Review History
RSOS-140377.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
The authors state in the manuscript that all microbial diversity data have been deposited in an online database and will be made available upon manuscript acceptance.

However, as far as I can tell, data on Bd infections of their samples have not been made available. This could be included in the supplementary material.
The current supplementary material is clear. I recommended that the collection summary table be moved to the main text.

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

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

Comments to the Author(s)
This manuscript investigates seasonal and ontogenetic variation in the amphibian skin microbiome and its relationship to natural disease dynamics in two species of amphibians. Overall, I think the study is very nice, and I recommend that it be published in Royal Society Open Science.

I have made extensive comments on the manuscript in the form of line edits. Within this, there are two substantial criticisms that I feel the authors should address before the manuscript is published:

First, I think the hypothesis is a bit of a stretch. I don’t think enough data is provided to establish a link between microbial diversity, infection, and immune function. Unless the authors can provide evidence of variation in infection and immune function coinciding with changes in microbial diversity in their samples, I recommend that they save their ponderings on the underlying cause of the differences for the discussion.

Second, I recommend that the final section of the results, addressing variation in just the Proteobacteria in one of the two amphibian species, be removed from the study unless the authors can provide a more convincing reason to include it. It just sort of comes out of nowhere because it isn’t set up or explained in the methods, and (if I’ve interpreted it correctly) I think the relationships between diversity and Bd load they find are rather weak in that it only captures a tiny fraction of the range in variation in infection in this species (1-4 zoospore equivalents).

Line Comments:

Title
I don’t think the title accurately reflects the study. I would suggest changing to something along the lines of “Seasonal and ontogenetic variation in the amphibian skin microbiome and relationship to natural disease dynamics in declining amphibians”

Also, just out of curiosity, what evidence is there that coquis are actually declining?

Abstract

Page 2, line 54. What do you mean by “thresholds”? I think you may want to use a different word here.

Page 2, lines 55-57. I would rephrase your hypothesis to make it more neutral regarding the relationship to Bd infection. As you yourself admit, it is difficult to determine, especially from the results of survey data, whether seasonal/developmental shifts in community structure lead to changes in pathogen presence, or whether it is seasonal/developmental variation in susceptibility
to invasion by the pathogen that causes changes in microbial community structure. I think this is best left for the discussion.

Page 2, lines 60-68. I think your wording is a bit misleading. I would rephrase so that it is clear that you assess seasonal variation in one species, and ontogenetic variation in the other.

Introduction

Page 3, line 79. Change “is often” to “has been”

Page 3, lines 81-83. I would rephrase as “Identifying conditions that disrupt stable…” (instead of support)

Page 3, line 94. Change “in determining” to “to determine”

Page 3, lines 97-98. This is a better way to think about framing the intro and your hypotheses. Much more in line with what you can say from your survey results.

Page 3, lines 98-99. Delete or move this sentence. It has nothing to do with disease, and so does not fit here.

Page 4, line 101, line 103, line 108. Lots of unnecessary punctuation (periods, commas) throughout manuscript. I haven’t taken the time to point out all of them, but this should be addressed by the authors before they resubmit.

Page 4, line 107. Do bacteria “replicate” or “reproduce”?

Page 4, line 107. Change “may predispose hosts for…” to “may predispose hosts to…” There are quite a few grammatical errors like this throughout the manuscript that should be addressed by the authors before resubmitting.

Page 4, line 113. Are these two species phylogenetic positions really contrasting?

Page 4, line 114-116. This is how you should explain what you did in the abstract.

Page 4, lines 117-125. I think this is a bit of a stretch. I don’t think you provide enough data to establish a link between microbial diversity, infection, and immune function or stress. I would save this for the discussion. You merely observe differences in microbial diversity that coincide with known differences in pathogen susceptibility in these species. You don’t even report whether you see these trends in infection your samples, something I think you should consider including in your results.

Page 5, line 126. You mention species and infection status here, but you haven’t set up these hypotheses yet.

Page 5, line 127. Throughout the methods and results, you keep referring to the “age” of E. coqui. This is incorrect. You should use “developmental stage”. Unless you know something more specific about the age of individuals, other than that they are juveniles or adults.

Page 5, lines 133-137. This was only done for E. coqui, right? Specify.

Page 5, lines 137-139. I think this sentence is more appropriate for the discussion. Also, I’m not convinced that you do provide any evidence for a protective role. You show that diversity changes between seasons and developmental stages and point out that previous work has shown that Bd infection changes between seasons and developmental stages. But you don’t directly link the two.
Methods

Page 5, line 141. Please add in a section that explains your sample collection. When did it occur? Where? I don’t think it’s enough to just keep referring to a table in the supplementary material.

Page 5, line 146. Please provide a little more detail for the swabbing methods. Did you use the exact methods of the citation you provide (i.e., number of strokes, location of strokes)?

Page 6, line 158. You should provide a summary of your Bd infection results. Were there differences in Bd infection across seasons/developmental stages for your samples? What range of Bd loads did you find across individuals?

Page 8, line 215. What were your thermocycler conditions?

Page 8, line 219. Where was your Illumina sequencing done?

Page 8, line 224. “Sequences were filtered…” not “are filtered”. There are several places throughout the manuscript, particularly in the Results, where verbs need to be changed to past tense. The authors should address this before resubmitting.

Page 8, line 225. Which version of the Greengenes database?

Page 9, lines 233-235. Now I’m confused. In the previous section, you state that you rarefied all samples to 5000 reads. Now, here, you state that you generated rarefied phylotype tables to different sequencing depths before computing alpha diversity metrics. Please clarify. Also, if you did rarefy to several different depths, what depths did you use?

Page 9, line 243. “guarantees that we are” should be past tense.

Page 9, line 240, line 245. Not “age classes”. Not “host age”.

Page 9, lines 246- 247. In the parenthetical statement, I think you mean OTUs, not “sequences”)

Page 9, lines 248-250. I cannot figure out what you mean here. Why were analyses not done at the OTU level? Please clarify.

Results

Page 10, line 254. This is the first time I’m aware that the samples are from multiple populations. This should be explained in the methods, in a section on sample collection.

Page 10, lines 256-259. If you are going to say “significantly”, you should provide some stats to back up your statement. Also, I think this is a bit misleading. Many individuals of L. yavapaiensis have communities that are just as diverse as those of E. coqui, it’s just that there are some low diversity individuals driving down the means.

Page 10, lines 259-260. Try to reword the results so that you are not reiterating the analyses you performed and then stating their results.

Pages 10-11, lines 275-277. This kind of sounds like methods. Consider moving.

Page 11, line 284. I think you want to avoid phrasing this as the “transition”. You aren’t really looking at the transition between the seasons, just the differences between the seasons.

Pages 11-12, lines 298-310. I would move this whole paragraph to the methods.
Page 12, line 311. “Collapsing the rarefied phylotype table …to species level…” What does this mean? I don’t follow.

Page 13, lines 327-328. Interesting. Coquis are highly dominated by a single taxa, whereas that is not the case in the other frog. I wonder why that is…

Page 13, line 329-333. These two sentences are a bit vague and I find them confusing. Particularly the last sentence of the paragraph.

Page 13, lines 336-337. I find the first sentence to be quite awkward. Reword.

Page 13, lines 341-342. To make this sentence more relevant, I would compare the dominant taxa in juveniles to that of adults, rather than just stating two taxa that were dominant on juveniles.

Page 13, lines 344-354. I don’t buy this section at all, and it just sort of comes out of nowhere because it isn’t set up or explained in the methods. Did Bd load really only range from 0-4 zoospore equivalents across individuals? If so, then there’s not a lot of variation in infection upon which to base your findings. Also, what points did you consider to be outliers? And what results do you report—those including or excluding the outliers? I would consider removing these analyses from the study entirely. If not, then you need to provide some discussion/justification for why your results are meaningful. Further, if I have interpreted the x axis correctly, and it only covers a very tiny range of the variation in infection possible, then it would be nice to see some discussion of what you think would happen at higher levels of infection.

Discussion

You really focus on variation in immune function as a cause of variation in microbial diversity between coqui life stages and yavapaiensis seasons. However, there are a number of other factors that could contribute to these differences—for example, differences in the microbial source pool available to colonize amphibians at different times of the year or life stages. It would be nice if you could address these other hypotheses, along with your immune function hypothesis.

Page 14, lines 368-370. Can you please reiterate exactly what the differences were here (just to make it easier for the reader)?

Page 14, line 375. Add “in L. yavapaiensis” to the section heading

Page 15, line 376. This is the first time you introduce the common name of L. yavapaiensis. For simplicity, I would just delete this and stick with L. yavapaiensis.

Page 15, line 377. You really don’t look at the transition between seasons at all—rather, you compare winter to summer. Please rephrase this sentence to reflect that.

Page 15, line 390. Is there supposed to be a hard return here? If so, then the next line should be indented.

Page 15, lines 393-396. This sentence is confusing; please reword.

Page 15, line 400. You didn’t actually specify whether diversity would increase or decrease in your hypothesis in the intro—you just thought it would differ between seasons. Please rephrase this sentence to reflect your hypothesis…or rephrase your hypothesis, with justification for why you anticipate an increase in diversity with increased infection.

Page 16, line 413. For clarity, I would keep your section headings consistent throughout the methods, results, and discussion: “Microbial shifts with developmental stage in E. coqui” or
“Ontogenetic shifts in microbial diversity in E. coqui”. I do think it is worthwhile to point out in this section that E. coqui is a direct developing species, though.

Page 17, lines 431-433. This statement seems more appropriate for the methods.

Page 17, line 448. What about “Is skin microbial diversity related to disease outcome?” I don’t think you can establish cause or effect with your results…

Page 17, line 449. This is just one way that skin microbiota can interact with Bd. What about producing antimicrobial metabolites?

Pages 18-19, lines 471-495. Again, I find this section and the results they are based on a bit wishy-washy…

Page 20, lines 502-507. As I explained in the intro, I don’t think the “disturbance” angle is the way to go in this manuscript. I like the way this paragraph started out—I think you can come up with a stronger way to conclude the manuscript.

Figure 3. Add “Adult” under the bar for L. yavapaiensis, if this bar only represents adults.

Decision letter (RSOS-140377)

19-Mar-2015

Dear Dr Longo,

The Subject Editor assigned to your paper ("Seasonality, development, and bacterial diversity: Consequences for natural disease dynamics in declining amphibians") has now received comments from reviewers. We would like you to revise your paper in accordance with the referee and Subject Editor suggestions which can be found below (not including confidential reports to the Editor). Please note this decision does not guarantee eventual acceptance.

Please submit a copy of your revised paper within three weeks (i.e. by the 11-Apr-2015). If we do not hear from you within this time then it will be assumed that the paper has been withdrawn. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office in advance. We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Editors, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers.

To revise your manuscript, log into http://mc.manuscriptcentral.com/rsos 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. Revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you must respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". Please use this to document how you have responded to the comments, and the adjustments you have made. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response.

In addition to addressing all of the reviewers’ and editor’s comments please also ensure that your revised manuscript contains the following sections before the reference list:
• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article’s supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors’ contributions
All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

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

Yours sincerely,
Emilie Aime
Senior Publishing Editor, Royal Society Open Science
openscience@royalsociety.org
Author’s Response to Decision Letter for (RSOS-140377)

See Appendix A.

RSOS-140377.R1 (Revision)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Yes. The authors have indicated that data will be made available in Dryad upon acceptance.

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

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

Comments to the Author(s)

Dear authors,

Very nice manuscript! I enjoyed reading the revised version. Your results are certainly very interesting. Unfortunately, when reading through the revised version of the manuscript, I found some small issues remained, mostly statements that require clarification and small grammatical mistakes (see my specific comments below). However, once these issues are addressed, I think the manuscript will be in good shape.

Page 4, line 118-133:
This section, which sets up the hypotheses, would benefit from a little more clarification and rearrangement of some of the information. I had to read it a few times before I got it. I suggest:

1. Start with:
   “By characterizing changes in microbial diversity across life histories or seasonal transitions, we can determine if periods of stress are associated with the occurrence of skin dysbioses…”
2. Then:
   I feel like there’s still a link missing in the train of thought here. How generally would you expect the occurrence of skin dysbiosis to affect microbial community diversity? I can envision a decrease in diversity, if the dysbiosis allows some sort of pathogenic bacteria to dominate the community, or an increase in diversity, if as a result of dysbiosis more transient bacteria are able to colonize the skin…It seems to me that your general expectation is an increase in alpha and beta diversity with increases in dysbiosis associated with stressful times…I think you need to add in a sentence explaining this.
3. Then:
“To investigate associations between infection dynamics and skin bacterial diversity, we focused on two important transitions…” or something like that.

4. Explain patterns of infection in E. coqui and L. yavapaiensis (higher in juveniles and higher in winter, respectively)

5. Then state your specific expectations for each species: (1) higher microbial diversity in juvenile coquis, winter-sampled yavapaiensis, and Bd-infected frogs and (2) changes in community composition and structure across life stages and seasons.

Page 6, lines 156-158:
By adding in the term “multiple populations” it now seems like the N for each species refers to the number of populations you sampled, which is incorrect. I would increase the information in the parenthetical statement to something like (N = 37 individuals across N populations) or (N=4 populations, X-X individuals per population)

Page 8, line 216:
It’s function ‘betadisper’, not betadisp.

Page 11, line 291: The first sentence of your results is redundant with information in the methods and can be deleted.

Page 12, line 301: Can you clarify this a bit more? It is clear that 90% of frogs were infected. What is not clear is if infections for all of these individuals was less than 100 zoospores? (i.e., 90% of frogs had infections ≤ 100 Bd zoospores) or (90% prevalence; 80/90 infected frogs with infections ≤ 100 Bd zoospores)

Page 13, line 332: This sentence is confusing. How can the total number of phylotypes for L. yavapaiensis be less than the number that were shared between the two species? Were there 754 unique phylotypes plus 1085 shared? Please clarify.

Also, how could L. yavapaiensis harbor greater diversity when there were far fewer phylotypes for this species (assuming 754 versus 2729 is correct)?

Page 13, line 355-357: I don’t quite understand this sentence. Do you mean that as similar proportion of phylotypes were accounted for by Gammaproteobacteria between species as well as within E. coqui? Please explain. Also, “phylotypes” is typed twice in a row in this sentence.

Page 14, line 365: You can delete “For instance,” since you are comparing the relative abundance for all 5 core phylotypes across developmental stages.

Page 14, line 371: insert “a” before “strong presence”.

Page 14, line 378: spelling “linage” should be “lineage”

Page 15, line 392: delete comma after “at least in part”

Page 15, line 399: “detected”, not “detect”

Page 16, line 417: “Bd” to “Bd’s upper temperature limit”

Page 17, line 434: “was” not “is not due to”
Page 17, line 435: “L.” yavapaiensis, not “R.” yavapaiensis.

Page 18, line 455: I would use “and” instead of “or”. I think you provide evidence of both.

Page 18, line 468: “age” or “developmental stage” of R. cascadae?

Page 19, line 499: “was” instead of “is more informative”

Page 21, line 532-534: This is the kind of statement I was looking for when you are setting up your hypotheses.

Page 21, lines 534-536: I don’t like this statement. I would suggest that you propose to address this more directly. Instead of quantifying Bd infection as some sort of proxy for immune function, why not just quantify immune function?

Page 21, lines 536-539: I don’t like this statement either. To me, this describes your study. You assessed ontogenetic and temporal changes in microbial community diversity as a step towards understanding links between microbial community dynamics and disease dynamics. I think what you mean here is that it will be important to disentangle whether the changes in microbial community structure observed between developmental stages or seasons is caused by disease, or by changes in environmental source pools of bacteria (or some else entirely). I suggest revising the second half of the sentence to reflect this: i.e., as a step towards elucidating the role of disease versus microbial source pools as the underlying causes of shifts in community structure. (If this is what you meant. If not, then revise the sentence to better clarify your meaning).

Table 2, caption: Is this mean relative abundance, averaged across all individuals/species? Clarify. The tables look much nicer in this revised version, by the way!

Same for Figure 3. Mean relative abundance?

Figure 4: How did you calculate % unique and shared phylotypes (i.e., 37% unique, 22% Shared 65%...)? I can’t figure it out—can you please explain what these numbers are referring to? Why are there two values for % Shared?

Decision letter (RSOS-140377.R1)

12-Jun-2015

Dear Dr Longo:

On behalf of the Editor, I am pleased to inform you that your Manuscript RSOS-140377.R1 entitled "Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

The reviewers and Subject Editor have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript.
• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors’ contributions
All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

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 (i.e. by the 21-Jun-2015). If you do not think you will be able to meet this date please let me know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under “Manuscripts with Decisions”. Under "Actions," click on "Create a Revision." You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referees and upload a file 'Response to Referees' in "Section 6 - File Upload". You can use this...
to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referees.

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

1) A text file of the manuscript (tex, txt, rtf, docx or doc), references, tables (including captions) and figure captions. Do not upload a PDF as your "Main Document".
2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format)
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript
5) Included your supplementary files in a format you are happy with (no line numbers, vancouver referencing, track changes removed etc) as these files will NOT be edited in production

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

Best wishes

Emilie Aime
Senior Publishing Editor
openscience@royalsociety.org

Author's Response to Decision Letter for (RSOS-140377.R1)

See Appendix B.
May 15, 2015

Emilie Aime  
Senior Publishing Editor, Royal Society Open Science

Dear Dr. Emilie Aime:

Enclosed you will find a revised version of our manuscript (RSOS-140377): “Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians”, submitted for consideration as a research article to Royal Society Open Science.

We have carefully considered all the comments from Reviewer #1, which improved and clarified our manuscript. Reviewer #1 raised some concerns about our hypothesis about the links among microbial diversity, *Bd* infection, and immune function. We have reworded several sections of the introduction and discussion taking into account Reviewer #1’s feedback. In addition, we have addressed the lack of integration of the Proteobacteria analyses.

In the following document, I address all the comments from Reviewer #1 and the corresponding changes in the revised manuscript. For ease of editing, any changes to the manuscript are shown in red.

Thank you for your consideration.

Sincerely,

Ana V. Longo on behalf of the authors
Response to Reviews:

1. First, I think the hypothesis is a bit of a stretch. I don’t think enough data is provided to establish a link between microbial diversity, infection, and immune function. Unless the authors can provide evidence of variation in infection and immune function coinciding with changes in microbial diversity in their samples, I recommend that they save their ponderings on the underlying cause of the differences for the discussion.

We partially agree with Reviewer #1 in that we lack specific immune response data to establish a direct link between microbial diversity, infection, and host responses. However, we based our hypotheses on well-documented patterns of susceptibility, and were very careful in our interpretations (lines 391-403, 418-420, 423-426, 531-534). These two host species have been well studied over the last 10 years (Longo et al. 2010; Savage et al. 2011; Savage and Zamudio 2011; Longo et al. 2013). Research shows that host ectotherm immune function is at its maximum during warmer temperatures (Maniero and Carey 1997, Raffel et al 2006), and also when hosts are fully developed (Rollins-Smith 1998). Our goal was to test for differences in microbial diversity across seasons and ontogenetic stages in two species that are persisting with enzootic Bd infections. Thus, we acknowledge that linking microbial diversity, Bd infection, and immune function still requires additional experiments, but these are beyond the scope of our manuscript. In this version of the manuscript we have toned down the link with immunity, while still maintaining immune response as a possible factor influencing infection dynamics in these two species. We now specifically state that studies of immune function in these species will be able to test this last link.

2. Second, I recommend that the final section of the results, addressing variation in just the Proteobacteria in one of the two amphibian species, be removed from the study unless the authors can provide a more convincing reason to include it. It just sort of comes out of nowhere because it isn’t set up or explained in the methods, and (if I’ve interpreted it correctly) I think the relationships between diversity and Bd load they find are rather weak in that it only captures a tiny fraction of the range in variation in infection in this species (1-4 zoospore equivalents).

We rewrote parts of the introduction, methods, results, and discussion to justify and integrate our analyses of Proteobacteria (lines 146-151, 270-276, 350-357). In short, Proteobacteria are the most common bacterial phylum isolated from amphibian skin. In fact, 77% of bacterial isolates from a recently released database of amphibian skin-associated bacteria are members of Proteobacteria (Woodhams et al. 2015). Reviewer #1 pointed out that the relationship between diversity and Bd load that we found is rather weak, but it is significant (all correlations are $P < 0.02$). To date, we have no knowledge of any other study of amphibians/Bd dynamics that has found a relationship between alpha diversity measures and Bd load, thus we consider it important to show these data, not only because they are interesting, but also to stimulate additional research about the mechanisms giving rise to these patterns. Reviewer #1 is correct that our discussion about the variation Bd load seemed misleading. The reason is that we omitted by mistake that the x-axis of Figure 1 represented a logarithm of qPCR zoospore counts. Thus, 1-4 zoospores actually represented a difference in orders of magnitude. We thank the Reviewer for noticing our mistake.
3. I don’t think the title accurately reflects the study. I would suggest changing to something along the lines of “Seasonal and ontogenetic variation in the amphibian skin microbiome and relationship to natural disease dynamics in declining amphibians”.

We modified our title as recommended to “Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians.” We do not use the word “microbiome” because our data includes both fingerprinting techniques and next-generation sequencing.

4. Also, just out of curiosity, what evidence is there that coquis are actually declining?

We added a sentence in the introduction describing past studies on population declines for both species in lines 114-115.

5. Page 2, line 54. What do you mean by “thresholds”? I think you may want to use a different word here.

Reviewer #1 asked for clarification of the word “thresholds” in the abstract. By threshold, we meant the limits in bacterial diversity or community composition can change the outcome from health to disease. We agree that this may be misleading, thus we changed the word to “characteristics”.

6. Page 2, lines 55-57. I would rephrase your hypothesis to make it more neutral regarding the relationship to Bd infection. As you yourself admit, it is difficult to determine, especially from the results of survey data, whether seasonal/developmental shifts in community structure lead to changes in pathogen presence, or whether it is seasonal/developmental variation in susceptibility to invasion by the pathogen that causes changes in microbial community structure. I think this is best left for the discussion.

We changed our hypothesis accordingly in lines 121-126. However, two recent studies indicated that Bd infection disturbs microbial communities (Jani and Briggs 2014; Becker et al. 2015). We added those studies as part of the framework leading to our hypothesis.

7. Page 2, lines 60-68. I think your wording is a bit misleading. I would rephrase so that it is clear that you assess seasonal variation in one species, and ontogenetic variation in the other.

We added a sentence to clarify this issue on lines 60-62.

8. Page 3, line 79. Change “is often” to “has been”. Done.


11. Page 3, lines 97-98. This is a better way to think about framing the intro and your hypotheses. Much more in line with what you can say from your survey results.

   We appreciate your constructive comment. However, since the initial submission of this manuscript, new studies have shown a relationship between Bd and microbial communities that further support our findings (Jani and Briggs 2014; Becker et al. 2015), and we have added them to the introduction and discussion.

12. Page 3, lines 98-99. Delete or move this sentence. It has nothing to do with disease, and so does not fit here.

   We moved this sentence to reflect what has been done with skin-associated microbial communities in amphibians.

13. Page 4, line 101, line 103, line 108. Lots of unnecessary punctuation (periods, commas) throughout manuscript. I haven’t taken the time to point out all of them, but this should be addressed by the authors before they resubmit.

   We have carefully checked the entire manuscript for these grammatical errors.

14. Page 4, line 107. Do bacteria “replicate” or “reproduce”?

   Reviewer #1 is correct. We changed the sentence to reflect that dysbioses might impede colonization, growth, and reproduction of protective microbes (lines 107-108).

15. Page 4, line 107. Change “may predispose hosts for…” to “may predispose hosts to…” There are quite a few grammatical errors like this throughout the manuscript that should be addressed by the authors before resubmitting.

   Thank you for pointing out this error. We have carefully checked the entire manuscript for these grammatical errors.

16. Page 4, line 113. Are these two species phylogenetic positions really contrasting?

   Yes. These two species contrast for many reasons: habitats (tropical vs. temperatre), reproductive mode (direct-development vs. aquatic), location (Puerto Rico vs. Arizona), phylogeny (Eleutherodactylidae vs. Ranidae). We mentioned all these differences in the manuscript lines 112-118.

17. Page 4, line 114-116. This is how you should explain what you did in the abstract.

   Thanks for the suggestion. We now incorporated a paraphrased version of this sentence in the abstract on lines 60-62.

18. Page 4, lines 117-125. I think this is a bit of a stretch. I don’t think you provide enough data to establish a link between microbial diversity, infection, and immune function or stress. I
would save this for the discussion. You merely observe differences in microbial diversity that coincide with known differences in pathogen susceptibility in these species. You don’t even report whether you see these trends in infection your samples, something I think you should consider including in your results.

We partially agree with Reviewer #1 in that we lack specific immune response data to establish a direct link between microbial diversity, infection, and host responses. As explained in item #1, research shows that host ectotherm immune function is at its maximum during warmer temperatures, and when hosts are fully developed. Our goal was to highlight differences in microbial diversity across seasons and ontogenetic stages. Thus, we acknowledge that linking microbial diversity, Bd infection, and immune function still requires additional experiments, but these are beyond the scope of our manuscript. In this version of the manuscript we have toned down the link with immunity, while still maintaining immune response as a possible factor influencing infection dynamics in these two species. Only by performing experiments, we will understand the function of these increases in microbial diversity and their relationships with host recovery after infection.

19. Page 5, line 126. You mention species and infection status here, but you haven’t set up these hypotheses yet.

We rewrote parts of the introduction to reflect our expectations about infection status and species in lines 125-126.

20. Page 5, line 127. Throughout the methods and results, you keep referring to the “age” of E. coqui. This is incorrect. You should use “developmental stage”. Unless you know something more specific about the age of individuals, other than that they are juveniles or adults.

Reviewer #1 is correct about the usage of the word “age”. We changed "age" to "developmental stage" throughout the manuscript.

21. Page 5, lines 133-137. This was only done for E. coqui, right? Specify. Done.

22. Page 5, lines 137-139. I think this sentence is more appropriate for the discussion. Also, I’m not convinced that you do provide any evidence for a protective role. You show that diversity changes between seasons and developmental stages and point out that previous work has shown that Bd infection changes between seasons and developmental stages. But you don’t directly link the two.

We modified this sentence to make it more neutral and specific about our findings in lines 148-151.

23. Page 5, line 141. Please add in a section that explains your sample collection. When did it occur? Where? I don’t think it’s enough to just keep referring to a table in the supplementary material.

We disagree with Reviewer #1 about adding a section that explains sample collection,
because we have a very detailed description in lines 158-166. However, we added more information about collection dates in the supplementary table that includes exact GPS locations and sample sizes.

24. Page 5, line 146. Please provide a little more detail for the swabbing methods. Did you use the exact methods of the citation you provide (i.e., number of strokes, location of strokes)?

We used standardized techniques for *Bd* detection from frog skin as described in lines 158-162.

25. Page 6, line 158. You should provide a summary of your *Bd* infection results. Were there differences in *Bd* infection across seasons/developmental stages for your samples? What range of *Bd* loads did you find across individuals?

Reviewer #1 suggested a summary of our *Bd* infection results. Presence/absence data are part of table 1, and infection intensity results were depicted on Fig. 5). Seasonality and ontogenetic patterns in *Bd* infection have been already discussed as part of other publications. Our objective was to highlight changes in microbial communities for categories with known differences in *Bd* infection.

26. Page 8, line 215. What were your thermocycler conditions? Reviewer #1 asked about the thermocycler conditions.

We added a sentence in lines xxx to specifically address our thermocycling protocol.

27. Page 8, line 219. Where was your Illumina sequencing done?

Illumina Sequencing was done at the Genomics Facility at Cornell University. We added the service provider in the manuscript in lines 233-235.

28. Page 8, line 224. “Sequences were filtered…” not “are filtered”. There are several places throughout the manuscript, particularly in the Results, where verbs need to be changed to past tense. The authors should address this before resubmitting.

We have carefully revised the entire manuscript for these grammatical mistakes.

29. Page 8, line 225. Which version of the Greengenes database?

We added the version of Greengenes database in line 247.

30. Page 9, lines 233-235. Now I’m confused. In the previous section, you state that you rarefied all samples to 5000 reads. Now, here, you state that you generated rarefied phylotype tables to different sequencing depths before computing alpha diversity metrics. Please clarify. Also, if you did rarefy to several different depths, what depths did you use?

Reviewer #1 correctly pointed out that we were missing some information about rarefactions.
We clarified this in lines 270-272.

31. Page 9, line 243. “guarantees that we are” should be past tense. Done.


33. Page 9, lines 246-247. In the parenthetical statement, I think you mean OTUs, not “sequences”). Done. Changed to “phylotypes”.

34. Page 9, lines 248-250. I cannot figure out what you mean here. Why were analyses not done at the OTU level? Please clarify.

   Our analyses were performed at the OTU level. We deleted this sentence to because it was misleading.

35. Page 10, line 254. This is the first time I’m aware that the samples are from multiple populations. This should be explained in the methods, in a section on sample collection.

   We mentioned in the methods that samples came from multiple populations (line 156); in addition, table S1 contained this information about sample sizes and exact localities.

36. Page 10, lines 256-259. If you are going to say “significantly”, you should provide some stats to back up your statement. Also, I think this is a bit misleading. Many individuals of L. yavapaiensis have communities that are just as diverse as those of E. coqui, it’s just that there are some low diversity individuals driving down the means.

   Reviewer #1 suggested that many individuals of L. yavapaiensis had communities that were just as diverse as those of E. coqui, and that some low diversity individuals were driving down the means. We believe that Reviewer #1 did not see the t-test results from Figure 1. We found significant differences between species, and these were not driven by outliers with low diversity.

37. Page 10, lines 259-260. Try to reword the results so that you are not reiterating the analyses you performed and then stating their results. Done.

38. Pages 10-11, lines 275-277. This kind of sounds like methods. Consider moving. Moved to methods.

39. Page 11, line 284. I think you want to avoid phrasing this as the “transition”. You aren’t really looking at the transition between the seasons, just the differences between the seasons.

   We agree with Reviewer #1 and modified the text in line 317.

40. Pages 11-12, lines 298-310. I would move this whole paragraph to the methods. Moved to methods.
41. Page 12, line 311. “Collapsing the rarefied phylotype table …to species level…” What does this mean? I don’t follow.

We rephrased this sentence. What we meant was that if we combined OTUs by their taxonomy at the species level we obtained a total of 457 phylotypes for *L. yavapaiensis* and 511 for *E. coqui* (see figure S2).

42. Page 13, lines 327-328. Interesting. Coquis are highly dominated by a single taxa, whereas that is not the case in the other frog. I wonder why that is…

Reviewer #1 seemed skeptical about the taxonomic composition in *E. coqui*. Our results are not that far different from others such as Kueneman et al. (2013). In their study, some Proteobacteria OTUs composed 19-24% of their total microbiomes. We cite these other comparative papers in the revised version of the manuscript.

43. Page 13, line 329-333. These two sentences are a bit vague and I find them confusing. Particularly the last sentence of the paragraph.

We rewrote the sentences, now in lines 350-357.

44. Page 13, lines 336-337. I find the first sentence to be quite awkward. Reword.

We rewrote the sentence, now in lines 360-361.

45. Page 13, lines 341-342. To make this sentence more relevant, I would compare the dominant taxa in juveniles to that of adults, rather than just stating two taxa that were dominant on juveniles. Changed.

46. Page 13, lines 344-354. I don’t buy this section at all, and it just sort of comes out of nowhere because it isn’t set up or explained in the methods. Did Bd load really only range from 0-4 zoospore equivalents across individuals? If so, then there’s not a lot of variation in infection upon which to base your findings. Also, what results do you report—those including or excluding the outliers? I would consider removing these analyses from the study entirely. If not, then you need to provide some discussion/justification for why your results are meaningful. Further, if I have interpreted the x axis correctly, and it only covers a very tiny range of the variation in infection possible, then it would be nice to see some discussion of what you think would happen at higher levels of infection.

We agree with Reviewer #1 in that this section required improvement and clarification. This section needed a better integration from the introduction to the discussion. We now clearly justify our reasoning behind our choice of analyses. Please refer to lines 146-148, 350-357. Reviewer #1 was right to point that infection intensity made little sense, because the X-axis of Fig. 5 was missing the log_{10} (Bd load + 1). That was our error and has now been corrected. We addressed the comments about outliers and variation in infection in line 376.
47. You really focus on variation in immune function as a cause of variation in microbial diversity between coqui life stages and yavapaiensis seasons. However, there are a number of other factors that could contribute to these differences—for example, differences in the microbial source pool available to colonize amphibians at different times of the year or life stages. It would be nice if you could address these other hypotheses, along with your immune function hypothesis.

Although variation in immune function is our main hypothesis, we discussed other hypotheses that could explain variation in microbial diversity. For example, on lines 418-420, 424-426 we discuss how temperature could alter particular members of the microbial community. In addition, we discussed how shifts in the age-structure of the population could have indirect effects on microbial turnover in adult skin on lines 434-439. We added the recommended explanation of changes in the microbial source pool on lines 419.

48. Page 14, lines 368-370. Can you please reiterate exactly what the differences were here (just to make it easier for the reader)? Added.

49. Page 14, line 375. Add “in L. yavapaiensis” to the section heading. Added.

50. Page 15, line 376. This is the first time you introduce the common name of L. yavapaiensis. For simplicity, I would just delete this and stick with L. yavapaiensis. Deleted.

51. Page 15, line 377. You really don’t look at the transition between seasons at all—rather, you compare winter to summer. Please rephrase this sentence to reflect that. Rephrased.

52. Page 15, line 390. Is there supposed to be a hard return here? If so, then the next line should be indented. Fixed.

53. Page 15, lines 393-396. This sentence is confusing; please reword. Reworked.

54. Page 15, line 400. You didn’t actually specify whether diversity would increase or decrease in your hypothesis in the intro—you just thought it would differ between seasons. Please rephrase this sentence to reflect your hypothesis…or rephrase your hypothesis, with justification for why you anticipate an increase in diversity with increased infection.

We reworded and justified our hypotheses in the introduction in lines 125-126.

55. Page 16, line 413. For clarity, I would keep your section headings consistent throughout the methods, results, and discussion: “Microbial shifts with developmental stage in E. coqui” or “Ontogenetic shifts in microbial diversity in E. coqui”. I do think it is worthwhile to point out in this section that E. coqui is a direct developing species, though.

We added the species name “E. coqui” to the section subheading.

56. Page 17, lines 431-433. This statement seems more appropriate for the methods.
We moved these sentences to the methods.

57. Page 17, line 448. What about “Is skin microbial diversity related to disease outcome?” I don’t think you can establish cause or effect with your results.

We changed the section subheading as recommended by Reviewer #1.

58. Page 17, line 449. This is just one way that skin microbiota can interact with Bd. What about producing antimicrobial metabolites?

We added other functions of skin microbiota including the one recommended by Reviewer #1 in line 475.

59. Pages 18-19, lines 471-495. Again, I find this section and the results they are based on a bit wishy-washy…

We recognize that our data are limited, yet the results are significant, thus these deserve to be discussed. As previously mentioned, the section on Proteobacteria required better integration.

60. Page 20, lines 502-507. As I explained in the intro, I don’t think the “disturbance” angle is the way to go in this manuscript. I like the way this paragraph started out—I think you can come up with a stronger way to conclude the manuscript.

We have modified our conclusion based on the recommendations of Reviewer #1.

61. Figure 3. Add “Adult” under the bar for L. yavapaiensis, if this bar only represents adults.

Done.

References


June 17, 2015

Emilie Aime
Senior Publishing Editor, Royal Society Open Science

Dear Dr. Emilie Aime:

Enclosed you will find a new revised version of our manuscript (RSOS-140377.R1): “Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians”, submitted for consideration as a research article to *Royal Society Open Science*.

I have included all the requested information and uploaded raw data to Dryad repository.

In the following document, I address all the comments from Reviewer #1 and the corresponding changes in the revised manuscript. For ease of editing, any changes to the manuscript are shown in red.

Thank you for your consideration.

Sincerely,

Ana V. Longo on behalf of the authors
Response to Reviews:

1. Page 4, line 118-133: This section, which sets up the hypotheses, would benefit from a little more clarification and rearrangement of some of the information. I had to read it a few times before I got it.

Reviewer #1 provided some good suggestions on how to improve our hypothesis framework. We rearranged some of the sentences and separated the section into two paragraphs. Please refer to lines 118-129.

2. Page 6, lines 156-158: By adding in the term “multiple populations” it now seems like the N for each species refers to the number of populations you sampled, which is incorrect. I would increase the information in the parenthetical statement to something like (N = 37 individuals across N populations) or (N=4 populations, X-X individuals per population).

We added the word individuals to accurately reflect that we refer to the total number of individuals.


4. Page 11, line 291: The first sentence of your results is redundant with information in the methods and can be deleted. Noted and deleted.

5. Page 12, line 301: Can you clarify this a bit more? It is clear that 90% of frogs were infected. What is not clear is if infections for all of these individuals was less than 100 zoospores? (i.e., 90% of frogs had infections ≤ 100 Bd zoospores) or (90% prevalence; 80/90 infected frogs with infections ≤ 100 Bd zoospores). Noted and clarified.

Reviewer #1 is correct. We fixed the sentence by adding the word unique. We found 754 unique phylotypes in *L. yavapaiensis* and 2729 unique haplotypes for *E. coqui* as shown in Figure S1. We based our statement of higher diversity in *L. yavapaiensis* on Fig. S2, for which its alpha diversity rarefaction curves are higher than for *E. coqui*. So, in terms of richness, yes, *E. coqui* has more distinct phylotypes. However, rarefactions showed consistent higher values in *L. yavapaiensis*. We discussed this as an artifact of low sample size in *L. yavapaiensis* (lines 339-344).

6. Page 13, line 332: This sentence is confusing. How can the total number of phylotypes for *L. yavapaiensis* be less than the number that were shared between the two species? Were there 754 unique phylotypes plus 1085 shared? Please clarify. Also, how could *L. yavapaiensis* harbor greater diversity when there were far fewer phylotypes for this species (assuming 754 versus 2729 is correct)?

Reviewer #1 is correct. We fixed the sentence by adding the word unique. We found 754 unique phylotypes in *L. yavapaiensis* and 2729 unique haplotypes for *E. coqui* as shown in Figure S1.

7. Page 13, line 355-357: I don’t quite understand this sentence. Do you mean that as similar proportion of phylotypes were accounted for by Gammaproteobacteria between species as well as within *E. coqui*? Please explain. Also, “phylotypes” is typed twice in a row in this sentence.
We reworded these two sentences to reflect that Gammaproteobacteria tend to dominate both unique and shared phylotypes (lines 358-354).

8. Page 14, line 365: You can delete “For instance,” since you are comparing the relative abundance for all 5 core phylotypes across developmental stages. Deleted.


14. Page 17, line 434: “was” not “is not due to”. Fixed.


16. Page 18, line 455: I would use “and” instead of “or”. I think you provide evidence of both. Fixed.

17. Page 18, line 468: “age” or “developmental stage” of R. cascadae? Changed to developmental stage.

18. Page 19, line 499: “was” instead of “is more informative”. Changed.

19. Page 21, line 532-534: This is the kind of statement I was looking for when you are setting up your hypotheses. We appreciate your comment.

20. Page 21, lines 534-536: I don’t like this statement. I would suggest that you propose to address this more directly. Instead of quantifying Bd infection as some sort of proxy for immune function, why not just quantify immune function?

We completely agree with Reviewer #1 in that quantifying immune function will be important to understand how host immune factors affect the skin microbiome. However, our statement was about incorporating microbiome data into studies of the ontogeny of immunity in amphibians, not about quantifying Bd infection as a proxy of immune function. We reworded this sentence to make it more explicit in lines 542-545.

21. Page 21, lines 536-539: I don’t like this statement either. To me, this describes your study. You assessed ontogenetic and temporal changes in microbial community diversity as a step towards understanding links between microbial community dynamics and disease dynamics. I think what you mean here is that it will be important to disentangle whether the changes in
microbial community structure observed between developmental stages or seasons is caused by disease, or by changes in environmental source pools of bacteria (or some else entirely). I suggest revising the second half of the sentence to reflect this: i.e., as a step towards elucidating the role of disease versus microbial source pools as the underlying causes of shifts in community structure. (If this is what you meant. If not, then revise the sentence to better clarify your meaning). Suggested change was incorporated in the MS.

22. Table 2, caption: Is this mean relative abundance, averaged across all individuals/species? Clarify. The tables look much nicer in this revised version, by the way! We appreciate your comment. We added the words mean relative abundance to the title.

23. Same for Figure 3. Mean relative abundance? We added the words mean relative abundance to the title.

24. Figure 4: How did you calculate % unique and shared phylotypes (i.e., 37% unique, 22% Shared 65%...)? I can’t figure it out—can you please explain what these numbers are referring to? Why are there two values for % Shared?

We added the formula that we used to calculate the % unique and shared phylotypes as part of the caption for Figure 4.