Dear Dr. Njal Rollinson:

Thank you for submitting your paper Manuscript ID ACV-05-21-OM-157 entitled "A 91% decline in a common anuran in an otherwise stable amphibian community inferred from 17 years of rapid road surveys." to Animal Conservation. We have now received two reviews for your paper, which are included at the bottom of this letter.

In view of the criticisms of the reviewers, we must decline the manuscript for publication in Animal Conservation at this time. However, the Editors would like to invite you to submit a new version of this manuscript which takes into consideration these comments.

Please note that resubmitting your manuscript does not guarantee eventual acceptance, and that your resubmission will be subject to re-review before a decision is reached.

ATTENTION: To resubmit your manuscript, please log into http://mc.manuscriptcentral.com/acv and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions". Under "Actions", click on "Create a Resubmission". Please be sure to provide a cover letter with your resubmission detailing how you have responded to the points raised by the reviewers and/or the Editors.

You will be unable to make your revisions on the original version of your manuscript. Instead, please revise your manuscript using a word processing program, and submit it following the instructions above.

I hope you find the reviewers' comments valuable and I look forward to receiving your resubmission.

Best wishes,

Dr. Elina Rantanen
Animal Conservation Editorial Office

on behalf of
Dr. Gurutzeta Guillera-Arroita
Editor, Animal Conservation

Dear Editor,

Thank you for continuing to consider our manuscript for publication in Animal Conservation. We apologize for the delay in resubmitting this manuscript, but the delay is commensurate with the depth of the revision we undertook, in direct response to reviewer suggestions.
We have re-analyzed all the data as the reviewers suggested, and we have revised the discussion so that it is less speculative; indeed, the re-analyses allowed us to rule out a few additional hypotheses and provided new insight into the decline. Yet, despite the effort we made in the revision, we still cannot pinpoint the exact cause of the amphibian decline.

We nevertheless believe that what we have documented over 17 years is nothing short of a remarkable change in the community composition of local amphibians, driven by an incredible decline in what was historically the most common amphibian. We have performed a very careful and nuanced analysis of these data, and we have not overreached in our speculation, and we have pointed out the shortcomings our study in the Discussion. We feel that our main finding is both surprising and concerning, and that the method used to achieve this result is simple and broadly applicable, such that we believe our work will be of interest to a broad readership. Thanks for your work handling this ms.

Associate Editor's Comments to the Author:

Both reviewers appreciate the potential interest of the study for readers of Animal Conservation, but also point out several issues that should be addressed in a thorough revision before the manuscript can be considered for publication. The manuscript is well written and reflects hard work by the authors, it presents valuable data and a potentially important [local] trend. However, in its current state, its inference and conclusions about the locally observed trend are not sufficiently justified, and its relevance for broader conservation [via the proposed roadside monitoring approach] limited or unclear.

I agree with Reviewer #1 that, while considering hypotheses about the observed patterns is appropriate and desirable, currently about half of the discussion is devoted to what is essentially speculation. Additional analyses, as suggested by both reviewers, would go a long way towards strengthening inference. While some may be impossible (e.g. further disease testing), some should be doable (e.g. including traffic variables). At the very least, the discussion could be tightened or made more robust. For example, the authors do not discuss contamination because of lack of data, but this does not mean it can be ruled out (l. 242); it is not discussed why metapopulation and land use dynamics would affect only leopard frogs and not other species (l. 244-267); as suggested by Reviewer #2, it is necessary to assess or at least discuss possible effects of traffic or other road-related dynamics. I am not a road ecology expert, but I was also surprised that while the survey protocol is highly recommended, there is scarce discussion of its pros and cons in the context of existing road ecology literature (see R#2’s comments).

Response (revised lines: XXX-XXX): We thank the editor and reviewers for their helpful
comments, and we agree with almost all of them. We have gone to considerable lengths to restructure our analyses, allowing us to further rule out certain hypotheses.

First, to investigate whether road mortality contributed to the decline, we added an analysis investigating whether the proportion of amphibians observed alive on the road (vs dead on the road) changed over time; this proportion did not change, allowing us to rule out road mortality as a main driver of the decline (revised lines: XXX-XXX).

Second, we included life stage as a fixed effect in our models, as the reviewer suggested, to investigate whether there were any life-stage-specific changes in the population over time (revised lines: XXX-XXX); this analysis shows that the decline occurs in both juveniles and adults.

Third, we used predicted values from the life stage analysis to better-investigate whether climate in the year prior contributed to the observed declines (revised lines: XXX-XXX); using this approach, we found that annual environmental variables were related to the abundance of adults and juveniles, but that a year effect was much stronger than environmental effects, strongly suggesting that the environmental variables we considered did not play a central role in the decline.

We continue to recommend our method of rapid road surveys, but we have toned down the “novelty” of this method as a selling point of the ms. Instead, we take the pragmatic approach of mentioning the rapid survey as a method in the introduction, describing the method in the Methods, and commenting on how the simplicity of this approach may be amenable to broad uptake, especially as citizen science initiatives.

While it remains true that we cannot test certain hypotheses due to a lack of data, we have updated the discussion to contain less speculation while ensuring that we consider alternative hypotheses that were not directly tested (revised lines: XXX-XXX).

Reviewer(s)' Comments to the Author:

Reviewer: 1

Comments to the Author
In this manuscript, the authors use a long time-series of amphibian counts to estimate changes in abundance of the amphibian community at a single site, and in a second analysis explore whether annual patterns can be explained by general climatic differences between years. The authors conclude that a strong decline in amphibian abundances is driven almost exclusively by a sustained crash in northern leopard frogs, which are the dominant species in the amphibian assemblage. They conclude that these declines cannot be contributed to climatic effects, and
speculate on other potential drivers of the observed pattern. In general, I think the manuscript is clearly written and well presented. However, I think there are several aspects of the manuscript which require elaboration or clarification in order to fully assess the manuscript. I elaborate below on my two main concerns with the manuscript in its current form, and then below provide some further comments on each of the sections of the manuscript individually.

<u>Major Concerns</u>

1. A key assumption for this work is that “on-road occurrence is known to reflect amphibian population sizes” (line 42). The whole premise of the manuscript is based on this statement, yet the two citations supporting this statement relate to studies of moose and bird densities, respectively. The effects of roads on amphibians are rather different to how I imagine these other taxa interact with roadways. Given the importance of this assumption for the framing of this manuscript, I feel that this statement really needs to be better supported by the citing literature.

Response (revised lines: XXX-XXX): To our knowledge, there are no studies that test the assumption that population sizes are reflective of on-road abundance in amphibians specifically, hence we must continue to use the citations we used in the previous version. However, in response to this concern, we suggest that unless leopard frogs (but not the other species) have changed the manner in which they interact with the road over time, then our assessment of relative abundance is valid. As such, we have reworded the statement to “Assuming that on-road occurrence reflects relative amphibian population sizes” (LN43). Further, in the discussion (and in response to another reviewer point), we have raised the possibility that leopard frogs (but not other species) have simply stopped using the road over time, as we cannot rule out this hypothesis.

2. In the second part of the analysis, various climatic variables are used to explain annual fluctuations in population abundances. As discussed further below, the initial analysis estimates different abundances per season. It is unclear from which season the response variable (mean abundance) is derived.

Response (revised lines: XXX-XXX): We address this point below in a similar comment from this reviewer.

Furthermore, there is not sufficient information provided in the manuscript as to exactly what climatic variables were used as predictors in this second part of the analysis (some cryptic figure labels in the SI and the sparse information in Table S2 are insufficient). Without this information it is really not possible to determine whether “our study can rule out a number of putative causes” (as the authors state in the abstract). I think there needs to be much more information on this part of the analysis if the authors wish to claim that patterns are not influenced by climate.

Response (revised lines: XXX-XXX): That’s a fair point; we did not focus enough on this analysis in the previous version. In the present version, we added an additional supplemental table to
describe the parameters used in model selection analyses, and the models. We also restricted our focus to leopard frogs in this version, as they are the only species that show a directional change in abundance. We are quite willing to add the supp table or more information to the main text, but we do not want to add too much length or detract from the thrust of the ms. Please advise if the present approach is unsatisfactory.

<u>Further comments</u>

<b>Introduction</b>

• Lines 59-60: this sentence is very unwieldy, I would recommend you try to rephrase it.

Response (revised lines: XXX-XXX): rephrased

• There seems to be some rather strange capitalisation of terms throughout the manuscript (e.g. Anurans/Caudates are capitalised in line 78, soay sheep and florida scrub jays in l93 are not). I recommend you go through the document and ensure that capitalisation of all terms follows the journal guidelines.

Response (e.g., revised lines: XXX-XXX): all capitals from names have been removed to follow the journal guidelines.

• Line 82. I know this is a rather pedantic point, but this sentence should read “sustained surveying can be used to distinguish natural …..”. The surveying itself does not do the distinguishing.

Response (revised lines: XXX-XXX): fixed

• Line 107: “a senior herpetologist”. Please change this wording. What is a reader supposed to infer from the use of senior- that the herpetologist is old? Presumably not- I assume this adjective is meant to infer long experience. Why not then be clear and write something like “experienced herpetologist”?

Response (revised lines: XXX-XXX): Oh dear. Yes, this is a good point. Thank you! We have changed this to “experienced herpetologist”

<b>Methods</b>

• Line 124-125: This sentence is rather imprecise- it would be helpful to have a summary of the average duration of surveys if this is available.

Response (revised lines: XXX-XXX): we thank the reviewer for this point, however this was not recorded during each survey and we cannot provide a more precise answer than 5 – 15mins. This will be considered for future studies.
• Line 126-127. It would be nice to have a visualisation of the distribution of surveys/day e.g. in the SI, particularly as this variable is effectively a measure of survey effort which will have a strong impact on your modelling results.

Response (revised lines: XXX-XXX): Good idea. We added an additional histogram in the supplemental material to show the distribution of surveys performed within a single day (fig S2). As these are count data, we natural log transformed the survey number predictor in each model so that predicted values could not range below zero.

• Line 130-131. I think it would be helpful to state how many live and road-killed animals in total are included in your sample (in addition to the detail provided as to how many roadkills couldn’t be identified).

Response (revised lines: XXX-XXX): Great point. We added a table in the supplemental material (Table S2) to summarize the number of alive on road (AOR) and dead-on road (DOR) observations. We also specified the number of observations where an AOR/DOR status was not recorded or the species identity could not be confirmed.

• Line 133-135: Do not provide this information if you don’t intend to discuss it in this manuscript.

Response (revised lines: XXX-XXX): Removed

• Line 140-141: How common was it that data were missing from the Kemptville weather station?

Response (revised lines: XXX-XXX): 76 days (out of 2388 days) were missing from the kemptville weather station. This information is summarized in supplemental table S3.

• Line 148: should read all of the winter surveys/ all winter surveys.

Response (revised lines: XXX-XXX): fixed

• It is stated that species with large sample sizes are treated differently to those with small sample sizes (5th/4th order splines). It would be helpful for you to indicate the boundary used to distinguish into which category each species falls.

Response (revised lines: XXX-XXX): this analysis was redone (see responses below), and the 5th order spline did not cause any overfitting issues in the new analysis. Thus, all models investigating species abundance over time contain a 5th order spline. Subsequent models investigating the proportion of AOR/DOR observations over time and abundance with respect to life stage contain a 4th order spline due to smaller sample sizes in the model and overfitting from additional fixed effects (e.g., interaction between year and lifestage). We do not feel that overfitting of splines is a serious concern in any event, as our goal is to accurately describe the
directional trend, and with large datasets like ours, judicious use of splines is very unlikely to give rise to erroneous temporal trends.

- I am not convinced that the approach used for mixed-effects models as described in lines 167-175 is actually doing what is desired. The inclusion of both year and season within the fixed effects structure implies that there is a mean abundance for each season-year combination which is being estimated (while accounting for the other continuous covariates described). Counts from individual days are therefore the non-independent observations, and I think the random effect should therefore be on a parameter such as Julian date, rather than year. This implementation may resolve the convergence issues described while acknowledging the hierarchical structure of your data.

  More generally, it seems strange to me that lines 167-175 start by explaining that random effects models are necessary to account for non-independence of observations, but then conclude by saying that those results are not presented in the main manuscript.

  Response (revised lines: XXX-XXX): Good point. We now use only random effects models, all of which are present in the main text. This is now possible because we natural log transformed covariates in each model due. After log-transforming, the mixed effect model (fit with an optimizer different than the default) did not create convergence issues. Thus, we chose to keep the analysis with the mixed effect model and keep year as a random effect. Adding Julian day as a random effect created singularity issues, as there was one observation per Julian day, and in this analysis, Julian day accounted for 0 variance.

  - Total daily number of surveys: This is an effective measure of sample effort, which I would expect to have a multiplicative interaction effect with your other covariates. Did you have a look at this?

    Response (revised lines: XXX-XXX): We struggled with this, as this raises the issue of “testing models before testing models” without a formal model selection framework. However, our sense is that the main thrust of the manuscript (a decline over time) does not change depending on the structure of the covariates. So, with this in mind, we ultimately decided to do as the reviewer suggested. In this version, we investigated the interaction between the number of surveys per day and the other covariates, prior to deciding on a final modelling framework. We found that the interaction between the number of surveys per day and temperature were significant predictors of abundance of “total amphibians”, and all subsequent models (i.e., species-specific models) contain this interaction, even if the interaction is not significant for a given species.

<b>Results</b>

- The models used season as a categorical variable- and as shown in Table 1, the season Autumn was used as the baseline level of this factor, which differences to the intercepts for the other seasons. Presumably the figures also show the abundances for autumn, which should be explicitly mentioned in the figures and in the text as necessary.

  Response (revised lines: XXX-XXX): To plot model predicted values (and create predicted values for subsequent analyses) we used the *ggemmeans* function which averages model predicted
values across all categorical levels; in other words, there is no "reference category", all levels of
the categorical variable are averaged. This has been added in the main text for clarity and figure
captions (fig 1).

• In a related way, why are abundances predicted to be higher in autumn than in spring or
summer? I would expect spring abundances to be highest.

**Response (revised lines: XXX-XXX):** we added an analysis that adds life stage as a fixed effect.
When controlling for differences in life stage, we see that abundance is slightly lower in the
spring, but that this result is non-significant (table S8). Since juveniles are significantly more
abundant in the fall (table S8), juvenile abundance is likely driving this trend in the original
models. While we do not directly address this point in the discussion, we made sure to mention
it in the results (revised lines: XXX-XXX).

**Discussion**

• The dataset appears to be dominated strongly by leopard frogs, and therefore it is
unsurprising that the overall trend for amphibians is similar to those of leopard frogs (that is not
to say that this pattern is not important). It would be useful for the authors to provide a
summary of the numbers of each species identified (perhaps in the SI) so that readers can assess
how much should be read into this relationship between the overall pattern and the trend for
leopard frogs.

**Response (revised lines: XXX-XXX):** sample size with respect to species has been added in table
S2. Additional tables that summarize the data used in each model has also been added (Table
S1, S4).

• In line 197 it is stated that the trend for species (excluding leopard frogs) remained
relatively stable. The strong differences in relative abundances for each species makes it rather
difficult to compare the degree of variation seen in trends for each species. Perhaps if you
calculate some standardised measure of the annual variation in each species (e.g. scaled to the
maximum observed abundance for the species) this would help hammer this point home.

**Response (revised lines: XXX-XXX):** we added an additional plot (fig 1b) that compares leopard
frog abundance to other species abundance to reflect the contrast But with the reviewer’s
comment in mind, we also changed the wording so that it reflects a ‘lack of directional change’
in abundance as opposed to a “stable” abundance.

• Lines 225-243: The following two papers discuss the same issues as this paragraph and I
think provide relevant information

  Grant et al (2016) Scientific Reports. “Quantitative evidence for the effects of multiple drivers on
  continental-scale amphibian declines”

  change in North American amphibian communities”
Response (revised lines: XXX-XXX): we thank the reviewer for these helpful resources, we have updated the discussion accordingly.

- As discussed above, I am not (yet) convinced that the analysis carried out provides sufficient evidence to rule out climatic drivers of amphibian trends (stated in lines 2234-236). The following several pages of text (approx. lines 244-290) offer speculation of other potential drivers of these patterns which have not been tested in the current study. This does seem like rather a large portion of the discussion to devote to such speculation.

Response (revised lines: XXX-XXX): we have updated the analysis to focus on leopard frogs and show that some climate variables influence leopard frog abundance. However, we also show that year as a fixed effect is the strongest predictor of abundance, indicating an additional factor independent of the climate factors we considered is influencing abundance.

- Lines 268-290 discuss the impact of Chytrid on amphibian communities across the study area, and this paragraph ends with the claim that the impacts of chytrid are unknown in the study region. I would point the authors to a recent study by McMillan et al (https://doi.org/10.1111/1365-2656.13170) which explicitly examine chytrid dynamics in this region, as well as the PhD thesis of the main author of that paper (Epidemiology of the amphibian pathogen Batrachochytrium dendrobatidis, across multiple spatial scales, McMillan 2017).

Response (revised lines: XXX-XXX): we thank the reviewer for this helpful comment and have updated the discussion accordingly.

- Lines 219-end: I think that the end of the manuscript makes a number of well-considered points and is well written.
- Line 318-319: I do not agree with this claim that most long-term monitoring studies receive funding only for one or a few years.

Response (revised lines: XXX-XXX): we have added an additional source that highlights the limitations of long-term studies and the challenges these studies face. One of these challenged relates to funding.

<b>Acknowledgements and Declarations</b>

- The acknowledgement of the territories in which the fieldwork was carried out is duplicated.

Response (revised lines: XXX-XXX): we have updated the acknowledgements accordingly

- The data availability statement appears to be in conflict with journal requirements.
Reviewer: 2

Comments to the Author

This is an interesting paper that documents population trends in an amphibian assemblage in Canada using rapid road surveys. Taking advantage of the long-term data collection applied by one of the authors, the team analysed the trends over time, concluding that one of the species (northern leopard frog) has experienced a decline of 91%. The population density was assessed using road-killed carcasses, a protocol that I recommend further argumentation and contextualisation (see below). Lastly, the authors discuss several hypotheses to justify the decline but don’t test any of them, which I found the weakest part of the paper. I pointed out a few more weaknesses in the design and narrative that I think can still be addressed.

Yet, I believe the study is within the journal’s scope and shows some relevance. While most of my comments and suggestion were made directly on the manuscript, I leave some additional considerations below:

Title: sure titles need to be informative and reflect the contents of the article, but this one is just too specific! I would suggest something like: "A steady decline in a common anuran in an otherwise stable community inferred from a long-term period of rapid road surveys."

Response (revised lines: XXX-XXX): We thank the reader for this suggestion, but we respectfully disagree. We feel that the current title succinctly captures the main result of the ms and will draw reader interest, likely increasing readership of this work.

Road mortality: if you find LESS road-killed frogs, does it mean the population decreased or simply that there is LESS traffic?

Response (revised lines: XXX-XXX): We added an analysis to investigate the proportion of Alive on Road (AOR) with respect to Dead on Road (DOR) leopard frogs over time. We found that the proportion of AOR to DOR observations did not exhibit a directional change, and we conclude that road mortality did not contribute substantially to the decline.

Do you have traffic-density information for the road in the study area? Other studies suggested counting the vehicles as they pass by the observer/ researcher during surveys (Matos et al. 2012.
Amphibia-Reptilia 33: 469-483). Do you have any way to suggest no change in traffic intensity over time?

Response (revised lines: XXX-XXX): we do not have traffic density information for the study area and can only provide information that it is situated in a rural village approximately 80km from the nearest city centre (Ottawa, Ontario, Canada). We also did not record traffic intensity during each survey and cannot show data that supports no trend in traffic intensity. However, we did add an analysis that shows the proportion of alive on road observations to dead on road observations, and we would expect a directional change if traffic intensity did change over time (e.g., higher proportion of DOR observations compared to AOR observations).

Additionally, other studies have shown that vegetation and changes in land use adjacent to the road lead to differential mortality (Ramp et al. 2005, 2006). You hypothesized that this decline could be associated (at least in part) with land-use change. But have you considered that an eventual land-use change could have benefited the frog population, i.e., led to a reduction in road mortality? For example, created ponds elsewhere or allowing vegetation to grow taller (after abandoned farmland), making the frog movement towards the road more difficult? This may not necessarily result in a population decline. Something to consider...


Response (revised lines: XXX-XXX): we have added to the discussion to reflect this consideration.

Statistics:
What about the inclusion of "life stage" as a fixed effect in the models? This could be particularly relevant when you talk about changes in dispersal patterns of juvenile leopard frogs in your Discussion.

Response (revised lines: XXX-XXX): Great point. We added an analysis that contains life stage as a fixed effect and used the results from this analysis to investigate whether climate contributed to observed changed in abundance.

All these points could be cleared out in the Methods section if more details are provided.

Discussion: the Chytridiomycosis hypothesis could certainly be more sustained if the authors presented prevalence data (at least for the leopard frogs). If they still have the collected carcasses, it would be great to screen for both Ranavirus and Chytrid fungus, which would
crystallise the argument presented by the authors. Although I completely understand that this may not be what the authors want or have in mind.

Response (revised lines: XXX-XXX): we thank the reviewer for this consideration and do not have data related to the presence of infectious disease in the population at the moment. However, this will be considered in future studies.

Given what we know about circulating pathogens in the region and how they interact with certain species, I would be more inclined to a Ranavirus hypothesis rather than chytrid (see the articles I suggested in the PDF, particularly the long term impact of FV3 Ranavirus by Teacher et al. 2010).

Response (revised lines: XXX-XXX): we thank the reviewer for this resource and have added to the discussion accordingly.

In the abstract, when you mention the "novelty of the method", what are you referring to? Having a single person counting carcasses over time? Or the actual use of amphibian carcass-counting to monitor population density? The latter is the actual novel aspect of your story and probably can be made more clear. This method has been used mainly in ungulates and other mammals. As I said in the pdf, I wouldn't take for granted that you can simply transfer this protocol from moose and apply it to the monitoring of amphibians... but it is something you can certainly discuss in your Discussion using your own data/results to justify it.

Response (revised lines: XXX-XXX): We have clarified this point in the abstract.

Lastly, when discussing this protocol and recommending it as a long-term tool, it is critical to highlight how important it is to also collect data on any potential factors that may act in concert to alter road-kills (not necessarily reflecting a change in population density). Some examples are given above.

Response (revised lines: XXX-XXX): we added to the discussion to reflect this point.

[please find more comments from the Reviewer on the attached PDF]

Response (revised lines: XXX-XXX): for comments addressing grammar or punctuation or wording, we have revised accordingly.

(line 101) well... this is not really what McCarthy et al. actually say. They used road-kills to predict species distributions, which is very different from inferring population density.

However, Rolandsen et al. do suggest that the number of Moose-car accidents increases with increasing density. Other authors have suggested the same for other ungulates (Seiler, (2004). Wildl Res 10:301–313).
Yet, there are not studies with amphibians (that I'm aware of) to support that. I'm not so convinced that you can directly transfer this rational to amphibian populations that naturally experience huge fluctuations in population density, often of different orders of magnitude (see eg., Loman & Andersson, 2006. Conserv Biol; or even the example of your Bullfrog population).

I would advise the authors to improve this argument. But maybe this can be done in the Discussion: making a case with your own study.

Response (revised lines: XXX-XXX): we thank the reviewer for this helpful point. we have revised and re-worded the goal of the study (lines XXX-XXX) and moved this citation to the discussion (lines XXX-XXX).

I would suggest to also include the following reference that is still among the best for this broad sentence: Stuart et al. (2004). Science 306: 1783-1786

Response (revised lines: XXX-XXX): added See the following paper:

Response (revised lines: XXX-XXX): added a new sentence elaborating on additional pollutants that could contribute to the decline.

This is very context and system dependent. Please see the following paper:
Echaubar et al. (2010). Context-Dependent Effects of Ranaviral Infection on Northern Leopard Frog Life History Traits. PLoS ONE
Moreover, FV3 Ranavirus (the same type found in Ontario) has been linked to long-term localized population declines in the UK (Teacher et al. 2010. Anim Conserv 13: 514-522) CMTV Ranavirus led to heavy community collapses in (for example) Iberia: Price et al. 2014 (Curr. Biol. 24: 2586-2591); Rosa et al. 2017 (Scientific Reports 7: 43260).

Response (revised lines: XXX-XXX): we thank the reviewer for these resources and have added to the discussion accordingly.

I would suggest the authors to have a look at these papers:
Intro
we thank the reviewer for these resources and have added to the discussion accordingly.

citizen science also present a lot of challenges:
- Roche et al. (2020). Front. Sociol.
This to say that "ideal" is a very strong and over optimistic word :) I would tone it down.

We added a sentence acknowledging the limitations that accompany citizen science based data.