waterfowl and Wetlands during Summer 2010 in California: Are Resident Birds a Potential Reservoir? PLoS ONE 7(2): e31471. https://doi.org/10.1371/journal.pone.0031471

METHODS
Line 109: How many vials were inoculated per site? Exact numbers can be reported in Results, but sample size is an important aspect of experimental design and should be reported in Methods.
Line 111: Paired swabs in 40mLs seems like a significant dilution. How many replicates were aliquoted per 40mL vial? This was confusing to me upon first read (reading the abstract led me to think you might have pulled samples periodically rather than only once), but upon a full read it's clear there were 2 replicates. It might be clearer to indicate duplicates or paired/matched replicates or refer to the samples as replicate 1 and replicate 2 as you do in Fig. 1.
Line 114: The text implies that the samples were tested prior to submerging the paired sample. If so, why submerge the negatives since only the positives were tested post recovery?
Line 117: Please specify what you mean by "appropriate control."
Line 134: Was the same vial used for all monthly sampling or multiple vials aliquoted at the outset? Standardize to either ml or mL throughout text/figs.
Line 142: Consider specifying what the positive control was (i.e., surface water spiked with H3N8).
Line 143: What was the dose of the inoculum? If you didn't titer the samples, you can at least specify VI sensitivity and/or the Ct values. Because the swabs were diluted into 40mL of surface water it seems that the inoculum may have been at a relatively low dose for at least some of the inoculums which is important for interpreting the challenge results. Maybe add Cts/dose information to Figure 5. This information seems particularly important given that most mallards shed viral RNA, but did not seroconvert. For low doses, the ducks have been able to clear the infection with only a cellular immune response before mounting an antibody response.
Line 145: Does paired OP/CL swabs mean both were collected or samples were placed in a single vial?
Line 146: Were paired swabs collected from each individual in a group?
Line 149: Please be more specific about which samples were selected for testing. The lowest Ct per individual/sample type/group?
Line 158: A single reference to the Supp Info at the outset of the methods would improve readability.

RESULTS
I found some of the results difficult to follow due to repetition of methods and distracting details. The comments below are potential instances where the results could be improved. These suggestions don't need to be addressed individually, but as a guide for potential improvements for readability/reducing redundancy.
Line 168: As all vials tested are replicate vials, calling them replicate vials doesn't add clarity without further characterization (e.g. Rep1 or Rep 2 or Initial vs Recovered/Submerged).
Line 180: Excessive detail in this sentence obscures the main result. "Using samples from our field experiment" could be deleted as these results are in the "Field Experiment" Section. Experimental/Sample details are well documented in the Methods/Supp Info/Table so don't need to be repeated; e.g., We obtained genomic information for all 51 viruses isolated in the initial screen of inoculated surface water samples and for 10 viruses isolated from the matched replicate samples that we recovered approximately 7 months later.

Line 191: Redundant details could be removed/generalized by describing these samples as virus isolates from submerged samples; e.g., There were no predominate combined HA-NA subtypes among isolates recovered from submerged samples at either location (Figure 2).
Line 195: Recommend limiting details; e.g., All isolates recovered from submerged samples shared >99.6% nucleotide identity at each viral gene segment with the virus isolated from the corresponding replicate sample.
Line 199: Details could be generalized for ease of reading; e.g. Fall for August or September 2018 Spring for April 2019. Same for Lines 205-206. Or timing detail could be generalized to the paired samples; i.e., as initial and recovered/submerged.
Line 216: Minimize reiteration of methods. That the samples were held between Sept-April isn't important since the samples were held at 4C.
Line 219: Don't need to repeat 40 samples as it was specified in Line 216.
Line 220: Rather than April 2019, the important result is the samples were still infectious by VI after appx. 7 months.
Line 224: Probably more straight-forward to say corresponding submerged samples rather than repeating that the samples are from MN and were recovered in April 2019.
Line 233: Redundant to restate that the viruses were recovered between 209-229 days.
Line 235: Redundant to restate the inoculum which was just identified in Line 233.
Line 238: Do not need a 3rd reiteration of the inoculum.
Line 239: This sentence is repetitive; all the specifics other than 99.8% nucleotide identity (which could be added parenthetically to the previous sentence) are in the Table/Figure.
Line 247: Details are all available in the Table/Figure; consider limiting reporting here to seroconversion was observed in the positive control and 3 of the 9 experimental groups.
Line 250: The main point is somewhat lost in details; e.g., Based on positive rRRT-PCR, VI, seroconversion, and > 99% nucleotide identity with the IAV isolated in the inoculum, one sample each from Alaska and Minnesota was infectious in a mallard model after being held at ambient temperature in the field for seven months (Figure 5).

RESULTS Other Comments
Line 164: Not sure you need to point out that you are presenting the results in descending order. Consider providing total sample numbers (e.g., positive samples in Minnesota (n = 65/208).
Line 168: Line 151 indicates an individual swab sample was tested but the earlier reference to then as paired might be interpreted as the pair was placed in a single tube.
Line 183: The sentence about GenBank submission could be moved to the end of the paragraph as it is more of a housekeeping detail and detracts from the results.
Line 217: The word monotonic might be clearer in place of successive.
Line 228: The information on this bird needs to be reported but might be better mentioned a bit later in the paragraph so as to not detract from the main results birds.
Line 236: Consider replacing the "or" with an "and."
Line 246: Missing the word "on" before 15 dpi?
Line 294: Including PCR result details might be informative with respect to the discrepancy between PCR results and seroconversion: were any/most of the birds PCR positive on more than 1 day post inoculation? Were the Cts relatively low/high? The inoculum dose might also be relevant if birds were able to clear/prevent infection for low exposure doses.

DISCUSSION
Line 272: Wouldn't 0.2micron filtration remove bacteria?
Line 273: The important discussion here should be whether the pH change was similar for submerged samples compared to the surrounding surface water and whether a difference would impact inference from results to natural systems.

TABLES & FIGURES
Table 1: The "Samples Collected" column heading should be renamed to "Paired swabs" or "Samples inoculated" since only water samples were collected. If the goal of the experiment was to test viral persistence, then the sample size is 51 (# of samples with infectious virus). If the goal is to test different water conditions, then the relevant sample size is 6. I appreciate the work that

went into the collection and testing of 686 samples, but that number deflates the assessment of viral persistence. A major result of the work is that nearly 20% of infectious samples tested in the fall were still infectious by VI 6-7 months later under field conditions, but that result gets lost in this table since the number of positives looks so low. Consider adding percentages to the table. Also, consider moving the deployment date column after the PCR and VI columns since the samples were tested and then deployed. For consistency and to be more specific, consider referring to the samples as submerged rather than deployed. Adding a column summarizing the number of days samples were submerged might be useful.
Figure 1: Maybe reword or switch 3 & 4 – it seems like samples would be collected before they were characterized. Clarify how 9 is different from 3? Laboratory Experiments – the methods indicate that the samples were maintained in their original tubes and not aliquoted. Challenge Study – "virus isolation positive contents" might be clearer to just say virus isolate from…
Figure 2: The figure is not a histogram; it is a bar chart. See: https://www.forbes.com/sites/naomirobbins/2012/01/04/a-histogram-is-not-a-bar-chart/#33ada0256d77 Consider replacing "environmental conditions" with ambient temperature. Or, it might be more informative to refer to them as submerged samples since maintaining them at environmental conditions could be done in the lab and you might want to emphasize that it was done in the field.
Figure 3: Define MN and AK in the figure legend as they are abbreviated on the Figure.
Figure 5 Legend: Consider specifying that the inoculum was contaminated surface water so readers don't assume it was propagated. The legend might be improved by emphasizing the primary goal of the figure and de-emphasizing details (e.g., 209-229 d could be appx. 7 months, the study sites could just be MN) in the first sentence. Consider replacing "environmental conditions" with ambient temperature.

Author's Response to Decision Letter for (RSPB-2020-0959.R0)

See Appendix A.

RSPB-2020-1680.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?
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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
I think that the authors have well addressed my concerns and I'm happy to recommend acceptance.

Decision letter (RSPB-2020-1680.R0)

10-Aug-2020

Dear Dr Ramey

I am pleased to inform you that your Review manuscript RSPB-2020-1680 entitled "Influenza A viruses remain infectious for more than seven months in northern wetlands of North America" has been accepted for publication in Proceedings B.

The referee does not recommend any further changes. Therefore, please proof-read your manuscript carefully and upload your final files for publication. 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 me know immediately.

To upload your 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 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, upload a new version through your Author Centre.

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. Please note that PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file from the main text and the file name should contain the author's name and journal name, e.g authorname_procb_ESM_figures.pdf
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 see: https://royalsociety.org/journals/authors/author-guidelines/

4) Data-Sharing and data citation
It is a condition of publication that data supporting your paper are made available. Data should be made available either in the electronic supplementary material or through an appropriate repository. Details of how to access data should be included in your paper. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

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=RSPB-2020-1680 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.

5) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit https://royalsociety.org/journals/authors/author-guidelines/.

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

Sincerely,
Professor Hans Heesterbeek
mailto:proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
Thank you for the very through revision of the paper. I think it is a really important contribution and really like the approach of using both lab experiment and field data. The referees are happy with the revision.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
I think that the authors have well addressed my concerns and I'm happy to recommend acceptance.

Decision letter (RSPB-2020-1680.R1)

11-Aug-2020

Dear Dr Ramey

I am pleased to inform you that your manuscript entitled "Influenza A viruses remain infectious for more than seven months in northern wetlands of North America" 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

Your article has been estimated as being 10 pages long. Our Production Office will be able to confirm the exact length at proof stage.

Open Access
You are invited to opt for Open Access, making your freely available to all as soon as it is ready for publication under a CCBY licence. Our article processing charge for Open Access is £1700. Corresponding authors from member institutions (http://royalsocietypublishing.org/site/librarians/allmembers.xhtml) receive a 25% discount to these charges. For more information please visit http://royalsocietypublishing.org/open-access.

Paper charges
An e-mail request for payment of any related charges will be sent out shortly. The preferred payment method is by credit card; however, other payment options are available.

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.

You are allowed to post any version of your manuscript on a personal website, repository or preprint server. However, the work remains under media embargo and you should not discuss it with the press until the date of publication. Please visit https://royalsociety.org/journals/ethics-policies/media-embargo for more information.

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,
Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Response to reviewers' comments for manuscript #RSPB-2020-0959

Dr. Hans Heesterbeek and Reviewers for *Proceedings of the Royal Society B*:

Thank you for the critical review of our recent submission entitled 'Influenza A viruses remain infectious for more than seven months in northern wetlands of North America'. Based upon the feedback received, we have revised our manuscript and specific changes are described below. We hope that you feel as though our revised submission now warrants publication in *Proceedings of the Royal Society B.*

Editor comments

Editor: Dear Dr. Ramey,

I am writing to inform you that your manuscript RSPB-2020-0959 entitled "Influenza A viruses remain infectious for more than seven months in northern wetlands of North America" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken after considering the advice of referees, who have recommended that revisions are necessary. The reviewers are positive overall though, as am I. 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.

(re-submission instructions omitted for brevity)

Response: We very much appreciate the helpful critiques provided through the review process and hope that our revision adequately addresses all Reviewer comments.

Reviewer comments

PRSB Reviewer #1: Ramey et al address the interesting question of viability of influenza viruses within the environment. To this extent, they perform field and laboratory experiments to determine that these viruses can remain infectious for up to 7 months, suggesting the importance of environmental transmission for avian influenza viruses.

I think this is an important study, combining both field and experimental study, which deserve to be published. Nevertheless, I have a couple of recommendations before.

Response: We thank the Reviewer for his/her critical review of our manuscript and for providing additional comments.

Reviewer #1: First, I clearly do appreciate the combination of field and experimental works. However, they feel quite disconnected in some way. The samples from the field have been used to do experimental work, but we do not know from the field study if the viruses found in water at the beginning of the season are the same than at the end of the season. Obviously, regarding the

huge diversity of AIV, it is not possible to determine if they are the same virus. But I think it could be extremely interesting to see if the decay in number of subtype isolates decreases through time at a speed compatible with the environmental viability measured in the lab. The subtype with a short experimental viability in the lab should disappear first from the field data. This nevertheless require to have subtype identification for all field isolates through time.

Response: We thank the Reviewer for his/her feedback and concur that it is extremely interesting and informative to utilize field and laboratory approaches in parallel to better understand the viability of influenza A viruses through time. This was the intention of our study, and therefore we perceive that there may be some misunderstanding of our methods and results. That is, we believe that our study provides information and inference towards the topics the Reviewer has provided comments on. For example, the Reviewer states, 'we do not know from the field study if the viruses found in water at the beginning of the season are the same than at the end of the season'. Respectfully, we would like to direct the Reviewer to final sentences under the sub-heading 'Field experiment' within the Materials and Methods section as well as the second-to-last paragraph under the same sub-heading in the Results. Upon careful review of these sections, we believe the Reviewer will find that we employed full genome sequencing in our study to assess whether specific viruses persisted through time. Indeed, we successfully cultured viruses from samples collected 'at the beginning of the season' that were genomically nearly identical to replicate samples recovered at 'the end of the season'.

Also, the Reviewer mentions that it would 'be extremely interesting to see if the decay in number of subtype isolates decreases through time at a speed compatible with the environmental viability measured in the lab.' While we acknowledge that our ability to assess the 'speed of decay' is imperfect based upon our study design (e.g., only two time points for field samples), a relatively small sample size, and lack of virus titers; we feel as though our study does incorporate meaningful comparisons between field and laboratory results that 'speak to' the Reviewer's comment. For example, as reported in the Results section under the sub-heading 'Laboratory experiment': 'Of the 40 samples from which IAVs were isolated upon initial testing, five (13%) yielded infectious viruses approximately seven months later at the time of final re-testing in April 2019. Three of the five samples that remained infectious for the duration of the lab experiment corresponded to replicate #2 samples that remained infectious after being held at field sites within Agassiz NWR, Minnesota as part of our field experiment (Reeves et al. 2020b). The other two IAVs that were consistently isolated throughout our laboratory experiment were not recovered from corresponding replicate #2 field samples (Reeves et al. 2020b).' That is, we compare the 'decay' of 40 viruses in the field and the lab over a period of seven months.

Furthermore, we interpret the results reported above in a context similar to what we understand the Reviewer to be seeking in his/her feedback. More specifically, we found the persistence of viruses to be generally concordant, albeit imperfect, between field and laboratory approaches. Again, we respectfully direct the Reviewer to the second paragraph in the Discussion as follows: 'When comparing the results of our field experiment in Minnesota to those obtained using a laboratory approach, we found results to be generally concordant. More

specifically, in our laboratory experiment, a slightly lower percentage of IAVs remained infectious for the duration of the approximately seven-month study period as compared to results obtained from Minnesota in our field experiment. Perhaps the lower percentage of IAVs remaining viable after approximately seven months in the laboratory could have been a function of the slightly warmer water temperature at which viruses were held, consistent with the general inverse relationship between duration of viral persistence and ambient temperature reported in previous controlled experiments (Stallknecht et al. 1990a, Brown et al. 2007, Brown et al. 2009, Keeler et al. 2014). Though most of the IAVs that remained infectious throughout the duration of the laboratory experiment also remained infectious in the field experiment in Minnesota, we also repeatedly isolated two viruses in our laboratory investigation throughout the approximately seven month study period that did not remain infectious after being held for a comparable time period in the field. This discrepancy may be due to experimental artifacts (e.g., unequal mixing of virus between our split field samples), differences among viruses with regard to the ambient temperature range at which they are able to remain viable, or potential isolation issues associated with the formation of viral aggregates through time. Additional comparative studies using parallel field and laboratory approaches may be useful for resolving this uncertainty.'

In summary, while we did not design our experiment to provide information in the exact format that we understand the Reviewer to envision (i.e., the same number of repeated measures in the field and the lab – a logistically challenging approach!) we do feel as though we have incorporated methods, results, and discussion that speak to the biological processes that the Reviewer identifies as being of particular interest. As such, we respectfully request the Reviewer to consider our manuscript in this context. We apologize if we have misunderstood the Reviewer's critique and therefore failed to adequately respond to his/her concerns.

Reviewer #1: I think also the consequences of this study are not elaborated enough. Regarding cross-immunity processes and evolutionary dynamics, it is non obvious that a longer environmental persistence would yield a larger diversity of strains within avian populations, neither in human populations

Response: We thank the Reviewer again for his/her comments and appreciate the suggestion to extend speculation to possible implications to cross-immunity processes and evolutionary dynamics. While we understand the viewpoint that some readers may find speculation regarding how environmental persistence influences broad evolutionary processes/dynamics as a useful basis for future hypothesis testing, we did not design our investigation to provide inference on these topics. As such, we feel that such speculation would be poorly supported by our data. We therefore respectfully decline to extend inference beyond the specific hypotheses that we set out to test in our project. Once again, we apologize to the Reviewer if we have misunderstood his/her critique and therefore failed to adequately respond to the feedback received.

PRSB Reviewer #2: This manuscript describes a field test of influenza A virus persistence for viruses collected from wild waterfowl. The experiments and data are very interesting and present a rigorous test of viral persistence in a field setting which is matched to a laboratory assessment and animal challenge. The finding that nearly 20% of isolated viruses are still viable by VI approximately 7 months after field exposure to ambient temperatures is an important demonstration that these viruses can overwinter in the environment. The test period encompassed fall-spring migration so the results have significance for our understanding of the natural ecology of these viruses. The experimental design, methods, and interpretation are sound and I do not have any major concerns. The primary suggestion I have for improving the manuscript is to edit for conciseness and clarity (primarily in the Results section).

Response: We thank the Reviewer for his/her critical review of our manuscript and for providing detailed comments.

Reviewer #2: ABSTRACT
Line 34: Not sure why there is a hyphen in far-less.

Response: Change made per the Reviewer's suggestion. We also note that we considerably shortened our abstract per the strict 200 word limit imposed by the journal outlet.

Reviewer #2: Line 38 "replicate samples of duck swabs in surface water" isn't immediately clear; might be clearer to say duck swabs stored in surface water or duck swabs inoculated into surface water?

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 40: I initially read this to mean that IAVs were molecularly detected directly from collected surface water samples (not samples spiked with swabs). It should be clarified that these are the same samples as described above; i.e., surface water inoculated with duck swabs.

Response: We have revised our text per the Reviewer's suggestion.

Reviewer #2: KEYWORDS
Reservoir might be useful as well.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: INTRODUCTION
Line 73: Potential reference: Hénaux V, Samuel MD, Dusek RJ, Fleskes JP, Ip HS (2012) Presence of Avian Influenza Viruses in Waterfowl and Wetlands during Summer 2010 in

California: Are Resident Birds a Potential Reservoir? PLoS ONE 7(2): e31471. https://doi.org/10.1371/journal.pone.0031471

Response: Change made per the Reviewer's suggestion.

Reviewer #2: METHODS
Line 109: How many vials were inoculated per site? Exact numbers can be reported in Results, but sample size is an important aspect of experimental design and should be reported in Methods.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 111: Paired swabs in 40mLs seems like a significant dilution. How many replicates were aliquoted per 40mL vial? This was confusing to me upon first read (reading the abstract led me to think you might have pulled samples periodically rather than only once), but upon a full read it's clear there were 2 replicates. It might be clearer to indicate duplicates or paired/matched replicates or refer to the samples as replicate 1 and replicate 2 as you do in Fig. 1.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 114: The text implies that the samples were tested prior to submerging the paired sample. If so, why submerge the negatives since only the positives were tested post recovery?

Response: We have clarified the timing of testing per the Reviewer's comment.

Reviewer #2: Line 117: Please specify what you mean by "appropriate control."

Response: All controls have been specified in the complete materials and methods. As such, we respectfully direct the Reviewer to Supplemental File S1. We simply do not have space to define and clarify all controls in the main text per strict length requirements imposed by the journal.

Reviewer #2: Line 134: Was the same vial used for all monthly sampling or multiple vials aliquoted at the outset? Standardize to either ml or mL throughout text/figs.

Response: The former information is reported in the preceding sentence and therefore no change was made. We standardized 'ml' throughout the manuscript and Figures per the Reviewer's suggestion.

Reviewer #2: Line 142: Consider specifying what the positive control was (i.e., surface water spiked with H3N8).

Response: Again, we respectfully direct the Reviewer to Supplemental File S1. We simply do not have space to define and clarify all controls in the main text per strict length requirements imposed by the journal.

Reviewer #2: Line 143: What was the dose of the inoculum? If you didn't titer the samples, you can at least specify VI sensitivity and/or the Ct values. Because the swabs were diluted into 40mL of surface water it seems that the inoculum may have been at a relatively low dose for at least some of the inoculums which is important for interpreting the challenge results. Maybe add Cts/dose information to Figure 5. This information seems particularly important given that most mallards shed viral RNA, but did not seroconvert. For low doses, the ducks have been able to clear the infection with only a cellular immune response before mounting an antibody response.

Response: Ct values have been reported in Figure 5 per the Reviewer's suggestion.

Reviewer #2: Line 145: Does paired OP/CL swabs mean both were collected or samples were placed in a single vial?

Response: We clarified that OP and CL swabs were placed in separate vials for testing as part of the challenge study in both the main text and Supplemental File S1 per the Reviewer's comment.

Reviewer #2: Line 146: Were paired swabs collected from each individual in a group?

Response: We clarified that OP and CL swabs were collected from each bird in both the main text and Supplemental File S1 per the Reviewer's comment.

Reviewer #2: Line 149: Please be more specific about which samples were selected for testing. The lowest Ct per individual/sample type/group?

Response: We appreciate the Reviewer feedback and recognize that our methods were not particularly clear. We have revised our text to more clearly convey 'All CL and OP swabs were screened for IAVs using rRT-PCR (Spackman 2014) and samples with rRT-PCR Ct values ≤ 38 were subjected to virus isolation as previously described.'

Reviewer #2: Line 158: A single reference to the Supp Info at the outset of the methods would improve readability.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: RESULTS
I found some of the results difficult to follow due to repetition of methods and distracting details. The comments below are potential instances where the results could be improved. These suggestions don't need to be addressed individually, but as a guide for potential improvements for readability/reducing redundancy.

Response: We thank the Reviewer for taking the time to provide detailed comments and therefore have taken the time respond to each suggestion individually.

Reviewer #2: Line 168: As all vials tested are replicate vials, calling them replicate vials doesn't add clarity without further characterization (e.g. Rep1 or Rep 2 or Initial vs Recovered/Submerged).

Response: We have carefully considered each and every use of 'replicate vials' to assess whether this term is informative in a given instance. We have also incorporated references to 'replicate #1' and 'replicate #2' samples throughout our manuscript per the Reviewer's suggestion.

Reviewer #2: Line 180: Excessive detail in this sentence obscures the main result. "Using samples from our field experiment" could be deleted as these results are in the "Field Experiment" Section. Experimental/Sample details are well documented in the Methods/Supp Info/Table so don't need to be repeated; e.g., We obtained genomic information for all 51 viruses isolated in the initial screen of inoculated surface water samples and for 10 viruses isolated from the matched replicate samples that we recovered approximately 7 months later.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 191: Redundant details could be removed/generalized by describing these samples as virus isolates from submerged samples; e.g., There were no predominate combined HA-NA subtypes among isolates recovered from submerged samples at either location (Figure 2).

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 195: Recommend limiting details; e.g., All isolates recovered from submerged samples shared >99.6% nucleotide identity at each viral gene segment with the virus isolated from the corresponding replicate sample.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 199: Details could be generalized for ease of reading; e.g. Fall for August or September 2018 Spring for April 2019. Same for Lines 205-206. Or timing detail could be generalized to the paired samples; i.e., as initial and recovered/submerged**.**

Response: We have carefully considered this suggestion and reviewed the text referenced. Based on the feedback received, we do not believe that the comment reflects any lack of understanding or clarity and rather represents stylistic preferences (i.e., generalization vs. precision). As such, no change has been made.

Reviewer #2: Line 216: Minimize reiteration of methods. That the samples were held between Sept-April isn't important since the samples were held at 4C.

Response: Changes made per the Reviewer's suggestion.

Reviewer #2: Line 219: Don't need to repeat 40 samples as it was specified in Line 216.

Response: We omitted repetition of 40 samples in consecutive sentences per the Reviewer's suggestion.

Reviewer #2: Line 220: Rather than April 2019, the important result is the samples were still infectious by VI after appx. 7 months.

Response: We have clarified that samples were infectious by VI after approximately seven months per the Reviewer's suggestion. We have retained reference to the timing of final measures as we feel some readers will find it pertinent that our lab study was conducted in parallel with the field experiment.

Reviewer #2: Line 224: Probably more straight-forward to say corresponding submerged samples rather than repeating that the samples are from MN and were recovered in April 2019.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 233: Redundant to restate that the viruses were recovered between 209-229 days.

Response: We have carefully reviewed the Reviewer's comment and the referenced text. In line 233, we do not state 'viruses were recovered between 209-229 days'. Rather, we, for the first time, clearly convey the molecular detection of viral RNA from mallards challenged with inoculum that was held under ambient temperatures for 209–229 days. As such, we do not

believe that information conveyed is redundant. Instead, we feel that this statement precisely conveys relevant information. We therefore respectfully decline to make changes to the text in response to this specific comment**.**

Reviewer #2: Line 235: Redundant to restate the inoculum which was just identified in Line 233.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 238: Do not need a 3rd reiteration of the inoculum.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 239: This sentence is repetitive; all the specifics other than 99.8% nucleotide identity (which could be added parenthetically to the previous sentence) are in the Table/Figure.

Response: We have carefully reviewed the Reviewer's comment and the referenced text. While we concur that much of the information presented in this sentence can be found in Figure 5 and the associated data release, we also feel as though it is appropriate to help guide readers through the results of our challenge study. While the Reviewer may find this guidance unnecessary, we feel it may be helpful to other readers. We therefore respectfully decline to make changes to the text in response to this specific comment.

Reviewer #2: Line 247: Details are all available in the Table/Figure; consider limiting reporting here to seroconversion was observed in the positive control and 3 of the 9 experimental groups.

Response: We have incorporated some changes per the Reviewer's suggestion but have also retained some details to help guide readers through our results. We appreciate that the Reviewer clearly understood our multi-faceted study, but we also feel that information may be less intuitive to other readers.

Reviewer #2: Line 250: The main point is somewhat lost in details; e.g., Based on positive rRRT-PCR, VI, seroconversion, and > 99% nucleotide identity with the IAV isolated in the inoculum, one sample each from Alaska and Minnesota was infectious in a mallard model after being held at ambient temperature in the field for seven months (Figure 5).

Response: We have carefully considered this suggestion and reviewed the text referenced. Based on the feedback received, we do not believe that the comment reflects any lack of understanding but rather represents stylistic preferences (i.e., generalization vs. precision). As such, no change has been made.

Reviewer #2: RESULTS Other Comments
Line 164: Not sure you need to point out that you are presenting the results in descending order. Consider providing total sample numbers (e.g., positive samples in Minnesota (n = 65/208).

Response: We have omitted text stating that results are presented in descending order per the Reviewer's suggestion. We have carefully considered the Reviewer's suggestion to incorporate denominators into the text (also presented in Table 1), but ultimately decided against doing so given that we feel a more complicated construction would be required to appropriately do so. That is, using the Reviewer's example, we feel it would be necessary to use a construction in the following format: 'positive samples in Minnesota (n = 65 of 208 total samples tested)'. We feel this more complicated construction would need to be replicated for each wetland complex. Given the general feedback received from the Reviewer on other areas of the manuscript (i.e., to simplify text and to minimize repetition/overlap with Tables and Figures), we feel as though the requisite construction to incorporate denominators into the text may, ultimately, not be viewed as an improvement.

Reviewer #2: Line 168: Line 151 indicates an individual swab sample was tested but the earlier reference to then as paired might be interpreted as the pair was placed in a single tube.

Response: We have revised our text to clarify that in our challenge study, paired OP and CL swabs were collected from each bird and placed in separate vials.

Reviewer #2: Line 183: The sentence about GenBank submission could be moved to the end of the paragraph as it is more of a housekeeping detail and detracts from the results.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 217: The word monotonic might be clearer in place of successive.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 228: The information on this bird needs to be reported but might be better mentioned a bit later in the paragraph so as to not detract from the main results birds.

Response: We thank the Reviewer for his/her comment and concur that the information referenced needs to be reported. We feel that many readers will first want to know if clinical signs were observed in our challenge study and others may be confused by an inverted duck silhouette in Figure 5 if we do not offer that one bird was euthanized one day post inoculation at the beginning of the Challenge study sub-section of the Results. Therefore, after careful

consideration of the Reviewer's suggestion, we respectfully decline to make this suggested change.

Reviewer #2: Line 236: Consider replacing the "or" with an "and."

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Line 246: Missing the word "on" before 15 dpi?

Response: We have revised the referenced text per the Reviewer's suggestion.

Reviewer #2: Line 294: Including PCR result details might be informative with respect to the discrepancy between PCR results and seroconversion: were any/most of the birds PCR positive on more than 1 day post inoculation? Were the Cts relatively low/high? The inoculum dose might also be relevant if birds were able to clear/prevent infection for low exposure doses.

Response: We are a bit confused by this feedback from the Reviewer, but have made our best attempt to respond to this critique. First, we note that Line 294 does not discuss any PCR results nor make any reference to seroconversion. Thus, we believe that this comment may apply to Line 249. Line 249 refers to seroconversion among groups of mallards experimentally inoculated with filtered water/swab samples that had overwintered at wetland field sites. Thus, we infer that the Reviewer is inquiring about PCR results that may be used to infer the detection of influenza A virus RNA among experimentally challenged birds. These results have now been made publicly available through the refenced data release which we acknowledge was not available to the Reviewer at the time of his/her review. Thus, we hope future readers will find this information easily accessible. Also, we have reported the Ct values the Reviewer inquired about in Figure 5 as previously suggested.

Reviewer #2: DISCUSSION
Line 272: Wouldn't 0.2micron filtration remove bacteria?

Response: Yes, filtration probably removed most bacteria; however, some bacteria can pass through such filtration (e.g., Hahn 2004; https://doi.org/10.1016/j.resmic.2004.05.003). We have revised our manuscript and included this reference to clarify.

Reviewer #2: Line 273: The important discussion here should be whether the pH change was similar for submerged samples compared to the surrounding surface water and whether a difference would impact inference from results to natural systems.

Response: We absolutely concur with the Reviewer that is important to discuss whether the pH change was similar for submerged samples compared to the surrounding surface water. Per this comment, we respectfully direct the Reviewer to a sentence preceding the referenced text: 'We note that a consistent decline in pH was observed among all filtered and non-filtered water samples collected from Alaska, Minnesota, and Louisiana wetlands between our initial sampling efforts in late summer and autumn 2018 and subsequent sampling/recovery efforts in spring 2019.' However, we also recognize that it is not-so-straightforward to project how differences in pH between sample vials and the surrounding water 'would impact inference from results to natural systems'. We have attempted to provide some additional speculation that we perceive the Reviewer may be seeking by slightly modifying the refenced text as follows: 'In the overwintering water bottles, this was likely caused by microbial oxidation of organic matter, which produced carbon dioxide and thus acted to decrease the pH of the samples. At field sites, the specific factors resulting in a reduction in surface water pH over the winter season could not be easily identified and may have included: (1) increased partial pressure of carbon dioxide due to heightened ecosystem respiration relative to primary production, (2) the accumulation of weakly ionizable organic acids, and/or (3) the influx of acidic meltwaters in the spring (Kratz et al. 1987, Baehr and Degrandpre 2002, Finlay et al. 2015). Future investigations focused on pH variability in both sample vials and the environment over time may provide new insights regarding how this water quality parameter acts to limit viral viability in wetland surface waters.

Reviewer #2: TABLES & FIGURES
Table 1: The "Samples Collected" column heading should be renamed to "Paired swabs" or "Samples inoculated" since only water samples were collected. If the goal of the experiment was to test viral persistence, then the sample size is 51 (# of samples with infectious virus). If the goal is to test different water conditions, then the relevant sample size is 6. I appreciate the work that went into the collection and testing of 686 samples, but that number deflates the assessment of viral persistence. A major result of the work is that nearly 20% of infectious samples tested in the fall were still infectious by VI 6-7 months later under field conditions, but that result gets lost in this table since the number of positives looks so low. Consider adding percentages to the table. Also, consider moving the deployment date column after the PCR and VI columns since the samples were tested and then deployed. For consistency and to be more specific, consider referring to the samples as submerged rather than deployed. Adding a column summarizing the number of days samples were submerged might be useful.

Response: Numerous changes have been incorporated in Table 1 per the Reviewer's suggestions.

Reviewer #2: Figure 1: Maybe reword or switch 3 & 4 – it seems like samples would be collected before they were characterized. Clarify how 9 is different from 3? Laboratory Experiments – the methods indicate that the samples were maintained in their original tubes and

not aliquoted. Challenge Study – "virus isolation positive contents" might be clearer to just say virus isolate from…

Response: Changes made per the Reviewer's suggestions.

Reviewer #2: Figure 2: The figure is not a histogram; it is a bar chart. See: https://www.forbes.com/sites/naomirobbins/2012/01/04/a-histogram-is-not-a-bar-chart/#33ada0256d77 Consider replacing "environmental conditions" with ambient temperature. Or, it might be more informative to refer to them as submerged samples since maintaining them at environmental conditions could be done in the lab and you might want to emphasize that it was done in the field.

Response: Changes made per the Reviewer's suggestions.

Reviewer #2: Figure 3: Define MN and AK in the figure legend as they are abbreviated on the Figure.

Response: Change made per the Reviewer's suggestion.

Reviewer #2: Figure 5 Legend: Consider specifying that the inoculum was contaminated surface water so readers don't assume it was propagated. The legend might be improved by emphasizing the primary goal of the figure and de-emphasizing details (e.g., 209-229 d could be appx. 7 months, the study sites could just be MN) in the first sentence. Consider replacing "environmental conditions" with ambient temperature.

Response: We thank the Reviewer for his/her suggestions and have clarified inoculum and replaced 'environmental conditions' with 'ambient temperature'. We respectfully decline to generalize information in our legend as we feel that some readers may find precision important (e.g., as the Reviewer found specifics of inoculum to be important to convey).