
**UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**
DOCTORADO EN CIENCIAS BIOMÉDICAS
CENTRO DE CIENCIAS GENÓMICAS

# Estudio de los factores bióticos y abióticos que influyen sobre el microbioma de la piel del ajolote de arroyo de montaña *Ambystoma altamirani*

Tesis
que para optar por el grado de:
Doctor en Ciencias

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Cuernavaca, Morelos. Octubre, 2023

Este trabajo se realizó en el laboratorio de Ecología Microbiana de Anfibios del Centro de Ciencias Genómicas de la UNAM, bajo la dirección de la Dra. Eria Alaide Rebollar Caudillo con el apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) IA201419 y IA200921.

Agradecimientos

A la UNAM y al Centro de Ciencias Genómicas que me han permitido continuar mi formación científica.

Al sistema de becas de la UNAM y México que me han permitido realizar estudios de posgrado.

A la Dra. Eria Rebollar, por la oportunidad de trabajar y aprender de ti, por tus ideas, apoyo y paciencia. Ha sido un honor colaborar contigo estos cinco años, muchas gracias.

A mi comité tutor, la Dra. Ana Escalante y la Dra. Ayari Fuentes, por sus comentarios y recomendaciones que siempre me impulsaron a ampliar mis perspectivas y a entender que hay que aprender a explorar las cosas desde un ángulo diferente.

A mi jurado de grado: Dra. María Esperanza Martínez Romero, Dr. Luis David Alcaraz Peraza, Dr. Lorenzo Patrick Segovia Forcella y Dr. José Jaime Zúñiga Vega por la revisión y los comentarios realizados a este documento.

A mi familia, por el cariño y el apoyo. A pesar de la distancia, sin ustedes nada sería posible, muchas gracias.

Y por último a Erika.... solo tú sabes lo que esto ha significado. Esto es de los dos.

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(*) El artículo que conforma este capítulo se encuentra en proceso de revisión en la revista Microbial Genomics por lo que está sujeto a cambios en su contenido.

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## Resumen

Los microbiomas de la piel de anfibios contribuyen a la sobrevivencia de sus hospederos ante la quitridiomicosis, una enfermedad infecciosa causada por hongos patógenos del género *Batrachochytrium*. Sin embargo, cambios en la diversidad taxonómica y funcional del microbioma de la piel a causa de la influencia de factores bióticos y abióticos se han asociado con el estado de salud de los anfibios ante esta enfermedad. El ajolote de arroyo de montaña (*Ambystoma altamirani*), es una especie endémica que se distribuye en la región central de México. Debido a sus características ecológicas, esta especie de ajolote es un modelo interesante para estudiar la influencia de diversos factores bióticos y abióticos, sobre la diversidad taxonómica y funcional del microbioma de la piel, y evaluar la relación que existe entre cambios en la diversidad del microbioma y el estado de infección ante la quitridiomicosis.

Mediante la secuenciación y análisis de datos genómicos (secuenciación de la región V4 del gen 16s rRNA y metagenomas) se describio la diversidad taxonómica y funcional del microbioma de la piel de *A. altamirani*. La diversidad bacteriana de la piel del microbioma de *A. altamirani*, mostro estar influenciada principalmente por, el estado metamórfico y la variación ambiental asociada a cambios estacionales y geográficos. Por su parte, la diversidad funcional del microbioma de la piel de ajolotes pre-metamórficos mostro estar influenciada por la variación estacional y geográfica. Además, se describió la presencia de diversos genes asociados a posibles funciones antifúngicas. Interesantemente, estos genes derivan de grupos bacterianos altamente abundantes sobre la piel de estos ajolotes. A pesar de la alta incidencia de la quitridiomicosis en las poblaciones de *A. altamirani* analizadas en este trabajo, únicamente se detectaron cambios significativos en la diversidad funcional entre ajolotes infectados y no infectados durante la estación de invierno.

Los resultados obtenidos como parte de este trabajo describen la influencia de distintos factores bióticos y abióticos sobre la diversidad taxonómica y funcional del microbioma de la piel de *A. altamirani*, y aportan evidencia que permite entender de mejor manera la contribución del microbioma de la piel de anfibios en la defensa de sus hospederos ante patógenos.

## Abstract

Amphibian skin microbiomes can contribute to hosts survival against chytridiomycosis, an infectious disease caused by fungal pathogens of the genus *Batrachochytrium*. However, variation in the taxonomic and functional diversity of the amphibian skin microbiome due to the influence of biotic and abiotic factors, is associated with differential health-disease outcomes against this disease. The mountain stream axolotl (*Ambystoma altamirani*) is an endemic and endangered species distributed in the central area of Mexico. Due to its ecological characteristics, this axolotl species represents an interesting model to study the influence of various biotic and abiotic factors on the taxonomic and functional diversity of the skin microbiome and its relationship with the infection status with pathogens of the genus Batrachochytrium.

Through sequencing and analysis of genomic data (sequencing of the V4 region of the 16s rRNA gene and metagenomes) it was possible to describe the taxonomic and functional diversity of the *A. altamirani* skin microbiome. The bacterial diversity of the *A. altamirani* skin microbiome of was influenced mainly by metamorphic status and environmental variation between seasons and geographical locations. Moreover, the functional genomic diversity of the skin microbiome of pre-metamorphic axolotls varied significantly throughout seasons and between sampling locations. Furthermore, various genes associated with possible antifungal functions were identified in *A. altamirani* skin microbiome, interestingly these belong highly abundant bacterial groups. Noteworthy, the presence of *Batrachochytrium* pathogens over the skin was linked to functional variation only during winter season.

The results obtained as part of this work describe the influence of different biotic and abiotic factors on the taxonomic and functional diversity of *the A. altamirani* microbiome and provide evidence that allows a better understanding of the contribution of the skin microbiomes in host defense against pathogens.

## Capítulo 1. Introducción general

### 1.1 El surgimiento de la ecología microbiana como disciplina y aspectos generales del estudio de las comunidades microbianas

La ecología de comunidades se define como la disciplina que busca entender las relaciones entre los organismos que conforman una comunidad biológica y su entorno[1,2]. Bajo este contexto, las comunidades biológicas se entienden como el objeto de estudio de la ecología de comunidades, estas se pueden definir como ensambles multi especie en el que los individuos que forman parte de ellas coexisten un mismo ambiente interactuando entre ellos [2]. La ecología de comunidades se desarrolló como disciplina mediante el estudio y descripción de comunidades biológicas de plantas y animales a través de la generación de catálogos de especies y la descripción de los patrones ecológicos de estas comunidades [1].

La clasificación de las especies y la descripción de sus patrones de diversidad son ejes centrales de la ecología de comunidades [2]. En el caso de las comunidades de microrganismo el estudio sobre estos dos ejes represento un reto para los primeros microbiólogos y ecólogos microbianos [3]. Si bien, las contribuciones de Leewenhoek y Hooek fueron esenciales para comenzar a clasificar de manera burda a los microorganismos [3,4], los estudios de científicos como Pasteur o Koch sesgaron el entendimiento de los microorganismos al considerarlos únicamente como agentes infecciosos [3,5].

Sin embargo, las contribuciones pioneras de Martinus Willem Beijerinck y Serguéi Winogradsky sobre el cultivo selectivo de microorganismos, representaron un cambio en el paradigma sobre entendiendo que se tenía de los

microorganismos [3,4]. Estos trabajaos permitieron describir la contribución de los microorganismos en los ciclos biogeoquímicos, lo que sentó la bases para plantear a la ecología microbiana como la disciplina encargada del estudio de las comunidades de microorganismos [3,4].

No obstante, por un periodo de casi 40 años (1930 a 1970) los ecólogos microbianos centraron la mayor parte de sus esfuerzos en la descripción taxonómica de los microorganismos [4]. En esa época, la clasificación taxonómica estaba basada en la descripción de características morfológicas (e.g. cocos o bacilos), y fisiológicas (e.g. presencia de flagelos o su capacidad para formar esporas) de las células microbianas [3,4]. Sin embargo, debido a que estas características no reflejaban la historio evolutiva de los microorganismos la clasificación taxonómica de estos fue un tema de controversia por cerca de 50 años [4].

No fue sino hasta mediados de la década de los 70's que se superaron algunas de las dificultades metodológicas para estudiar las comunidades de microorganismos. Carl Woese, propuso una estrategia de clasificación molecular basada en la comparación de secuencias de genes conservados entre todos los organismos del planeta. Específicamente, Woese propuso el uso de genes ribosomales como el 16S rRNA para clasificar a los microorganismos [4,6]. En la actualidad gracias a su trabajo pionero, es posible describir la diversidad taxonómica de las comunidades de microorganismos y explorar a profundidad la ecología de las comunidades microbianas.

Se ha planteado que la ecología microbiana debería buscar responder tres preguntas esenciales i) ¿Cuál es la estructura de las estas comunidades

biológicas?, ii) ¿Cuál es la función de estas comunidades?, y iii) ¿Cómo varia la estructura y la función de las comunidades a través del tiempo y el espacio? [7]. En la actualidad, para tratar de responder estas preguntas los ecólogos microbianos emplean estrategias de secuenciación masiva independiente de cultivos para describir la diversidad, abundancia y patrones de distribución (espacial y temporal) de las comunidades de microorganismos [3,8].

Estas estrategias, se basan principalmente en la amplificación y secuenciación de regiones genéticas conservadas en todos los organismos, como lo son las regiones hipervariables de genes ribosomales como el 16S rRNA, 18S rRNA, espaciadores internos de estos mismos genes (ITS1 e ITS2), [9–12], o mediante la secuenciación completa del material genético de las comunidades microbianas y su posterior ensamble. En conjunto estas estrategias permiten describir la diversidad taxonómica y funcional de las comunidades de microorganismos y evaluar la influencia de diversos factores bióticos y abióticos sobre las comunidades microbianas [13–15].

La descripción de patrones de diversidad y distribución de especies es uno de los objetivos principales de la ecología de comunidades [1,16], en el caso de las comunidades de animales y plantas la generación de catálogos de especies y la comparación de diversas métricas de diversidad entre comunidades se consideran una de las herramientas principales para la ecología de comunidades [1,2]. En el caso particular de los microorganismos, específicamente las bacterias, en donde el concepto de especie es controversial [17–19] se ha optado por generar catálogos basados en unidades taxonómicas operativas (OTU por sus siglas en inglés) [20–22], que se generan mediante el agrupamiento de

secuencias obtenidas de experimentos de secuenciación de genes ribosomales [21].

En años recientes el uso de OTU como unidad básica de clasificación bacteriana ha sido remplazado por el uso de secuencias de variantes de amplicones o ASV (por sus siglas en inglés) [23–25]. De manera general, los OTU y ASV se pueden definir como clústers de secuencias que comparten cierto porcentaje de similitud entre ellas. En el caso de los OTU, el porcentaje de similitud pude variar del 97% al 99% con base a los distintos algoritmos y criterios de agrupamiento, por su parte, los ASV son clústers compuestos por secuencias con una similitud del 100% [23].

Las estrategias metodológicas para agrupar secuencias obtenidas de experimentos de secuenciación de genes ribosomales, difieren en el tipo de algoritmos que emplean [26]. Estos algoritmos pueden estar basados en, la comparación de métricas de distancia entre secuencias como es el caso de la distancia de Hamming [24], o en el cálculo de modelos de probabilidad basados en la calidad, distribución y abundancia de las secuencias obtenidas de un experimento de secuenciación [25].

Una vez definidos algunos puntos importantes sobre las estrategias que se empelan actualmente para describir y clasificar a las comunidades de microorganismos, es importante mencionar que los ecólogos microbianos, han acuñado términos específicos para referirse a estas comunidades. Específicamente, los términos microbiota y microbioma se han popularizado desde inicio de los años 2000 [5]. El termino microbiota hace referencia al conjunto de bacterias, arqueas, eucariotas microscópicos (e.g. hongos, algas,

protozoarios) y partículas virales que coexisten con un hospedero [27–29]. Por su parte el termino microbioma se ha usado para hacer referencia a la microbiota en conjunto con la totalidad de su repertorio genético [30–32].

## 1.2 Microbiomas asociados a hospederos: importancia y características de estas interacciones

Las plantas y los animales han evolucionado en un mundo dominado por microorganismos, estableciendo a lo largo de su historia evolutiva múltiples interacciones simbióticas con diversas comunidades de microorganismos a las que hoy en día denominamos microbiomas [27,33]. Se ha llegado a estimar que, en el humano existe una relación 1:1 entre el número de células microbianas (pertenecientes a su microbioma) y las células humanas [34]. Además, se estima que el repertorio genético del microbioma humano puede llegar a ser hasta 100 veces mayor al del genoma humano [35]. Actualmente, se reconoce que los microbiomas contribuyen a la sobrevivencia y evolución de sus hospederos [36] participando y facilitando funciones relacionadas con la adquisición de nutrientes [37], la reproducción [38,39], el desarrollo de órganos [40,41], la maduración del sistema inmune [42] o la protección contra patógenos [43–45].

Por ejemplo, se ha descrito que la diversidad de la microbiota intestinal varía con relación al tipo de alimentación de sus hospederos [46]. En el caso específico de animales clasificados como herbívoros, se sabe que diversas bacterias del microbioma intestinal participan en la degradación de celulosa mediante la producción complejos enzimáticos llamados celulosomas, que permiten la obtención de nutrientes mediante la degradación de la fibra vegetal [47,48]. Específicamente los microorganismos del intestino metabolizan la fibra vegetal

y producen ácidos grasos de cadena corta que son metabolizados por las células intestinales de los hospederos [49].

Por otra parte, se ha reportado que los microbiomas contribuyen al éxito reproductivo de sus hospederos [50]. Por ejemplo, en machos de la especie *Zonotrichia capensis* conocida comúnmente como copetón, se ha correlacionado un aumento de la diversidad bacteriana en las cloacas de estas aves con un incremento en los niveles de testosterona una horman asociada a la actividad sexual [51].

Si bien, diversos trabajos han descrito la contribución que los microbioma a la sobrevivencia y evolución de sus hospederos, es importante tener en cuenta que, las interacciones entre los microbiomas y sus hospederos están sujetas a diversos mecanismos de control. Estos mecanismos influyen sobre la diversidad taxonómica de las comunidades microbianas y por ende en las funciones que estas comunidades despeñan cuando interactúan con un hospedero [52]. Se han propuesto diversos modelos que explican el tipo de interacción que existe entre un hospedero y su microbioma tomando en cuenta el tipo y grado de control que el hospedero ejerce sobre los microorganismos (Figura 1) [52].

Estos modelos se han clasificado como:

A) Ecosistemas a raya: En este modelo los hospederos interactúan con comunidades de microorganismos que se caracterizan por presentar una alta diversidad, además se asume que los integrantes de estas comunidades interactúan entre ellos y otorgan algún beneficio al hospedero. Por su parte, los hospederos ejercen diversos mecanismos de control sobre los microorganismos mediante la dieta o el sistema inmune que permiten controlar la diversidad de las

comunidades microbianas. Los microbiomas intestinales son un ejemplo de este modelo, los hospederos ejercen control sobre los microorganismos mediante la acción de células o moléculas inmunes y el tipo de dieta [53–55], por su parte los microbiomas, contribuyen a la degradación o conversión de componentes de la dieta [47,56] y la producción de nutrientes como ácidos de cadena corta que pueden ser metabolizados por las células del hospedero [56–58] (Figura 1A).

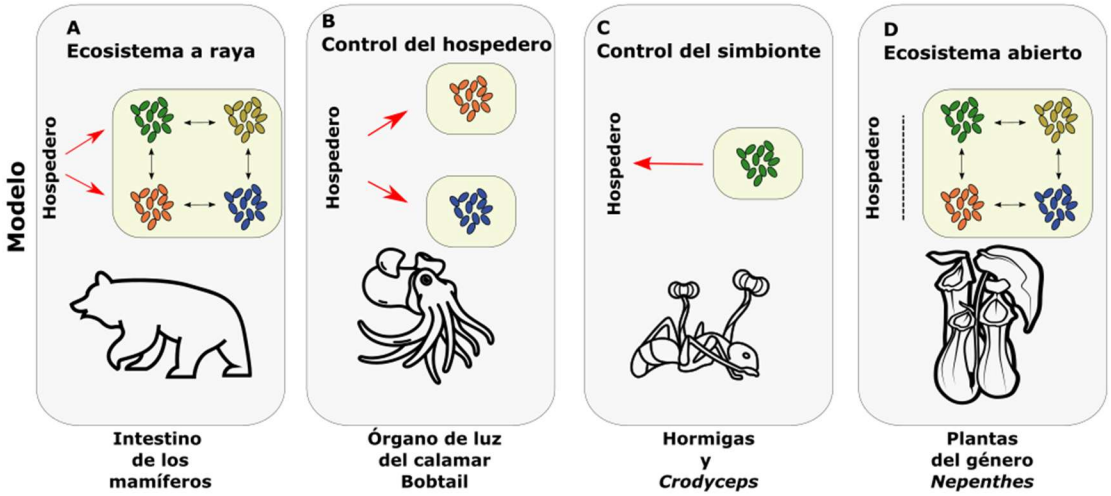


Figura 1. Modelos de interacción hospedero - microbioma. Las flechas negras indican la direccionalidad de las interacciones ecológicas. Las flechas rojas indican mecanismo de control. A) Ecosistema a raya. B) Modelo de control por el hospedero. C) Modelo de control por los simbiontes. D) Modelo de ecosistema abierto. Figura modificada de Foster et al. 2017. Los vectores de los hospederos usados para generar la figura fueron tomados de la web Noun Project y fueron creados por los usuarios Kevin[US], Oleksandr Panasovskyi,Olena Panovska[UA] y Cassandra Bachman.

B) Ecosistemas controlados por el hospedero: En este modelo los hospederos interactúan con comunidades microbianas caracterizados por una baja diversidad, comúnmente estos microbiomas están dominados por una o unas pocas especies microbiana. Además, en este modelo los hospederos han desarrollado mecanismos de control especializados en reclutar y controlar los niveles poblacional de sus simbiontes. Un ejemplo de este modelo, es la interacción entre *Euprymna scolopes* (calamar hawaiano) y comunidades

simbiontes de vibrios [59]. Los vibrios que se encuentran en baja proporción en el ambiente donde habita este calamar, son capaces de colonizar órganos especializados del calamar en donde crecen de manera selectiva sobre la mucosa de su hospedero. La colonización de las mucosas está controlada por el hospedero, que sintetiza una serie de compuestos antimicrobianos con la capacidad de inhibir el crecimiento de bacterias con la excepción de los vibrios. A su vez, los vibrios que colonizan la mucosa de órganos especializados conocidos como órganos de luz son capaces de generar bioluminiscencia, que permiten a su hospedero camuflarse en su ambiente natural [60–62] (Figura 1B).

C) Ecosistemas controlados por los simbiontes: Este modelo propone que los microorganismos ejercen mecanismos de control sobre su hospedero, con el fin de garantizar su sobrevivencia, las comunidades simbiontes de este modelo se caracterizan por presentar una baja diversidad y estar formadas por una solo especie microbiana. Un ejemplo de este modelo, es la interacción que ocurre entere los hongos patógenos del género *Crodyceps* y las hormigas u otros insectos que se ven afectados por estos patógenos [63–65] (Figura 1C).

D) Ecosistemas abiertos: Este modelo se caracteriza por presentar comunidades microbianas con una alta diversidad, sin embargo, propone que los hospederos no ejercen ningún tipo de control sobre las comunidades de microorganismos. El rol de hospedero se basa únicamente en compartamentalizar el sitio de la interacción, y este no recibe ningún beneficio o se ve afectado por la interacción. Un ejemplo de este modelo es el de la plantas carnívoras del género *Nepenthes* [66,67], que cuentan con un compartimento especializado donde ocurre la digestión de sus presas, mismo en el que en este se establecen las comunidades microbianas (Figura 1D).

Es importante mencionar que, si bien estos modelos resumen diversos escenarios de interacción entre los hospederos y su microbioma, no toman en consideración la influencia que el ambiente tiene sobre ambos participantes de esta interacción. En secciones posteriores se abordará la influencia que tiene la variación ambiental sobre la diversidad, estructura y función de la microbiota de la piel de anfibios.

### 1.3 Microbiomas y su importancia en la conservación de especies

El objetivo principal de la biología de la conservación es el de minimizar o mitigar aquellos problemas relacionados con la pérdida biodiversidad [68]. Actualmente, gran parte de los problemas relacionados con la pérdida de biodiversidad están relacionados con actividades humanas, como el cambio en el uso de suelo, la contaminación o el cambio climático [69]. Adicionalmente, la aparición de diversas enfermedades emergentes, provocadas en su mayoría por patógenos fúngicos [70], representan una amenaza para diversas especies de animales de vida libre como el caso de los murciélagos [71], serpientes [72] y anfibios [73] e incluso para cultivos de interés alimenticio para el hombre, como el trigo [74].

Debido a la contribución que los microbiomas tienen en funciones asociadas a la adquisición de nutrientes, la maduración del sistema inmune o la protección contra patógenos [5,27,50], se ha propuestos que los microbiomas sean considerados como una frontera en el conocimiento de disciplinas como la biología evolutiva [27] y la biología de la conservación [69,75,76]. Se ha demostrado que la manipulación de los microbiomas intestinales representa una alternativa para facilitar estrategias de translocación de especies amenazadas o

para favorecer el desarrollo y mejoramiento de programas de conservación mediante su uso como agentes probióticos [69,75–79].

Existen algunos ejemplo de éxito de estas estrategias, por ejemplo, la manipulación de la microbiota intestinal a través de la dieta en etapas del desarrollo temprano de esturiones (*Acipenser dabryanus*) criados en cautiverio, contribuye a una mayor sobrevivencia post translocación en ambientes naturales [80]. Por otra parte, la capacidad inhibitoria de ciertos miembros del microbioma de la piel de murciélagos o anfibios ante los patógenos que provocan enfermedades como el síndrome de la nariz blanca o la quitridiomicosis, han impulsado la investigación y búsqueda de estrategias basadas en la manipulación del microbioma de la piel para a proteger a las especies susceptibles a estas enfermedades [81–83].

En las siguientes secciones se discutirá el impacto que la quitridiomicosis ha tenido sobre la diversidad global de anfibios y como las comunidades microbianas de la piel de los anfibios contribuyen a la sobrevivencia de sus hospederos ante esta enfermedad.

### 1.3.2 Quitridiomicosis y su impacto en la diversidad de anfibios

Los hongos del filo Chytridiomycota, presentan un amplio rango de características morfológicas, metabólicas, y de historia de vida. Sin embargo, la gran mayoría comparte un ciclo de vida similar caracterizado por tres etapas principales: una etapa como zoospora móvil que posteriormente se convierte en un talo para finalmente pasar a un estadio de zoosporangio, que se caracteriza por ser una estructura esférica en donde se forman y maduran nuevas zoosporas [84–86]. Durante la etapa de zoospora, las células presentan un tamaño de entre

2 a 10 micras y se caracterizan por poseer un flagelo que les brinda movilidad, lo que les permite responder a diversos estímulos ambientales relacionados principalmente con la percepción de nutrientes [85,87,88].

Los miembros del orden Rhyzophydiales que forma parte del filo Chytridiomycota, presentan un amplio rango de estrategias ecológicas. Algunas especies son consideradas como saprobias, creciendo en sustratos ricos en polen, queratina, celulosa o quitina; otras especies son consideradas como parásitos de algas, invertebrados o plantas. Vale la pena mencionar que *Batrachochytrium dendrobatidis* (Bd) y *Batrachochytrium salamandrivorans* (Bsal) son los únicos hongos de este filo considerados como parásitos de vertebrados siendo los anfibios los hospederos principales de estos hongos [84].

Bd y Bsal son reconocidos como los agentes etiológicos de la quitridiomicosis, una enfermedad infecciosa de la piel que ha tenido un gran impacto en la reducción de la diversidad de anfibios a nivel mundial [73,89]. Bd fue identificado entre los años de 1998 y 1999 mediante el análisis microscópico y molecular de muestras de tejido de ranas de los géneros *Dendrobates* y *Litoria* que presentaban signos de enfermedad como aletargamiento y ulceraciones en la piel [90,91]. Por su parte, Bsal fue identificado en 2013 en muestras de tejido obtenido de individuos de *Salamandra salamandra* que habían muerto a causa de la infección [92].

Bd y Bsal se encuentran normalmente como zoosporas móviles en cuerpos de agua, en donde perciben a sus hospedero mediante mecanismos de quimiotaxis (específicamente detectan la hormona tiroidea) [87,88]. Una vez sobre la piel de su hospedero las zoosporas móviles pierden, su flagelo y forman

un talo, que les permite penetrar las capas superficiales de la piel de los anfibios. Una vez que infectan las capas superficiales de la piel, comienzan a formar zoosporangios, formando nuevas zoosporas de estas estructuras que más adelante serán liberadas sobre la piel del hospedero infectado o al ambiente en el que se encuentre el hospedero [93,94] (Figura 2).

Mediante análisis histológicos, se ha demostrado que Bd y Bsal infectan principalmente las regiones queratinizadas de la piel. En los anfibios estas regiones se encuentran en las capas superficiales de la epidermis en adultos y en regiones cercanas a la boca en anfibios en estadios larvarios [93]. Los anfibios infectados por Bd y Bsal presenta una serie de complicaciones que desencadenan un desbalance osmótico, que a la larga provoca un arresto cardiaco y por ende la muerte del hospedero infectado [95,96].

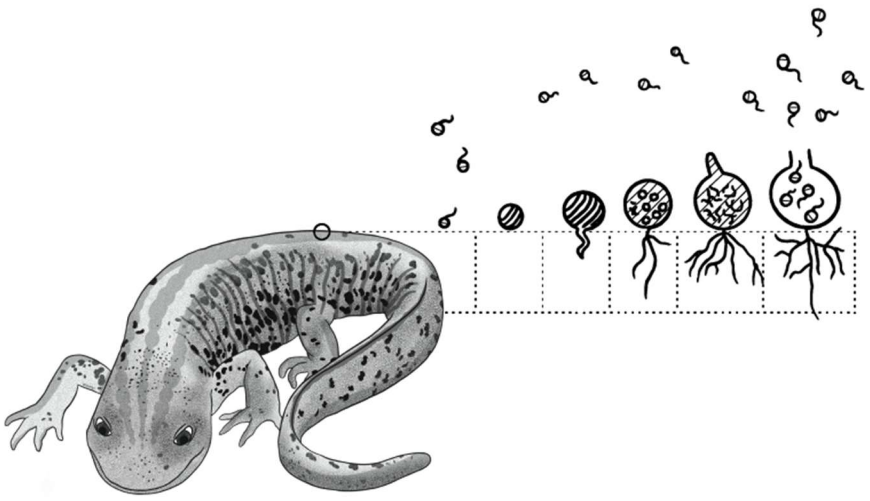

Figura 2. Ciclo de infección de *Batrachochytrium dendrobatidis* sobre la piel de su hospedero. El ciclo de infección incluye tres etapas, i) zoosporas móviles, 2) invasión mediada por la formación de un talo, y iii) formación de un zoosporangio, maduración y liberación de nuevas zoosporas. Figura modificada de Van Rooij et al. 2015.

Se ha estimado que cerca de 500 especies de anfibios han sido afectadas por la quitridiomicosis alrededor del mundo. Entre estas, 90 especies se consideran extintas en la actualidad a causa de esta enfermedad [73]. Debido al

impacto que la quitridiomicosis ha tenido sobre la diversidad de anfibios en el mundo, esta se ha considerado como un de las causas de la sexta extinción masiva [97,98]. Específicamente, los anfibios que habitan en las regiones de Australia y Centroamérica han mayormente afectados por esta enfermedad. Por otro lado, según la literatura, en Asia no existen reportes sobre declives poblacionales a causa de la quitridiomicosis [73]. Interesantemente, diversos estudios moleculares sugieren que Bd y Bsal tienen un origen común en Asia [73,89,99].

En América, los declives poblaciones de diversas especies de anfibios a causa de la quitridiomicosis se comenzaron a registrar a finales de los años 80 [100]. Estos declives poblacionales continuaron durante los años 90, específicamente en las regiones centrales del continente [101,102]. En México, se ha registrado la presencia de Bd en más de 50 especies de anfibios [103–105], y mediante el análisis de especímenes de museo se sabe que Bd está presente en el país desde finales del siglo XIX [106].

Es importante aclarar que la mayoría de los declives poblaciones registrados a la fecha se han atribuido a Bd. Se reconoce que este hongo tiene seis linajes genéticos diferenciados distribuidos en diversas regiones del planeta [107–109]. Entre los linajes de Bd que se conocen, se ha reportado que el linaje pandémico global (BdGPL) está distribuido por todo el mundo [108,110,111]. BdGPL es reconocido como el linaje genético más virulento de Bd, y se le han atribuido la mayoría de los declives poblacionales de anfibios [112–114]. Por su parte, el resto de linajes tienen un rango de distribución limitado a ciertas regiones del planeta: BdASIA1 y BdASIA3 se distribuyen únicamente en Asia, BdASIA2/BdBRAZIL se encuentra en regiones de Asia y Brazil, BdCH solo ha

sido identificado en regiones de Europa, y por ultimo BdCape se distribuye en los continentes de África, Europa y América [110,111].

A diferencia de Bd, Bsal presenta una distribución restringida a algunas regiones de Asia y Europa. Este patógeno es especialmente virulento para anfibios del orden Caudata (salamandras y tritones) [92,107,115,116]. Si bien este patógeno no está presente actualmente en Norteamérica [117,118], se han estimado mediante el modelado de nichos ecológicos que, en México existen regiones con condiciones climáticas idóneas para que Bsal se establezca en el país [119]. Estas mismas predicciones indican que regiones en el país como: el Eje Neovolcánico, la Sierra Norte de Oaxaca o la Sierra Madre del Sur en Guerrero y Chiapas, se verían gravemente afectadas por la llegada de Bsal ya que son consideradas como puntos de alta diversidad de salamandras [119].

A pesar del impacto que la quitridiomicosis ha tenido sobre muchas especies de anfibios, es importante mencionar que no todas las especies presentan el mismo grado de susceptibilidad ante esta enfermedad [120–123]. Algunas especies de anfibios son consideradas como tolerantes o resistentes a Bd y Bsal [99,124,125]. En este sentido, la evidencia acumulada hasta el día de hoy sugiere que, el grado de susceptibilidad frente a Bd y Bsal esta influenciado por diversos factores como: i) variación en alelos del complejo mayor de histocompatibilidad (MHC) [122], ii) la composición peptídica de la mucosa de la piel [123], iii) la historia evolutiva de los hospederos [99,112] o iv) la composición del microbioma de la piel [123,126].

Se ha descrito que, los anfibios son capaces de montar diversas respuestas inmunes para combatir la infección contra Bd o Bsal empleando mecanismo

inmunes mediados por la producción de anticuerpos o la síntesis de péptidos antimicrobianos [127,128]. Se ha demostrado que, estos patógenos son capaces de evadir y contrarrestar la respuesta inmune de los anfibios mediante la producción de metabolitos que inducen la apoptosis de linfocitos o inhiben la respuesta inflamatoria [120,128–132]. Adicionalmente, aunado a las estrategias para evadir el sistema inmune se ha demostrado que la variación ambiental asociada a cambios estacionales a factores ambientales influye sobre la respuesta inmune de los anfibios frente a la quitridiomicosis [133–135].

### 1.3.3 El microbioma de la piel de los anfibios y su rol en la defensa contra la quitridiomicosis

Diversos estudios han demostrado que los microorganismos simbiontes de la piel de los anfibios desempeñan un rol importante en la protección de su hospedero frente a Bd y Bsal [79,126,136–138]. Los primeros reportes sobre la capacidad inhibitoria de integrantes de la microbiota de la piel de anfibios datan de mediados de los años 90. En estos trabajos se demostró que bacterias como *Lysobacter gummosus* o *Janthinobacterium lividum* aisladas de la piel de la salamandra *Plethodon cinereus* eran capaces de inhibir el crecimiento de Bd en [137].

Posteriormente, se demostró que la capacidad inhibitoria de estas bacterias se debe que estas sintetizan metabolitos antifúngicos como el 2,4-diacetilfloroglucinol sintetizado por *L. gummosus* [139] o la violaceina y el indol-3-carboxaldehido que producidos por *J. lividum* [140]. A la fecha solo se han caracterizado un puñado de metabolitos sintetizados por bacterias de la piel de anfibios como es el caso de la prodigiosina sintetizada por bacterias del género *Serratia* [83], el triptofol identificado en co-cultivos de *Bacillus* sp y *Chitinophaga*

*arvensicola* [141] y la vsicosina sintetizada por *Pseudomonas cichorii* [142] Adicionalmente, se ha reportado que bacterias de los géneros *Janthinobacterium* y *Serratia* producen compuestos volátiles con la capacidad de inhibir el crecimiento de Bd [79]. Adicionalmente, análisis genómicos han demostrado que bacterias de los géneros *Pigmentiphaga* [143], Pseudomonas [144] y *Acinetobacter* [145] aisladas de la piel anfibios poseen clústeres biosintéticos que codifican para la producción de con actividad antibacteriana [143,144] y antifúngica [145].

Derivado de los reportes que señalaban que diversas bacterias presentes en los microbiomas de la piel de anfibios tenían la capacidad de inhibir el crecimiento de Bd, se planteó el uso de estas como probióticos para proteger a especies susceptibles a la quitridiomicosis [79,146]. Estas estrategias son dependientes del aislamiento e inoculación directa [147] o indirecta (mediante bioaumentación [146,148]) de bacterias capaces de inhibir a Bd. A la fecha, existen algunos ejemplos de éxito en especies como *Rana muscosa* y *P. cinereus*. Los primeros ensayos que se realizaron demostraron que, anfibios previamente inoculados con *J. lividum* presentaban un mayor porcentaje de sobrevivencia en ensayos de infecciones experimentales con Bd [147,149,150]. Además, se demostró que *J. lividum* era capaz de colonizar y persistir sobre la piel de los anfibios en, lo que permitía sugerir que esta bacteria podría proteger de manera persistente a sus hospederos [148].

Sin embargo, a pesar del éxito inicial de estos experimentos, estudios posteriores demostraron que la inoculación de *J. lividum* no es suficiente para evitar la mortalidad por Bd en especies susceptibles a la quitridiomicosis como la rana arlequín (*Atelopus zeteki*) [151]. Adicionalmente, en otras especies de

ranas como como *Lithobates clamitans,* se observó que *J. lividum* puede colonizar la piel de esta rana, sin embargo la abundancia de esta bacteria disminuye con el paso del tiempo [152].

Vale la pena mencionar que, hasta el año 2015, se habían aislado cerca de 2,000 cepas bacterianas con la capacidad de inhibir el crecimiento de Bd y Bsal en ensayos *in vitro* [153]. A la fecha, se reconoce que la capacidad inhibitoria contra los patógenos que causan la quitridiomicosis es un rasgo común entre los miembros del microbioma de la piel de los anfibios. Específicamente, se ha reportado que bacterias aisladas de la piel de anfibios pertenecientes a los fila Actiniobacteria, Alfaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes y Firmicutes son capaces de inhibir el crecimiento de Bd y Bsal [153–155].

Sin embargo, la diversidad y abundancia de las bacterias inhibitorias varia de manera significativa entre distintas especies de anfibios y con relación a diversos factores bióticos y abióticos [79,154,155]. Por ejemplo, se ha descrito que una disminución en la abundancia de Betaproteobacterias a lo largo del desarrollo de la rana toro (*Anaxyrus boreas*) se correlaciona con un aumento en la abundancia de Actinobacterias y hogos sobre la piel de este anfibio [126]. De la misma manera se ha descrito que una baja diversidad bacteriana, se correlaciona con una alta abundancia de bacterias del orden Burkholderiales al que pertecne géneros como *Janthinobacterium* [138].

## 1.4 Factores que influyen en la diversidad, estructura y función del microbioma de la piel de los anfibios

La piel es un órgano que actúa como barrera protegiendo a los organismos contra diversos factores bióticos y abióticos [156]. En el caso de los anfibios, la piel cumple funciones relacionadas con el balance osmótico, el intercambio de gases, la absorción de agua, o la producción de moléculas inmunes contra depredadores y patógenos [156–160]. Además de las diversas funciones fisiológicas que cumple la piel, este órgano permite a los organismos interactuar con su entorno y se puede considerar como un ecosistema complejo en el que habitan una gran diversidad de microorganismos [161,162].

Se ha demostrado que las comunidades de microorganismos que habitan sobre la piel de los anfibios son susceptibles a diversos factores bióticos y abióticos que influyen sobre la diversidad, estructura y función de las comunidades simbiontes de la piel [163–169]. Estos factores pueden clasificarse como: i) asociados a los hospederos, ii) asociados al microhábitat, y iii) asociados a factores biogeográficos y climáticos (Figura 2) [163]. En las siguientes secciones se discutirán las principales evidencias que demuestran la influencia de estos factores sobre las comunidades microbianas simbiontes de la piel de los anfibios.

### 1.4.1 Factores asociados al hospedero

Se ha demostrado que las comunidades de microorganismos ambientales son la principal fuente de diversidad del microbioma de la piel de los anfibios y que estos son capaces de seleccionar a los microorganismos pueden colonizan su piel [148,170,171]. Para entender cómo funcionan los mecanismos de

selección del microbioma que empelan los anfibios, es importante conocer las características fisiológicas de la piel de estos animales.

Los anfibios poseen sobre su piel dos tipos de glándulas especializadas conocidas como: gandulas de mucosa y glándulas granulares [172–174]. Específicamente, las glándulas de mucosa se encargan de producir y liberar secreciones hidrofílicas ricas en proteínas, y se ha propuesto que estas secreciones facilitan el intercambio de gases en la piel [172,174].

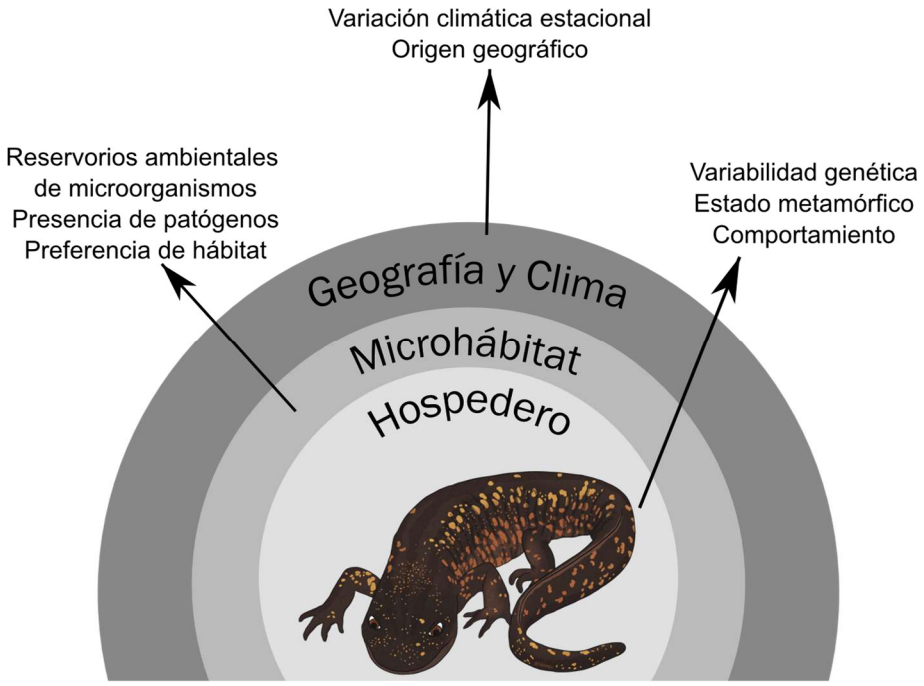


Figura 3. Factores bióticos y abióticos que influyen sobre la diversidad y estructura de la microbiota de la piel de los anfibios. Figura modificada de Rebollar et al 2020.

Por su parte, dentro de las glándulas granulares se sintetizan y almacenan diversos metabolitos como toxinas o péptidos antimicrobianos (AMP's) que cumplen funciones relacionadas con la defensa ante depredadores y patógenos [172,175,176]. Se sabe que la abundancia y la actividad de estas glándulas secretoras varía con relación a la especies [174], estadio del desarrollo [177–

179] y sexo de los anfibios [173]. Se ha demostrado que diversos estímulos mecánicos y químicos promueven la liberación del contenido de estas glándulas [176].

Además de actuar como moléculas inmunes implicadas en la defensa contra microorganismos patógenos [160,176,180,181], evidencia reciente seguiré que los AMP's sintetizados dentro de las glándulas granulares de la piel de los anfibios, tienen un papel importante en el reclutamiento de bacterias simbiontes [182]. Específicamente, se ha reportado que ciertos AMP's actúan como promotores de crecimiento para grupos específicos de bacterias que forman parte de la microbiota de la piel de los anfibios y estas bacterias, a su vez, inducen cambios en los perfiles de AMP's producidos por los hospederos [182]. Además, estudios genómicos proponen que la presencia de bombas de flujo en los genomas de bacterias simbiontes de la piel de anfibios podría estar relacionada con la resistencia que tienen las bacterias simbiontes ante los AMP's secretados por sus hospederos [144].

Por otra parte, se ha demostrado que la diversidad y estructura de la microbiota de la piel de los anfibios difiere de manera significativa entre especies de anfibios [121,168,183,184]. Por ejemplo, se ha observado que entre especies simpátricas de anfibios, la estructura y diversidad de las comunidades bacterianas de su piel varia de manera significativa con relación a la especie de hospedero, estas diferencias se asocian a cambios en la abundancia de diversos grupos bacterianos [121,168,183,185]. Adicionalmente, cuando se compara la diversidad bacteriana del microbioma de la piel entre anfibios simpátricos pertenecientes a distintos ordenes, se ha reportado que las comunidades

bacterianas de la piel de anfibios del orden caudata (salamandras) presentan una menor diversidad comparada con los anuros (ranas y sapos) [183–185].

El estado metamórfico de los anfibios es otro de los factores que influye de manera importante sobre la diversidad y estructura del microbioma. Los anfibios presentan dos estrategias principales de desarrollo, el desarrollo directo y la metamorfosis [186]. La metamorfosis es un proceso del desarrollo por el que pasan y que conlleva una serie de cambios fisiológicos como: la restructuración de tejidos, cambios en los perfiles transcripcionales de órganos como el corazón, el hígado o la piel [19,20], e incluso el rearreglo del sistema óseo (perdida de huesos craneales y rearreglo del sistema dental) [187].

Además, cuando los anfibios pasan por un proceso de metamorfosis, deben suprimir algunas de las respuestas inmunes mediadas por linfocitos para no interferir con los rearreglos fisiólogos de sus tejidos [178]. Es importante mencionar que las glándulas secretoras de la piel maduran al completar el proceso de metamorfosis [177], lo que explica las diferencias que existen en la composición química de la mucosa entre anfibios pre-metamórficos y metamórficos [179].

Aunado a los diversos cambios fisiológicos por los que pasan los anfibios durante la metamorfosis, estos también presentan cambios ecológicos. Específicamente en especies que pasan por la metamorfosis, los estadios larvarios y juveniles se encuentran en hábitats acuáticos como ríos, lagos o pozas y arroyos temporales. Al completar la metamorfosis la mayoría de las especies de anfibios pasan a ocupar hábitats terrestre [188,189].

De manera general, se ha reportado que la microbiota de la piel de los anfibios difiere de manera significativa entre individuos pre-metamórficos (juveniles) y metamórficos (adultos) [126,166,190,191]. Estas diferencias se pueden explicar mediante el análisis de los patrones de abundancia de ciertos grupos bacterianos, por ejemplo, individuos pre-metamórficos de *Anaxyrus boreas* presentan una alta abundancia de Betaproteobacterias, mientras que en individuos metamórficas las Actinobacterias son el grupo bacteriano más abundante [126], estos patrones se han descrito en otras especies de anfibios en vida libre y cautiverio [166,190]

Finalmente, vale la pena mencionar que, si bien, se reconoce que la microbiota de la piel de los anfibios se ensambla mediante el reclutamiento de microorganismos presentes en el ambiente, algunas observaciones que sugieren que en ciertas especies de anfibios podrían ocurrir eventos de transmisión vertical de miembros de la microbiota, debido a comportamientos relacionados con el cuidado parental de las puestas de huevos [137,192–194].

### 1.4.2 Factores asociados al microhábitat.

En la sección anterior se mencionó que las comunidades ambientales de microorganismos, son la principal fuente de diversidad de la microbiota de la piel de los anfibios [171]. En este sentido, es importante tener en cuenta que los anfibios pueden ocupar diversos microhábitats y, con base en su preferencia de hábitat se pueden clasificar como: i) acuáticos (ocupan microhábitats como estanques, lagunas, o arroyos), ii) terrestres (habitan principalmente entre la materia vegetal en descomposición como hojarasca y troncos de árboles), iii) arborícolas (se encuentran en el dosel vegetal), iv) riparios (se encuentran entre la transición de los arroyos y el hábitat terrestre inmediato) y v) fosoriales (estas

especies habitan dentro de cuevas o debajo de la tierra en madrigueras) [125,188,189].

Se ha demostrado que la microbiota de la piel de los anfibios varía de manera significativa entre especies de hospederos que habitan en microhábitats distintos [121,195–197]. Incluso, algunas observaciones sugieren que ciertos grupos de bacterias tienen a ser más abundantes sobre la piel de los anfibios con relación al microhábitat que estos ocupan. Por ejemplo, en un estudio donde se comparó la diversidad de la microbiota de la piel entre especies de ranas que habitan en microhábitats contrastantes se observó que, los géneros bacterianos *Pigmentiphaga*, *Agrobacterium* y *Methylotenera* estaban diferencialmente enriquecidos sobre la piel de ranas arborícolas, terrestres y acuáticas respectivamente [169].

Por su parte, la variación ambiental asociada a ciertos factores fisicoquímicos como la temperatura [164,165,198], el pH [164,199] o la salinidad [200] también influyen de manera significativa sobre la diversidad de la microbiota de la piel de los anfibios. En este sentido, es importante considerar que la variación de estos factores influye directamente sobre la fisiología de los microorganismos, y por ende en su posible función inhibitoria ante los patógenos Bd y Bsal [201,202]. Mediante ensayos *in vitro* se ha demostrado que la capacidad inhibitoria frente a Bd, difiere de manera significativa cuando las bacterias inhibitorias crecen a distintas temperaturas (e.g. 12 ºC – 18 ºC) [202], presentado una reducción considerable de su actividad inhibitoria en temperaturas bajas (por debajo de 13ºC) [203].

Por su parte, se ha descrito que la presencia de Bd puede influir de manera significativa sobre la diversidad y estructura de la microbiota de la piel [204,205]. En este sentido, es importante mencionar que las infecciones por Bd pueden ser: enzoóticas (presencia continua de individuos infectados en las poblaciones) o epizoóticas (presencia esporádica de individuos infectados en las poblaciones) [73,206,207], siendo los eventos epizoóticos lo que han asociado con mayor frecuencia a declives poblacionales de especies de anfibios [206,207].

Diversos estudios han demostrado que anfibios en cautiverio [205] y vida libre [204,208,209] que sobrevivieron cuadros epizoóticos por Bd presentan cambios significativos en la diversidad y estructura de la microbiota de la piel. Estas observaciones sugieren que las comunidades de bacterias de la piel presentan una baja resiliencia después de los eventos de infección [205,208]. Adicionalmente, se ha reportado que la intensidad de la infección por estos patógenos está asociada a la baja abundancia de taxa bacterianos como Proteobacterias y los Bacteroidetes [209].

En el caso de poblaciones de anfibios que pasaron por cuadros enzoóticos, se han reportado resultados contrastantes sobre la influencia que tiene Bd sobre la diversidad y la estructura de la microbiota de la piel. Algunos estudios demuestran que la presencia de Bd no influye de manera significativa sobre la diversidad de la microbiota de la piel entre individuos infectados y no infectados [164,195,210]. Por otra parte, se ha reportado que la presencia de Bd en poblaciones con altos índices de infección esta correlacionada con microbiomas dominados por algunos taxa bacterianos y que además presentan una baja diversidad, sin embargo, los taxa dominantes pertenecen a grupos bacterianos con la capacidad de inhibir a Bd [138].

### 1.4.3 Factores biogeográficos y climáticos

Entender los patrones de distribución geográfica de los microorganismos y la influencia que los cambios ambientales a gran escala tienen sobre las comunidades de microorganismos son temas de creciente interés en la ecología microbiana [211–213]. Se ha reportado, que la diversidad taxonómica y funcional de las comunidades de bacterias presentes en el suelo varía de manera significativa entre regiones templadas, tropicales y árticas. Estos cambios están asociados a la variación de diversos factores climáticos entre regiones [214].

En el caso de la microbiota de la piel de los anfibios, se ha demostrado que la variación estacional de factores ambientales como la temperatura [164,198], precipitación [166,209] e incluso el origen geográfico de los hospederos [167,215], influyen de manera importante sobre la diversidad y estructura de las comunidades bacterianas de la piel de los anfibios. Por ejemplo, en algunas especies de tritones la variación estacional de la temperatura influye de manera significativa sobre la diversidad de la microbiota [198].

Por otra parte, diversos estudios han demostrado que la microbiota de la piel de los anfibios varia de manera significativa a los largo de gradientes geográficos, específicamente a lo largo de gradientes altitudinales [167,191,216,217] y de urbanización [218,219]. En estos trabajos también se ha descrito que la capacidad inhibitoria del microbioma frente a Bd varia a lo largo de estos gradientes [191,219].

## Capítulo 2. ¿Por qué estudiar el microbioma de *Ambystoma altamirani*?

### 2.1 Características generales del género *Ambystoma*

Dentro del género *Ambystoma* se pueden enlistar treinta y tres especies, conocidas comúnmente como ajolotes, estos anfibios tiene un rango de distribución que se extiende desde Alaska hasta el centro de México [189,220]. En México habitan diecisiete de las treinta y tres especies de ajolotes que se conocen, cabe la pena mencionar que dieciséis de estas especies son endémicas para México [188,220] y según la NOM-059-SEMARNAT-2010, quince de estas especies están enlistadas bajo alguna categoría de protección especial [220,221]. En cuanto a sus hábitos alimenticios, los ajolotes son considerados como depredadores principalmente de moluscos e insectos [220]. Sin embargo existen reportes que indican que diversas especies presentan comportamientos de canibalismo e incluso pueden llegar a depredar ranas pequeñas [188,222].

Entre las especies de ajolotes que se encuentran en México, tres son consideradas como neoténicas obligadas [223–225], lo que implica que, de manera general (en vida libre) estas especies no pasan por un proceso metamórfico y conservan características juveniles a lo largo de su ciclo de vida [187,226]. Cabe mencionar que en estas especies la metamorfosis puede ser inducida mediante la exposición a la hormona tiroidea [190,223,227]. El resto de especies de ajolotes que habitan en México son consideran como neoténicas facultativas, lo que implica que algunos individuos de estas especies pueden o no pasar por el proceso de metamorfosis [188,220,228–230].

Los anfibios en estadio de neotenia se caracterizan por conservar características morfológicas juveniles como la presencia de branquias, sin embargo estos son capaces de reproducirse al igual que individuos metamórficos [177,226,231]. Si bien en las especies consideradas como neoténicas facultativas algunos individuos pasan por el proceso de metamorfosis, no se conoce con certeza que factores biológicos y ecológicos inducen este proceso. Sin embargo, en especies neoténicas obligadas se ha reportado que deficiencias en la producción de la hormona tiroidea [226].

Las especies de anfibios del género *Ambystoma*, cuentan con especies consideradas como neoténicas facultativas [223,232,233] u obligadas [225,227]. Estudios realizados en especies neoténicas facultativas, demuestran que la metamorfosis en estas especies es dependiente de la concentración de hormona tiroidea en estadios específicos del desarrollo [234]. Específicamente, se ha reportado que en *Ambystoma tigrinum* los niveles de esta hormona en plasma incrementan durante la metamorfosis [234], además el aumento de esta hormona depende de la correcta estimulación de la glándula pituitaria por parte de otras hormonas como la corticotropina [233]. Por su parte, se ha demostrado que la neotenia obligada en *Ambystoma mexicanum* se relaciona con una baja concentración de la horman tiroidea en plasma [235,236].

Es importante mencionar que a diferencia de muchas especies de anfibios que durante sus estadios larvarios ocupan hábitats acuáticos ([188,237,238] y hábitats terrestres después de la metamorfosis [188,232,239], la mayoría de las especies Mexicanas del género *Ambystoma* permanecen en los cuerpos de agua después de la metamorfosis [220,224,228,229], lo que implica que los individuos

juveniles, neoténicos y adultos coexisten en el mismo hábitat durante su ciclo de vida completo,

## 2.2 Estudios sobre la microbiota de la piel de los anfibios del género *Ambystoma*

Hasta antes de este trabajo, solo se había descrito la microbiota de la piel de tres de las diecisiete especies de ajolotes. Estos estudios se han centrado en describir la diversidad bacteriana de la piel *A. tigrinum* [183] y *A. rivulare* [185] en individuos de vida libre y *A. mexicanum* [190] en cautiverio. Estos trabajos demostraron que la diversidad de las comunidades bacterianas de la piel de los ajolotes esta influenciada por factores como: el estado metamórfico del hospedero [190] o por cambios ambientales entre estaciones asociados a la precipitación [185]. Otro factor que influye de manera significativa sobre la diversidad de la microbiota de la piel de los ajolotes, es la identidad del hospedero. Específicamente, se ha reportado que la diversidad bacteriana del microbioma de la piel de anfibios del género *Ambystoma* difiere de manera significativa de la diversidad bacteriana de la piel de anfibios simpátricos de los ajolotes [183,185].

Los estudios sobre la microbiota de la piel de ajolotes revelan que las Proteobacterias y Bacteroidetes son los fila bacterianos más abundantes sobre la piel de estos anfibios [183,185]. Adicionalmente, se ha predicho mediante la comparación de secuencias ribosomales con bases de datos de bacterias inhibitorias, que diversos grupos bacterianos presentes sobre la piel de los ajolotes podrían tener un papel importante en la protección de su hospedero frente a los patógenos que causan la quitridiomicosis [185].

## 2.3 *Ambystoma altamirani* como modelo de estudio

*A. altamirani* conocido comúnmente como ajolote de arroyo de montaña, tiene un rango de distribución acotado a los estados de Morelos, Estado de México y Ciudad de México, específicamente esta especie se distribuye en la región de la Sierra de Cruces [238], habitando dentro de arroyos ubicados en pastizales de montaña o bosques de pino u oyamel [220,238].

Debido a que el rango de distribución de *A. altamirani* traslapa con una de las regiones más pobladas del país, esta especie enfrenta graves problemas de reducción de hábitat por lo que esta enlistada bajo el estatus de amenazada en la NOM-059-SEMARNAT-2010 y clasificada bajo el estatus EN (en peligro) por la IUCN (Unión Internacional para la Conservación de la Naturaleza) [220,221,238]. Adicionalmente, el bajo flujo genético entre las poblaciones aunado a un alto porcentaje de endogamia podrían representar futuros problemas para la conservación de esta especie [240,241].

Con respecto a sus características ontogénicas, *A. altamirani* es considerada como neoténica facultativa, por lo que, en poblaciones de esta especie se pueden encontrar individuos adultos metamórficos y pre-metamórficos (individuos neoténicos) e individuos juveniles pre-metamórficos [229,230,242]. Se ha descrito que el periodo de tiempo en el que un individuo juvenil de *A. altamirani* completa el proceso de metamorfosis va desde los seis meses hasta un año y, a diferencia de otras especies de anfibios que adquieren un estilo de vida terrestre al completar la metamorfosis, los individuos metamórficos de *A. altamirani* permanecen dentro en los cuerpos de agua [229,230,242].

Si bien no existen registros sobre declives poblacionales relacionados con la quitridiomicosis en esta especie, si se ha reportado la presencia de Bd en

poblaciones de *A. altamirani* [243–245]. Cabe mencionar que las muestras colectadas como parte del primer artículo de este trabajo (Capítulo 3) se usaron para caracterizar las dinámicas de infección por Bd en esta especie de ajolote [243]. Basanta y colaboradores (2022), mostraron que Bd tiene una prevalecía del 70% en las muestras colectadas como parte de este trabajo (los detalles del muestreo de estos ajolotes están descritos en tercer capítulo de este trabajo). Los resultados de Basanta y colaboradores, sugieren que las poblaciones de *A. altamirani* que se analizaron en este trabajo se encuentran en un estado enzoótico, presentando un mayor índice de infección durante el invierno [243].

Adicionalmente, se han reportado casos aislados de ajolotes de esta especie muertos en campo (observaciones personales publicadas en [246]), y mediante estudios histológicos y moleculares se confirmó la presencia de Bd en los ajolotes muertos [246]. En conjunto, estas observaciones sugieren que *A. altamirani* es una especie tolerante a la quitridiomicosis, sin embargo, hasta el momento se desconoce bajo qué condiciones los individuos infectados por Bd no son capaces de tolerar la infección.

## Capítulo 3. Objetivos

*A. altamirani* fue seleccionado como modelo de estudio debido a que los individuos metamórficos y pre-metamórficos de esta especie coexisten en un mismo hábitat durante su ciclo de vida completo por lo que están expuestos a la misma fuente de microorganismos ambientales, y a la misma variación ambiental. Además, a pesar de la alta incidencia de Bd en las poblaciones *A. altamirani*, esta especie es considerada como tolerante a la quitridiomicosis.

### 3.1 Objetivo general

Considerando los antecedentes presentados en los capítulos anterior, este trabajo se planteó con el objetivo de evaluar ¿Qué factores bióticos y abióticos influyen sobre la diversidad taxonómica y funcional del microbioma de la piel de *A. altamirani*?

#### 3.1.1 Objetivos específicos

1. Describir la diversidad bacteriana del microbioma de la piel de A. altamirani.
2. Evaluar la influencia de diversos factores bióticos y abióticos sobre la diversidad taxonómica del microbioma de la piel del ajolote de arroyo de montaña.
3. Caracterizar la diversidad funcional del microbioma de la piel de ajolotes pre-metamórficos de *A. altamirani* y evaluar cambios en los perfiles funcionales del microbioma con relación a la estacionalidad, elevación y estado de infección por Bd.
4. Evaluar la posible contribución de grupos específicos de bacterias en la defensa contra los patógenos que causan la quitridiomicosis.

## Capítulo 4. La microbiota de la piel del ajolote *Ambystoma altamirani* está altamente influenciada por la metamorfosis y la estacionalidad, pero no por la infección del patógeno Bd

### 4.1 Resumen

En este trabajo se evaluó la influencia de distintos factores bióticos y abióticos sobre la diversidad y estructura de las comunidades bacterianas de la piel del ajolote *A. altamirani* mediante la secuenciación de amplicones del gen 16s rRNA. Se realizó un muestreo longitudinal que abarcó las cuatro estaciones del año (verano 2019, otoño 2019, invierno 2020 y primavera 2020) en cuatro localidades ubicadas en la Sierra de Cruces en los municipios de Isidro Fabela y Tlazala en el Estado de México. Durante cada colecta se tomaron muestras de la microbiota de la piel de individuos metamórficos (sin branquias) y pre-metamórficos (con branquias), así como muestras de agua y de sedimento de los arroyos donde estos ajolotes habitan. Adicionalmente, en cada localidad se registraron diversos parámetros fisicoquímicos del agua como la temperatura, el pH, la conductividad y el nivel de oxígeno disuelto.

Los resultados mostraron que las comunidades bacterianas de la piel de los ajolotes difieren significativamente de las comunidades bacterianas de las muestras ambientales (agua y sedimento). Además, se observó que la diversidad y estructura de la microbiota difiere de manera significativa entre ajolotes metamórficos y pre-metamórficos, estas diferencias se vincularon a cambios en la abundancia de familias de bacterias como Chitinophagaceae y Burkholderiaceae. Al comparar la diversidad alfa y beta entre cada una de las transiciones estacionales, se observó que las comunidades bacterianas de la piel de ajolotes pre-metamórficos varían de manera significativa entre otoño –

invierno e invierno – primavera. En el caso de los ajolotes metamórficos solo la transición estacional entre invierno – primavera influyo de manera significativa sobre la diversidad bacteriana de la piel. Adicionalmente, se observó que la microbiota de la piel de ajolotes pre-metamórficos varia de manera significativa entre las distintas localidades de muestreo.

Estos resultados indican que la microbiota bacteriana de la piel de ajolotes pre-metamórficos esta influenciada en mayor medida por cambios ambientales entre estaciones y localidades cuando se compara con las comunidades bacterianas de los ajolotes metamórficos. Por otra parte, se describió que los patrones de variación estacional observados en la diversidad beta de la microbiota de la piel están asociados a cambios estacionales de factores fisicoquímicos del agua como el pH y la temperatura.

A pesar de la alta prevalencia de Bd en las muestras analizadas en este trabajo, no se observó que la presencia de Bd influyera de manera significativa sobre la diversidad de las comunidades bacterianas de la piel de *A. altamirani*. Sin embargo, se detectó que la abundancia de ciertos ASV bacterianos esta correlacionada con la intensidad de la infección por Bd.

Los resultados presentados como parte de este capítulo contribuyen a un mejor entendimiento sobre la influencia de diversos factores bióticos y abióticos sobre la microbiota de la piel de los anfibios. Usando como modelo al ajolote *A. altamirani* demostramos que la microbiota de la piel de *A. altamirani* varia de manera significativa en relación con el estatus metamórfico del hospedero y a cambios ambientales entre estaciones.

Animal Microbiome

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# The skin microbiota of the axolotl *Ambystoma altamirani* is highly influenced by metamorphosis and seasonality but not by pathogen infection

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## Abstract

**Background:** Microbiomes have been increasingly recognized as major contributors to host health and survival. In amphibians, bacterial members of the skin microbiota protect their hosts by inhibiting the growth of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Even though several studies describe the influence of biotic and abiotic factors over the skin microbiota, it remains unclear how these symbiotic bacterial communities vary across time and development. This is particularly relevant for species that undergo metamorphosis as it has been shown that host physiology and ecology drastically influence diversity of the skin microbiome.

**Results:** We found that the skin bacterial communities of the axolotl *A. altamirani* are largely influenced by the metamorphic status of the host and by seasonal variation of abiotic factors such as temperature, pH, dissolved oxygen and conductivity. Despite high Bd prevalence in these samples, the bacterial diversity of the skin microbiota did not differ between infected and non-infected axolotls, although relative abundance of particular bacteria were correlated with Bd infection intensity.

**Conclusions:** Our work shows that metamorphosis is a crucial process that shapes skin bacterial communities and that axolotls under different developmental stages respond differently to environmental seasonal variations. Moreover, this study greatly contributes to a better understanding of the factors that shape amphibian skin microbiota, especially in a largely underexplored group like axolotls (Mexican *Ambystoma* species).

**Keywords:** Skin microbiota, Amphibians, Metamorphosis, Seasonality

## Background

Host associated microbiomes are vital for host health and survival, as they play relevant functions linked to nutrition, reproduction, behavior, defense against pathogens or predators [1–5]. Specifically, some animal associated microbiomes contribute to host health due to their ability to inhibit the growth of pathogens responsible for infectious diseases threatening diverse host species such as bats, snakes, or amphibians [6–8]. For instance, it has been shown that some members of the amphibian skin microbiome inhibit the growth of the lethal pathogens *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* [9–12], which have caused amphibian populations declines and extinctions worldwide [13].

Studies accumulated over the past two decades showed that the amphibian skin microbiome is influenced by

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host associated factors (host genetics and development) [14–16], microhabitat related factors (environmental microorganisms, habitat abiotic conditions and pathogen presence) [17–21], and climatic and geographical factors (seasonality, precipitation, temperature or land use) [14, 22–25].

In the case of host-associated factors, it has been shown that the skin microbiota of amphibians (specifically frogs) changes across development and particularly before and after metamorphosis [26–28]. During metamorphosis amphibians in larval stages transition to adults following a series of physiological rearrangements such as tail reabsorption, limb development and remodeling of muscles, heart, intestine brain, and skin [29]. Metamorphosis also induces immunosuppression in response to thyroid and corticosteroid hormone signaling and eventually the immune system reorganizes and gradually matures in newly metamorphosed adults [30].

Along with physiological rearrangements, many amphibian species go through behavioral and lifestyle changes, while larval stages inhabit aquatic environments, adults become terrestrial and only return to water environments in the reproductive season [31–33]. These changes in microhabitat occupancy could influence skin microbial composition since the environmental microbial communities are one of the main sources of microbial diversity [16, 17].

In the case of climatic factors, temporal variation of abiotic factors [34] such as temperature and precipitation have a strong influence over amphibian skin microbial community structure [22, 35]. For example, in tropical regions microbial diversity on the amphibian skin differs between wet and dry seasons [19, 26, 36]. In temperate regions, where the four seasons are well defined through the year, seasonal changes have been linked to the temporal dynamics of the amphibian skin microbiota [22, 37–39]. In addition, it has been shown that spatial variation such as elevation gradients [40–42] or distinct microhabitats [43] influence the skin microbial diversity of amphibians.

Bd influence over the amphibian skin microbiota has been described in amphibian species with contrasting Bd infection status (infected–non-infected [19] and high Bd prevalence—low Bd prevalence [44]). These studies showed that disruption of skin microbiota following Bd infection can influence host survival and that the final outcome of the infection depends on the interplay between host, microbiome and the environment [21, 23, 45].

Here we analyzed the skin bacterial diversity of the axolotl *Ambystoma altamirani,* a stream dwelling salamander endemic to conifer and oak-pine forest from the central region of Mexico [46]. *A. altamirani* is a facultative paedomorphic species in which, metamorphic (without gills) and pre-metamorphic (with gills) individuals inhabit the same streams all year long [47, 48], allowing us to evaluate how metamorphosis and seasonality influence the skin microbiota in a species living in the same aquatic environment across time and development. In addition, we evaluated if skin microbiota differs from environmental bacterial communities and if Bd presence and infection intensity influence the skin microbiota of *A. altamirani*. We hypothesized that *A. altamirani* skin microbiota would (a) differ from environmental bacterial communities, (b) vary between metamorphic and pre-metamorphic salamanders, (c) change across seasons and (d) differ according to Bd infection status.

## Results

We sampled a total of 279 *A. altamirani* individuals (85 metamorphic and 194 pre-metamorphic) at four locations across four seasons. Additionally, 159 environmental samples from sediment (80) and water (79) were collected. After quality control and rarefaction at 10,000 reads per sample, 13 samples were discarded and the remaining 438 samples were used to perform all diversity analyses (Table 1). A final table with a total of 72,408

**Table 1** Final list of collected samples that passed bioinformatic filters

| | Metamorphic | Pre-metamorphic | Sediment | Water | Total samples (N) |
|---|---|---|---|---|---|
| Summer (July 2019) | 25 | 41 | 19 | 20 | **105** |
| Autumn (October 2019) | 28 | 29 | 20 | 20 | **97** |
| Winter (January 2020) | 9 | 66 | 20 | 20 | **115** |
| Spring (April 2020) | 23 | 58 | 20 | 20 | **121** |
| Total samples (N) | **85** | **194** | **79** | **80** | **438** |

Numbers in bold indicate the total number of samples collected for each sample type or season

Number of samples from the skin of *A. altamirani* individuals (metamorphic and pre-metamorphic) and environmental samples (sediment and water)

amplicon sequence variants (ASVs) was obtained including all samples.

### *A. altamirani* skin microbiota differs from environmental bacterial communities

When comparing the number of unique and shared ASVs across sample types, we found that each sample type harbored many unique ASVs and only 2408 ASVs (3.32% of the total) were shared among the four sample types (Fig. 1A). Sediment and water samples were the samples with highest numbers of unique ASVs (20,031 and 9902 respectively), while metamorphic and pre-metamorphic samples had 8916, and 6650 unique ASVs respectively. Interestingly only 677 ASVs (1.16% of the total ASVs) were shared between metamorphic and pre-metamorphic salamanders.

Taxonomic results showed that, Burkholderiaceae was the most abundant bacterial family in all four sample types accounting for 32.6% and 51.1% of the relative abundance in metamorphic and pre-metamorphic samples respectively, and 14.6% and 40.8% of sediment and water respectively (Additional file 1: Figure S1). For the axolotl samples we found that Chitinophagaceae and Pseudomonadaceae varied in relative abundance according to host metamorphic status, with Chitinophagaceae showing a higher abundance in pre-metamorphic axolotls (metamorphic 2.7%/pre-metamorphic 27%) and Pseudomonadaceae being more abundant in metamorphic samples (metamorphic 18.1%/pre-metamorphic 6.4%).

Bacterial alpha diversity was significantly different between sample types (metamorphic, pre-metamorphic, sediment and water) as measured by ASV richness (Kruskal–Wallis (KW), $\chi 2 = 278.46$, $p$-value $< 0.001$, df $= 3$), Shannon index (KW, $\chi 2 = 276.28$, $p$-value $< 0.001$, df $= 3$) and Faith´s phylogenetic diversity (PD) (KW, $\chi 2 = 286.91$, $p$-value $< 0.001$, df $= 3$) (Fig. 1B). Post hoc pairwise comparisons for each alpha diversity index showed significant differences among all sample types (Additional file 2: Table S1) except for metamorphic salamanders and water in ASV richness (Wilcoxon, $p$-value $= 0.48$) and Shannon diversity index (Wilcoxon, $p$-value $= 0.66$). Sediment samples showed the highest alpha diversity values while pre-metamorphic salamanders always had the lowest values.

Bacterial community composition based on the weighted UniFrac distance matrix varied significantly among sample types (PERMANOVA, pseudo-F $= 64.76$, $p$-value $< 0.001$, df $= 3$) (Fig. 1C, Additional file 2: Table S2). Dispersion significantly differed among sample types according to the permutational test (PERMPUTEST, F $= 34.5$, $p$-value $= 0.001$, df $= 3$) (Fig. 1D, Additional file 2: Table S3).

### The skin bacterial composition of *A. altamirani* is mainly influenced by metamorphosis

Clear differences in skin bacterial alpha and beta diversity were found between metamorphic and pre-metamorphic salamanders (Fig. 1B, C, D). To look deeper into the bacterial taxa driving these differences we used an analysis of composition of microbiomes (ANCOM) which identified 45 bacterial families (out of 392 families in the axolotl skin samples) that were differentially abundant between metamorphic and pre-metamorphic samples (Fig. 2). Most of these bacterial families (40 out of 45) were enriched in metamorphic samples, being Verrucomicrobiaceae, Caulobacteraceae and Sphingomonadaceae the families with higher W values. In contrast, five bacterial families were enriched in pre-metamorphic samples with Burkholderiaceae, Chitinophagaceae being the families with higher W values.

We identified the core skin bacteria in metamorphic and pre-metamorphic *A. altamirani* axolotls, as those ASVs shared in $\geq 90\%$ of the samples on each specific morph. Four ASVs represented the bacterial core of metamorphic axolotls accounting for a cumulative relative abundance of 16.26% of the ASVs. Meanwhile, two ASVs represented the bacterial core of pre-metamorphic axolotls accounting for 45.78% of the relative abundance (Table 2).

We also identified the core bacteria of environmental samples (Additional file 2: Table S4) and we found that metamorphic axolotls shared two core ASVs with the water core and another one with the sediment core. Core bacteria of pre-metamorphic axolotls are not present in the core of environmental samples.

### Seasonality and location differentially influence skin bacterial diversity in metamorphic and pre-metamorphic axolotls

Physicochemical variables measured at each sampling location (pH, conductivity, dissolved oxygen, maximin, minimum mean and delta seasonal temperatures) varied significantly across seasons (MANOVA, Wilks $= 0.002$, $p$-value $< 0.001$, df $= 3$) and sampling locations (MANOVA, Wilks $= 0.0009$, $p$-value $< 0.001$, df $= 3$). While all physicochemical variables varied across seasons, dissolved oxygen was the only variable that did not vary between sampling locations (Additional file 2: Table S5).

Alpha PD of metamorphic axolotls varied significantly across seasons (KW, $\chi 2 = 13.69$, $p$-value $= 0.003$, df $= 3$) (Fig. 3A) and post-hoc pairwise comparisons showed that only the transition between winter-spring was significant (Wilcoxon, $p$-value $= 0.005$) (Additional file 2: Table S6). In contrast, PD of pre-metamorphic *A. altamirani*

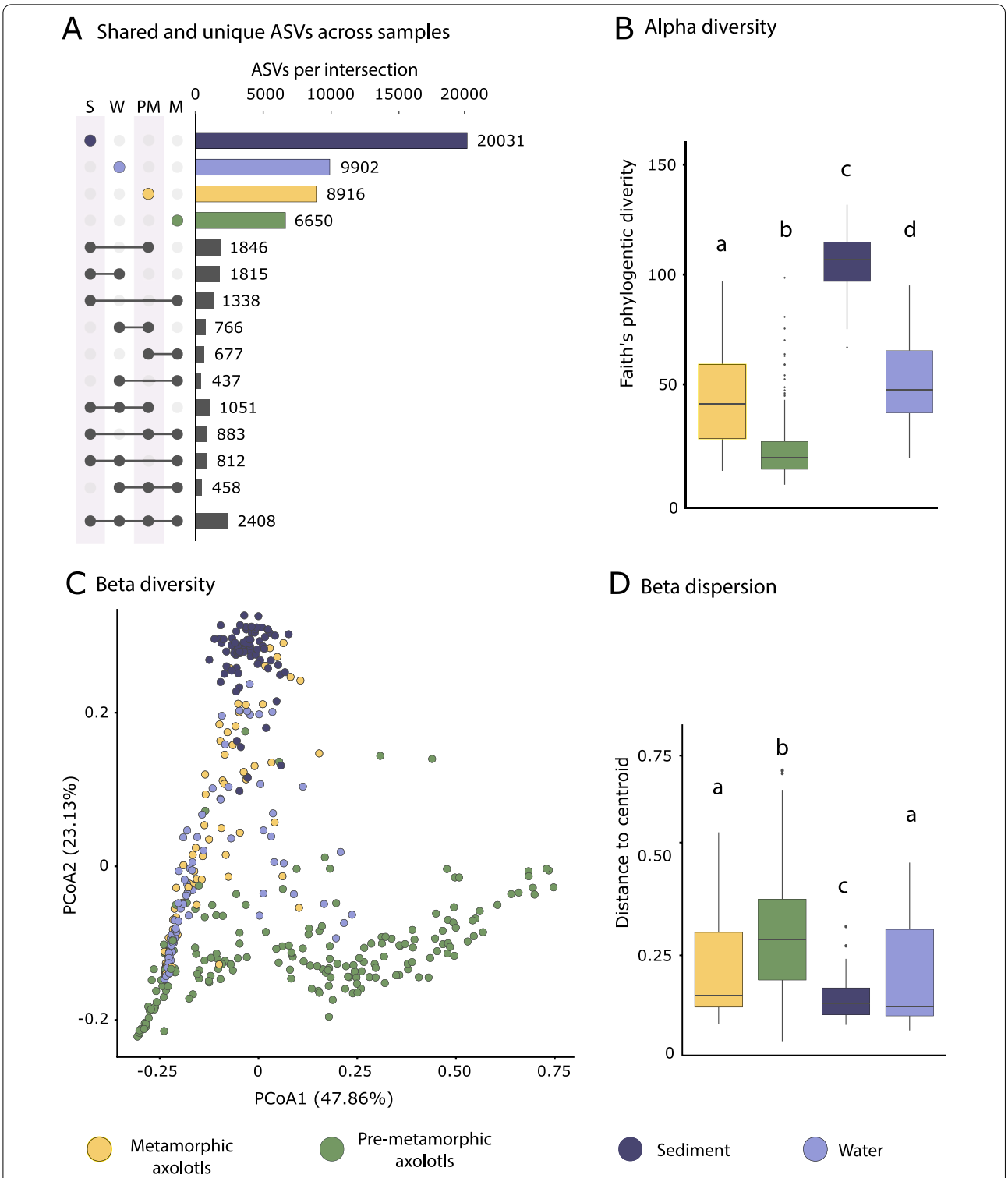


**Fig. 1** Bacterial diversity of *A. altamirani* skin and environmental samples. **A** Upset plot illustrating the number of unique and shared ASVs. Numbers aside the color bars indicate how many ASVs were present on each sample type (color bars) and shared between sample types (gray bars). **B** Alpha Faith's Phylogenetic diversity (PD) across sample types. **C** Principal coordinate analysis (PCoA) based on weighted UniFrac distances across sample types. **D** Beta dispersion using Analysis of multivariate homogeneity of groups dispersions. Letters a–d indicate statistically significant comparisons

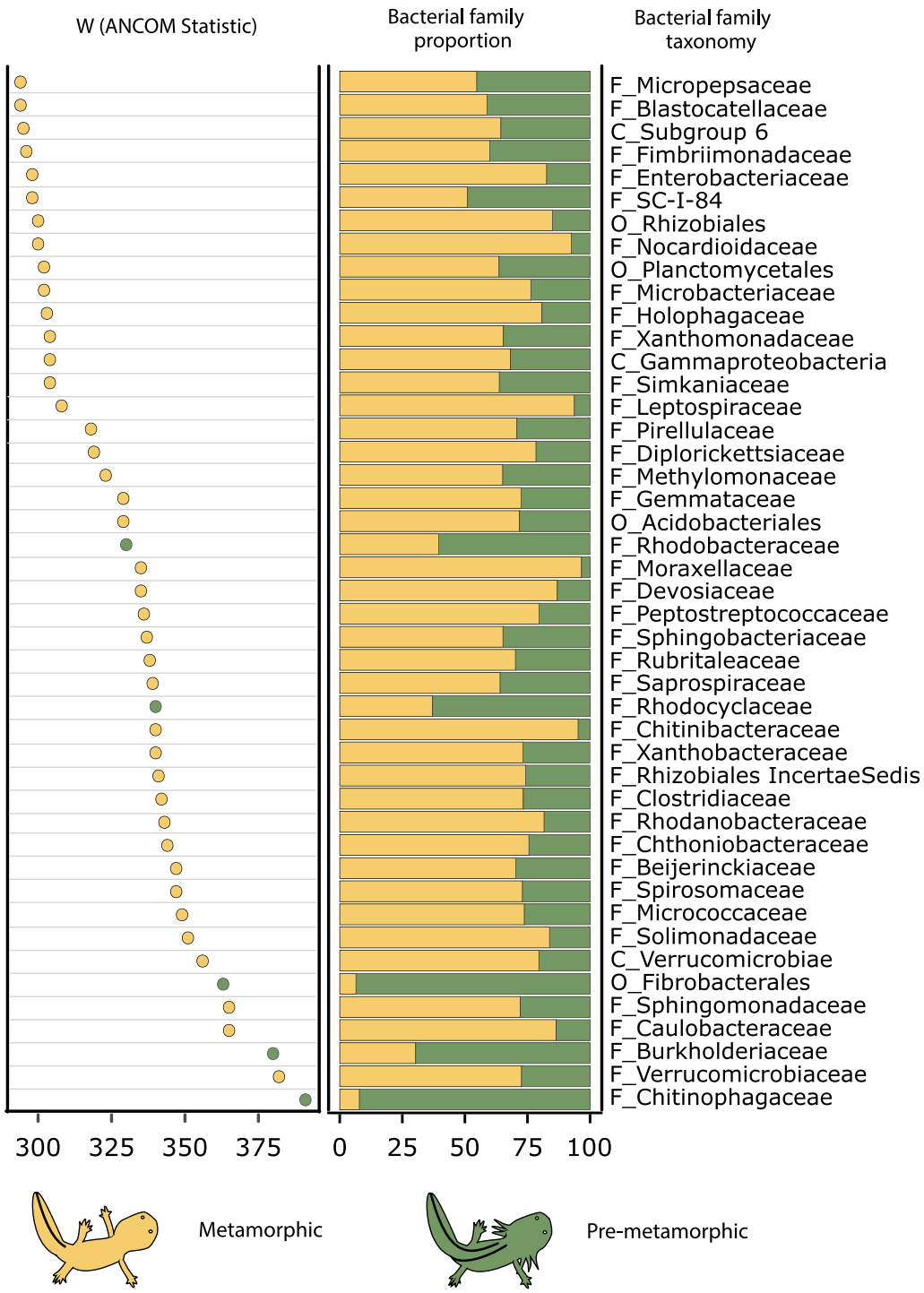


**Fig. 2** ANCOM results showing differentially abundant bacterial families between metamorphic and pre-metamorphic axolotls. Left panel shows ANCOM W values, the middle panel shows the relative proportion for each bacterial family, and the right panel shows the best taxonomic assignments according to the SILVA database at order (O), class (C) or family (F) level. Circles and bars are color-coded according to the host metamorphic status

**Table 2** Amplicon sequence variants (ASVs) defining the bacterial core of the microbiota of metamorphic and pre-metamorphic *A. altamirani*

| | ASV ID | Taxonomy at family level | Relative abundance | Persistence |
|---|---|---|---|---|
| Metamorphic | 9936daae333af6e517a9deb4b9e18ffa | Pseudomonadaceae | 13.81 | 95.12 |
| | 6d0c9d0395e6a2a7667eb0b07c17a275 | Burkholderiaceae | 1.40 | 97.56 |
| | 17d60505100c3cf44d4f9fad620d1636 | Pseudomonadaceae | 0.73 | 93.90 |
| | be8eb25874b4202cf98050dbadeeb7ce | Burkholderiaceae | 0.33 | 93.90 |
| Pre-metamorphic | 3c28f0caf9183357de05d1882a943f8e | Chitinophagaceae | 25.03 | 96.84 |
| | ed5a79897d0f82525c3854759d384c26 | Burkholderiaceae | 20.75 | 98.42 |

ASVs were considered part of the skin bacterial core if they were present in ≥ 90% of the samples of metamorphic or pre-metamorphic axolotls

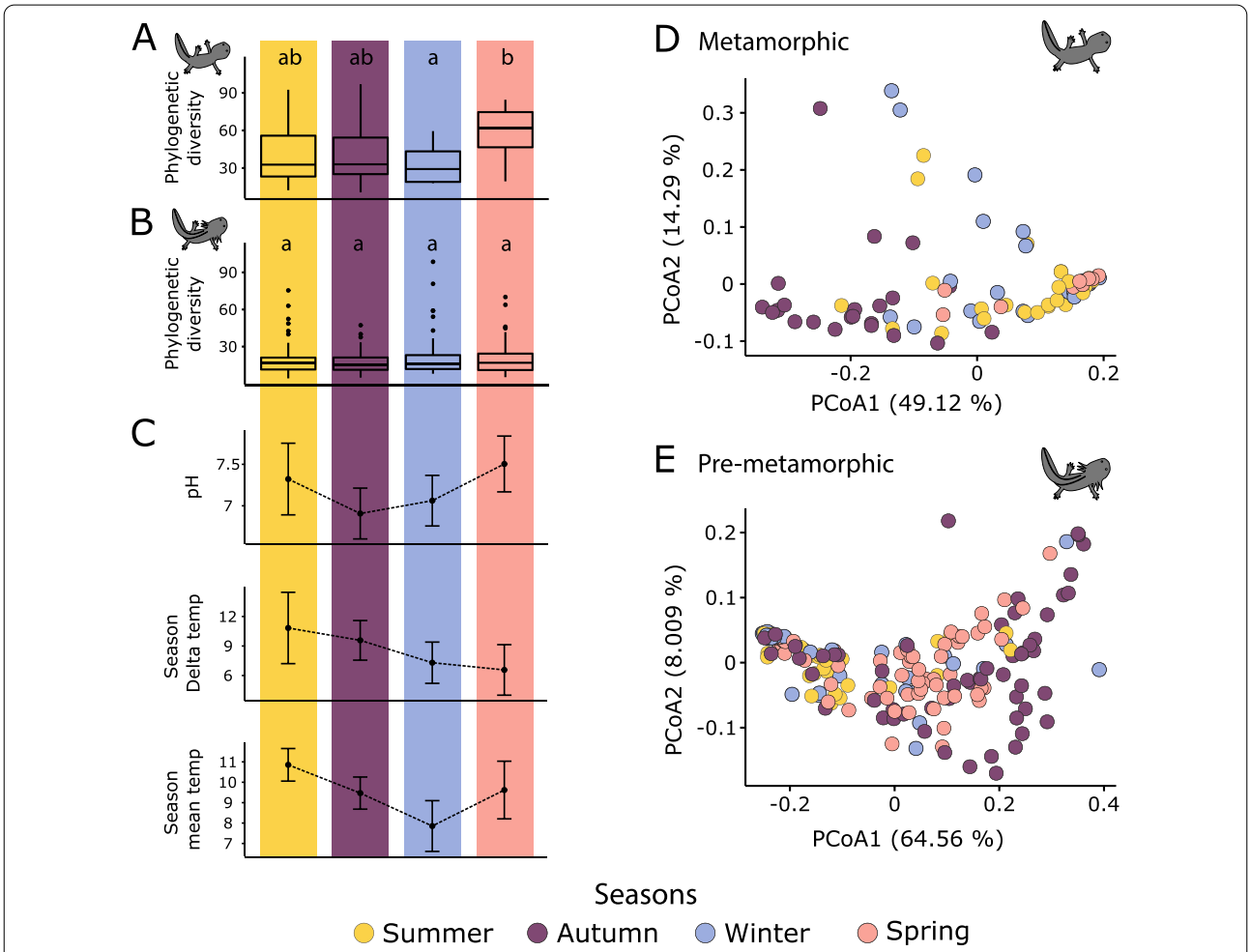


**Fig. 3** Seasonal influence over metamorphic and pre-metamorphic skin bacterial diversity. **A** Phylogenetic diversity (PD) across seasons in metamorphic samples. Letters a–d indicate statistically significant comparisons. **B** PD across seasons in pre-metamorphic samples. **C** Seasonal variation of pH, delta temperature and mean temperature of the stream water. **D** Principal coordinate analysis (PCoA) based on weighted UniFrac distances across seasons of metamorphic samples. **E** PCoA based on weighted UniFrac distances across seasons in pre-metamorphic samples. Circles in **D** and **E** panels are color-coded by season

(Fig. 3B) did not differ across consecutive seasons (KW, $\chi 2 = 0.21$, $p$-value = 0.97, df = 3) (Additional fiie 2: Table S6).

Additionally, we found that seasonality significantly influenced skin bacterial community composition (PERMANOVA, pseudo-F = 12.37, $p$-value < 0.001, df = 3) (Fig. 3D, Additional file 2: Table S7), but not dispersion (PERMUTEST, F = 1.4, $p$-value = 0.24, df = 3) (Additional file 2: Table S8) of metamorphic axolotls. Seasonality also influenced skin bacterial community composition (PERMANOVA, pseudo-F = 15.69, $p$-value < 0.001, df = 3) (Fig. 3E, Additional file 2: Table S9) of pre-metamorphic axolotls, in addition we found that dispersion significantly differed between seasons (BETADISPER, F = 2.7, $p$-value = 0.038, df = 3) (Additional file 2: Table S10). Specifically, pairwise PERMANOVAs showed that metamorphic samples differed between winter-spring seasons (PERMANOVA, pseudo-F = 14.92, $p$-value = 0.001, df = 1), while pre-metamorphic skin microbiota differed between autumn–winter (PERMANOVA, pseudo-F = 13.47, $p$-value < 0.001, df = 1) and winter-spring seasons (PERMANOVA, pseudo-F = 12.61, $p$-value < 0.001, df = 1).

Three bacterial families were identified by ANCOM as differentially abundant in metamorphic samples between winter-spring seasons (Fig. 4). In the case of pre-metamorphic individuals, ANCOM identified three bacterial families that were differentially abundant between autumn–winter and eleven families differentially abundant between winter-spring (Fig. 4). Pseudomonadaceae, Aquaspirillaceae and Shewanellaceae were significantly enriched in both metamorphic and pre-metamorphic axolotls during winter and spring seasons. However, Pseudomonadaceae was more abundant in metamorphic axolotls during spring and more abundant in pre-metamorphic axolotls during winter. Shewanellaceae was more abundant in winter, and Aquaspirillaceae was present in the winter and completely absent in the spring for both metamorphic and pre-metamorphic axolotls.

When analyzing the effect of location in the skin bacterial diversity, we found that PD of metamorphic samples differed significantly between sampling locations (KW, $\chi 2 = 9.69$, $p$-value = 0.02, df = 3), however post hoc paired test showed that PD only differed significantly between sites 2 and 3 (Additional file 1: Figure S2A). Bacterial PD of pre-metamorphic samples also varied significantly between sampling locations (KW, $\chi 2 = 40.9$, $p$-value = 6.71e-9, df = 3). Post hoc test showed that most pairwise comparisons were significant with the exception of sites 1 and 3 and sites 2 and 3 (Additional file 1: Figure S2C, Additional file 2: Table S11). Skin bacterial community composition was also influenced by sampling location in metamorphic (PERMANOVA, pseudo-F = 2.71, $p$-value = 0.006, df = 3) and pre-metamorphic samples

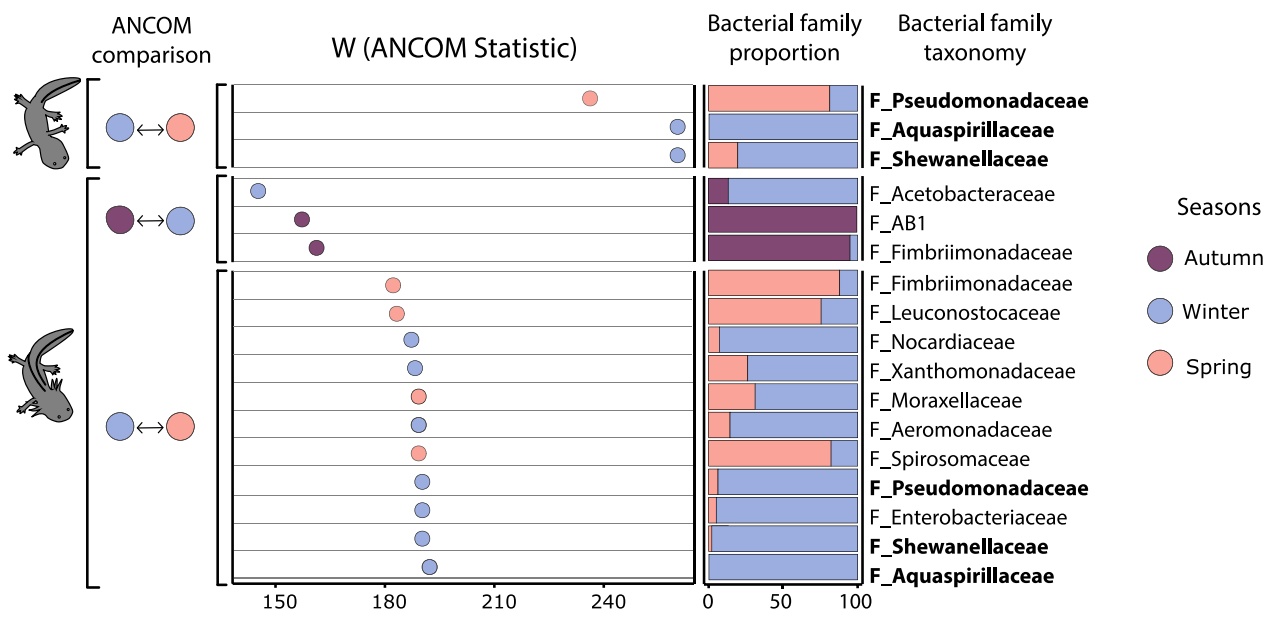


**Fig. 4** ANCOM results showing differentially abundant bacterial families in metamorphic and pre-metamorphic axolotls across consecutive seasons: autumn to winter seasons in pre-metamorphic axolotls, and winter to spring seasons for metamorphic and pre-metamorphic axolotls. From left to right: ANCOM comparisons color-coded by season, ANCOM W values, the relative bacterial family proportion and the best taxonomic assignment according to SILVA at order (O), class (C) or family (F) level. Circles and bars are color-coded by season. Shared bacterial families between metamorphic and pre-metamorphic axolotls between winter and spring seasons are shown in bold

(PERMANOVA, pseudo-F = 31.34, *p*-value = 0.001, df = 3) (Additional file 1: Figure S2B, D). Pairwise comparisons showed that bacterial community composition only differed between sites 2 and 3 in metamorphic axolotls (Additional file 2: Table S12), while community composition differed between all sampling locations for pre-metamorphic samples (Additional file 2: Table S13). Interestingly dispersion did not vary across localities for metamorphic axolotls (PERMUTEST, F = 0.29, *p*-value = 0.8, df = 3) (Additional file 2: Table S14), but we found significant differences in dispersion for pre-metamorphic axolotls (PERMUTEST, F = 6.68, *p*-value = 0.01, df = 3) (Additional file 2: Table S15).

### Biotic and abiotic factors influence the skin bacterial community structure of *A. altamirani*

Our results showed that bacterial community composition of *A. altamirani* skin is influenced by seasonality and location. To assess the specific influence of all the biotic and abiotic factors measured in this study we performed a distance-based Redundancy Analysis (dbRDA) on the skin bacterial beta diversity. After forward model selection, that incorporates all the variables measured, only the biotic and abiotic factors that better explained community composition were included in the dbRDA regression model: host metamorphic status, host weight, pH, dissolved oxygen, conductivity, mean temperature, season delta temperature (difference between the maximum and minimum seasonal temperature) and site elevation.

The dbRDA calculated eight canonical components for the PCA, however anova.cca (by = axis) showed that only four of these canonical components were statistically significant. These four statistically significant canonical axes explained 26.47% of the variation in the weighted UniFrac distance matrix (Fig. 5, Additional file 2: Table S16). Permutational analysis (anova.cca, by = terms) over each variable in the model showed that the metamorphic status of the host (PERMANOVA, pseudo-F = 39.1, *p*-value = 0.001) had the greatest effect-size over the variation, followed by seasonal delta temperature (PERMANOVA, pseudo-F = 19.8, *p*-value = 0.001), pH (PERMANOVA, pseudo-F = 15.85, *p*-value = 0.001) and seasonal mean temperature (PERMANOVA, pseudo-F = 12.05, *p*-value = 0.001) (Fig. 3C, Table 3).

### Skin bacterial diversity of *A. altamirani* is not influenced by Bd infection status but specific bacterial taxa abundances correlate with infection intensity

Pathogen prevalence and infection intensity were conducted by Basanta et al. [49]. Briefly we found a Bd prevalence of 70.3% across all samples specifically 54 (out of 85) metamorphic and 142 (out of 194) pre-metamorphic axolotls resulted positive for Bd infection.

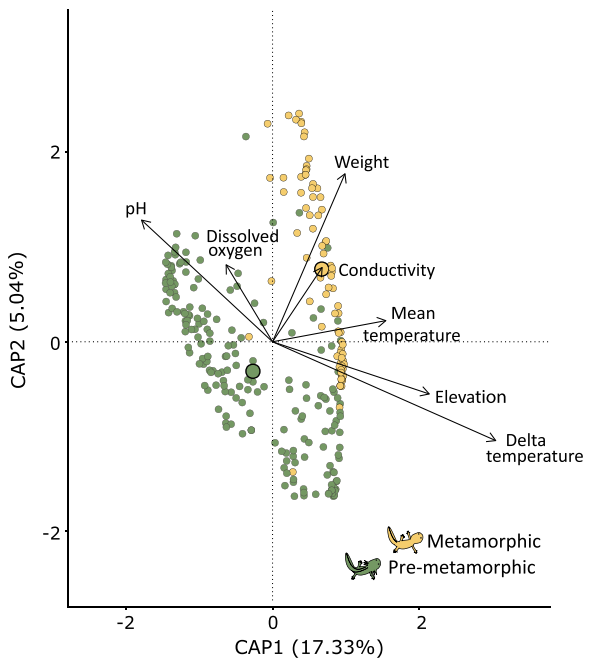


**Fig. 5** Distance based redundancy analysis of *A. altamirani* skin bacterial communities. Distances in the PCA are based on a weighted UniFrac distance matrix. Vector directions indicate the type of correlation of each predictor variable. Distance of each sample with respect to vectors highlight the weight of the correlation with a given predictor variable. Non quantitative variables are represented as centroids (outlined circles larger). Circles are color-coded by host metamorphic status

**Table 3** Permutational like ANOVA results of each variable introduced in the dbRDA regression model

| | F | *p*-value |
|---|---|---|
| Developmental stage | 39.121 | **0.001** |
| Delta temperature | 19.889 | **0.001** |
| pH | 15.854 | **0.001** |
| Mean temperature | 12.053 | **0.001** |
| Elevation | 5.334 | **0.001** |
| Dissolved oxygen | 4.478 | **0.002** |
| Conductivity | 3.470 | **0.005** |
| Weight | 2.604 | **0.018** |

Columns indicate: F statistic, p-values calculated by Permutational like ANOVA for each variable

Numbers in bold indicate significant *p*-value

Alpha PD did not differ between infected and non-infected samples in both metamorphic (KW, $\chi 2 = 0.09$, *p*-value = 0.76, df = 1) (Fig. 6A) and pre-metamorphic (KW, $\chi 2 = 0.51$, *p*-value = 0.47, df = 1) *A. altamirani* samples (Fig. 6C). Additionally, beta diversity based on the weighted UniFrac distance matrix did not vary between

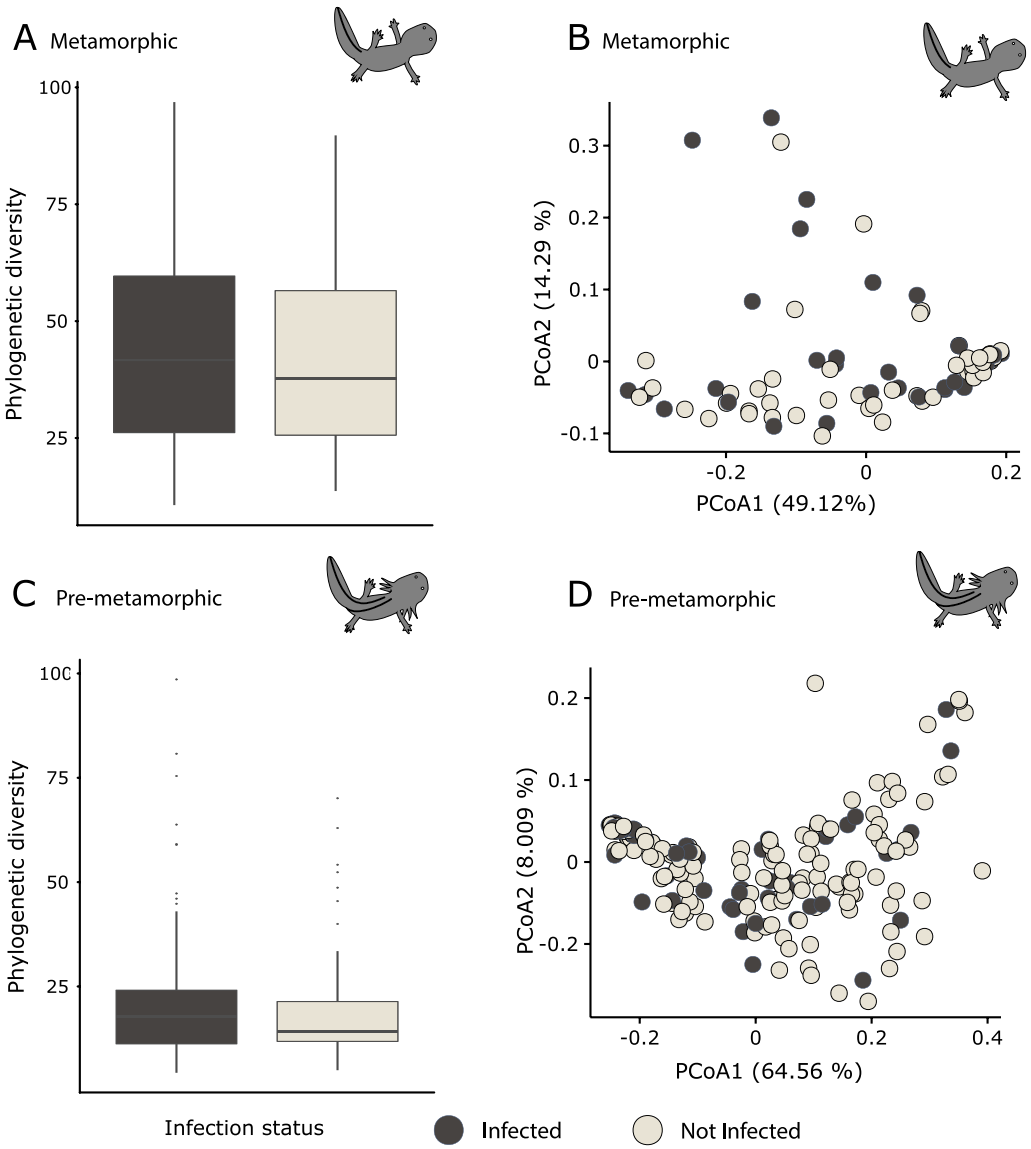


**Fig. 6** *A. altamirani* skin bacterial diversity with respect to Bd infection status. **A** Alpha phylogenetic diversity (PD) between infected and non-infected in metamorphic axolotls. **B** Principal coordinate analysis (PCoA) based on weighted UniFrac distances in infected vs non-infected metamorphic axolotls. **C** PD between infected and non-infected in pre-metamorphic axolotls. **D** PCoA based on weighted UniFrac distances in infected vs non-infected in pre-metamorphic axolotls. Circles are color-coded by Bd infection status

infected and non-infected samples for metamorphic (PERMANOVA, pseudo-F = 1.37, $p$-value = 0.19, df = 1) (Fig. 6B) and pre-metamorphic axolotls (PERMANOVA, pseudo-F = 2.45, $p$-value = 0.08, df = 1) (Fig. 6D).

Even though alpha and beta diversity did not vary according to Bd infection status, Kendall's correlation test showed that the relative abundance of 139 and 129 ASV present in infected metamorphic and pre-metamorphic samples respectively, significantly correlated with pathogen infection loads (Additional file 1: Figure S3). Specifically, 116 (out of 139) and 52 (out of 128) bacterial ASVs had positive correlations with pathogen infection loads in metamorphic and pre-metamorphic samples, respectively.

Almost all the ASVs that correlated with pathogen load in metamorphic samples had low relative abundances ranging from 0.001 to 0.67% (Additional file 1: Figure S3A), while in pre-metamorphic samples, correlated ASVs ranged from 0.001 to 28.5% (Additional file 1: Figure S3B). Among the ASVs that correlated with pathogen

infection intensities, twelve of them were shared between metamorphic and pre-metamorphic axolotls and half of these ASVs had a differential type of correlation between morphs (e.g., positive in metamorphic and negative in pre-metamorphic) (Additional file 2: Table S17). Two of these ASVs were classified as members of the Chitinophagaceae family and both were positively correlated with Bd infection intensity in metamorphic axolotls and negatively correlated in pre-metamorphic axolotls.

## Discussion

The aim of this study was to evaluate the influence of metamorphosis, seasonality and pathogen presence over the skin microbiota of the axolotl *A. altamirani*. Since this is the first study exploring the skin microbiota of *A. altamirani*, we also evaluated if skin bacterial diversity differed from environmental bacterial communities of the streams where this species inhabits.

Consistent with previous studies showing differences between amphibian skin microbiota and their surrounding environmental bacterial communities [20, 50], we found that *A. altamirani* skin bacterial microbiota significantly differed from environmental samples, and that a great portion of the ASVs were unique to each sample type (ie, sediment, water, metamorphic and pre-metamorphic axolotls, Fig. 1A), supporting the idea that the amphibian skin hosts a distinctive bacterial repertoire compared to the environmental samples [18, 35, 37, 43, 51]. Our results highlight differences on the microbial diversity between the bacterial communities of the skin and the environment. We identified differences in alpha and beta diversity compared to water and sediment. Also, we identified core bacteria that were unique to axolotls or were clearly enriched on their skins compared to the environment.

Several studies have shown that amphibian skin microbiota varies significantly across host development [26, 27, 41]. These studies focused on amphibian species that transition from an aquatic larval stage to a terrestrial adult stage [22, 36, 37, 52], making it difficult to tease apart the effects of host development stage and habitat conditions on skin microbial diversity [17, 18]. For species where adult and larval stages coexist in the same aquatic environment (i.e., newts), host developmental stage has had contrasting results in different species; for example adult and larvae of *Lissotriton boscai* showed clear differences in skin bacterial community composition, however this pattern was not observed in *Triturus marmoratus* [52].

In this study, we evaluated the influence of metamorphosis over skin bacterial diversity on a paedomorphic salamander species (axolotl) in which metamorphic and pre-metamorphic stages coexist in permanent streams along their life cycle [47, 53]. Our results showed that *A. altamirani* skin bacterial communities are strongly shaped by metamorphosis. Specifically, we found that pre-metamorphic individuals harbor less diverse and more variable skin bacterial communities compared to metamorphic individuals.

These differences could be explained by differences in skin mucus composition, immune response, or gene expression before and after metamorphosis as it has been proposed that mucus chemical composition (e.g., production of antimicrobial peptides) play a critical role in shaping the skin microbiota as well as in defense against pathogens [30, 54, 55]. Antimicrobial peptide repertory of the skin changes through development in some frog species [56], and some bacteria can induce the synthesis of specific antimicrobial peptides [51]. In addition, the number and distribution of Leydig cells, which have been associated with the secretion of mucus [57], changes across urodele development [58, 59].

In addition, the core microbiota analysis and ANCOM results shown here highlighted differences in composition between metamorphic and pre-metamorphic axolotls. Specifically, pre-metamorphic skin microbiota was composed by fewer core members and had less differentially abundant bacterial ASVs when compared to metamorphic skin microbiota. It is interesting to highlight that both analyses identified that families Chitinophagaceae and Burkholderiaceae were enriched in pre-metamorphic samples, specially two ASVs from these families conform the core microbiota of pre-metamorphic axolotls which account 45.7% of relative abundance in these samples.

Bacteria from the family Chitinophagaceae and Burkholderiaceae have been isolated from other amphibian hosts and have shown the ability to inhibit Bd [60–62]. Moreover, some members of the Chitinophagaceae family such as *Chitinophaga pinenis* can degrade chitin [63] which is a main component of fungal cell wall. In our study, the high prevalence of these bacterial families on the skin of *A. altamirani* could suggest that these bacteria play a protective role against chytrid pathogens.

Temporal and spatial dynamics of amphibian skin microbiota have been linked to variation in environmental factors such as temperature, precipitation or elevation [25, 26, 34, 35, 52]. Specifically, temperature fluctuations over short periods of time [22] and seasonal variations (dry–wet) [38] have been linked to differences in bacterial skin diversity on amphibians inhabiting aquatic environments. Our results showed that seasonal variation of temperature (delta temperature and mean temperature), pH, conductivity, and dissolved oxygen influence axolotl skin bacterial diversity.

Previous studies have shown that spatial variation has an influence on skin bacterial diversity of terrestrial

salamanders [14, 41, 64]; for example populations of *Ensatina eschscholtzii* from different geographic locations vary in bacterial community composition [15]. In this study we found that sampling location significantly influences skin bacterial diversity, and this effect is stronger in pre-metamorphic axolotls. Considering that the main source of diversity of the skin microbiota are the environmental microbial communities and that they vary in response to environmental variation [65–67] it is likely that the skin microbiota reflect to some extent the environmental variations across localities as seen in the case of pre-metamorphic axolotls. It has been shown that skin bacterial diversity vary in response to precipitation [19, 23] temperature [22] or elevation gradients [24, 41, 42]. However, genetic differences across populations could also explain some of our results, since a previous study showed that *A. altamirani* populations of sites 2 and 3 are genetically differentiated [68]. Additional work is needed to tease apart the effects of environment and host genetics on the skin microbial diversity of *A. altamirani*.

Stability as a characteristic of an ecological community could be defined as the response to disturbance, comprising resilience and resistance against external disturbances [69, 70]. It has been shown that the stability of the amphibian skin microbiota can change after the experimental exposure to fungal [71] and viral pathogens [72]. Also, it has been shown that skin microbiomes with higher diversity are less stable to a pathogen induced disturbance [72].

Environmental variation across seasons is a different kind of perturbation for microbial communities, and it has been shown that seasonality influences microbial communities of soil [73–75], water [76, 77] and host-associated microbiomes [78, 79]. We found that skin bacterial communities of *A. altamirani* vary across seasons, particularly in pre-metamorphic axolotls which have a lower bacterial diversity compared to metamorphic axolotls.

Together our results suggest that more diverse bacterial communities (as the ones present in metamorphic axolotls) allow for a more stable microbiota that could be either more resistant or resilient to the environmental variation. Similar patterns of diversity—stability trough time have been described in populations of *Rana sierrae* [39]. Further studies are needed to evaluate if these patterns of stability across seasons influence the function of the skin bacterial communities of *A. altamirani*, as it has been shown that less stable bacterial communities show less functional redundancy [80].

Disruption of the skin microbiota following Bd infections has been previously documented in naive amphibian populations before and after Bd infection [20, 21], and in populations with different pathogen intensities where Bd seems to be present in an enzootic stage [23, 44]. Even when Bd was highly prevalent in *A. altamirani* populations [49], we did not find any significant influence of Bd presence over the skin bacterial diversity.

Of the 279 axolotls sampled only two individuals exhibited clear signs of infection [81] (lethargy, skin ulceration and extreme skin sloughing) and they died soon after we sampled them. Apart from these two cases, the remaining individuals showed no signs of infection. These observations suggest that this population is able to tolerate Bd infection. Further studies testing the survival rates of *A. altamirani* against Bd are needed to elucidate if this species is resistant or susceptible to chytridiomycosis.

It has been shown that Bd presence have contrasting effects over skin microbiota diversity inducing changes in skin microbiota composition following infection [21, 23, 71] or not influencing diversity of skin microbial communities [42, 44] as we found in this study. However, it also has been shown that relative abundances of some bacterial members of the skin microbiota correlates with chytrid infection intensity [19, 44, 45] and its suggested that according to the type of correlation these groups could act as anti Bd bacteria [19]. We identified several bacteria with positive and negative correlations with Bd infection intensities and most of these ASVs exhibited low relative abundances.

Observations in several amphibian species indicate that certain bacteria with properties such as biofilm formation [82] or putative inhibitory ability [55] are positively or negatively corelated with a decrease of Bd prevalence. Thus, we expect that bacteria with negative correlations to infection intensity could be important in the defense against Bd in *A. altamirani*. However, these Bd-inhibitory bacteria exhibited reduced abundances over the amphibian skin [83, 84].

Inhibitory potential against Bd has been described for several bacterial isolates mainly form Burkholderiaceae, Yersiniceae, Pseudomonadaceae or Xanthomondaceae families [60, 85–88], We found that Burkholderiaceae and Chitinophagaceae were highly abundant over *A. altamirani* skin. In line with our results, high abundance of Burkholderiaceae in *Anaxyrus boreas* skin microbiota correlated with reduced fungal presence over the skin during early life stages [27]. Additionally, populations of *R. sierrae* with contrasting Bd loads (high vs low) exhibited differential abundances of Burkholderiaceae [21, 44]. In the case of Chitinophagaceae little is known about their inhibitory ability against Bd with only few isolates considered as Bd-inhibitory strains [25], and further work is needed to elucidate if members of this bacterial family present on *A. altamirani* skin display inhibitory functions against Bd.

## Conclusion

Our results show that host metamorphic status is a major determinant of *A. altamirani*, influencing diversity and structure of skin symbiotic bacterial communities. To our knowledge this study is the first to address how the effects of environmental variation over the skin bacterial communities are dependent on the amphibian developmental stage; we demonstrate that seasonal environmental variation significantly influences bacterial skin diversity of *A. altamirani*, and that metamorphic and pre-metamorphic axolotls respond differently to environmental variation. Despite a growing body of literature suggesting that Bd influences skin bacterial diversity we did not find such an effect. Nonetheless, we found that particular bacterial taxa are likely interacting with Bd. Further studies using metagenomics and cultivation techniques could elucidate if changes in skin microbiota across development and across seasons are reflecting functional differences regarding Bd inhibition or other host symbiotic traits [89, 90].

## Methods

### Sample collection

Skin samples were collected during four sampling periods at three-month intervals (July 2019, October 2019, January 2020, and April 2020) spanning all the seasons of a whole year at four localities at La Sierra de Cruces, Estado de México, México (Table 1, Additional file 2: Table S18). Individuals of *A. altamirani* were captured at each location using dip nets and held individually in sterile plastic containers filled with stream water until swabbing. Sampling occurred for three consecutive hours across a 150 m transect along each stream. Each captured salamander was manipulated with sterile nitrile gloves, rinsed with 25 ml of sterile deionized water to remove transient microorganisms from the skin and swabbed 30 times (five times in their ventral and dorsal surface each and five times in each limb joint) using sterile rayon swabs (MWE, Corsham UK). Swabs were stored in 1.5 ml microcentrifuge tubes containing 170 µl of DNA/RNA Shield (Zymo Research, Irvine, USA) and kept at 4 °C during field work. Once in the laboratory tubes were stored at − 80 °C until processing. Immediately after swabbing morphometric measurements of weight, tail and body length were registered for each individual. Once all axolotls were swabbed and measured, they were released at the same site of capture. Sampling was approved by Subsecretaría de Gestión para la Protección Ambiental under the permit number: SGPA/DGVS/5673/19.

For the purposes of this work, we classified axolotl samples as metamorphic and pre-metamorphic according to the presence or absence of gills as reported previously [59]. Recognizing that gilled individuals of *A. altamirani* could be either juvenile or paedomorphic adults, we classified non-gilled axolotls as metamorphic and gilled axolotls as pre-metamorphic respectively in order to evaluate the effect of the metamorphic status of the host.

Additionally, five samples of sediment and water were collected at each location in all sampling periods. Water samples were obtained by submerging a sterile rayon swab at approximately 20 cm deep inside water for 10 s, and sediment samples were obtained by submerging swabs inside the bottom sediment of the stream for 10 s [50].

### Environmental characterization

Stream water temperature was recorded at 1 h intervals during one year at each sampling location using Onset HOBO dataloggers (Onset Computer Corporation, Bourne, USA) from June 2019 to April 2020. Additionally, pH, dissolved oxygen and conductivity of the water was registered using a HANNA multiparameter HI98194 (HANNA Instruments, USA) during each sampling. Measurements were taken at each location in triplicate across 10 m transects. To evaluate if these physicochemical variables vary between seasons and sampling location, we applied a two-way MANOVA test in R (v 4.0.2).

### DNA extraction and sequencing

Amplicon libraries of the 16S rRNA gene spanning the V4 region were constructed using 515F/806R primers following the Earth Microbiome Project standard protocol (www.earthmicrobiome.org) and previously published studies [50, 91]. In brief, DNA was extracted from skin and environmental swabs using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, USA) following manufacturer instructions with an initial lysozyme incubation step at 37° for 1 h. Samples were PCR amplified in triplicate plus one negative control per sample, PCR products and negative controls were verified in 1% agarose gels, and PCR products were pooled in one tube per sample. Pools were quantified using a Qubit 4.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, USA), samples were pooled in two amplicon libraries at a concentration of 240 ng per sample (221 and 217 samples each). Each pool was cleaned using the QIAquick PCR clean up kit (Qiagen, Valencia, USA). 16S amplicon libraries were sequenced in two sequencing runs (250 single end) using v2 Illumina chemistry at Dana-Farber Cancer Institute of Harvard University.

### Bioinformatic pipeline

Sequences were processed using Quantitative Insights Into Microbial Ecology (QIIME v2-2020.2) [92]. A total

of 8,434,775 and 8,821,621 demultiplexed raw sequences were obtained from the sequencing facility for each sequencing run respectively. Prior to quality control primers were trimmed from the sequences using the cutadapt plugin in Qiime2 then sequences were quality filtered and denoised independently for each run using the DADA2 plugin to obtain two single feature table. Feature tables obtained for each sequencing run were merged to generate a final Amplicon Sequence Variant (ASV) table containing 14,415,727 reads with a mean read depth of 32,900 reads per sample.

A phylogenetic tree was generated using the representative sequences of the ASV table using the q2-phylogeny plugin which first uses mafft to perform sequence alignment and then generate a phylogeny using FastTree. Samples were rarefied at 10,000 reads per sample according to observed ASV rarefaction curves in order to preserve the largest number of samples and sequences. After denoising and rarefaction at 10,000 reads per sample, seven axolotl (out of 279, three from metamorphic and four from pre-metamorphic) and eight environmental (out of 159, 3 from sediment and five from water) samples were discarded due to low read counts (< 10,000 reads per sample). The rarefied table containing 10,000 reads per sample was used for all further analyses including to calculate alpha and beta diversity metrics using the q2-diversity plugin. Taxonomy was assigned using a naive Bayesian classifier pre-trained for the V4 region (515F/806R 16 s rRNA) on the SILVA 132 99% database [93].

#### Microbial diversity and composition analyses

Statistical analyses for alpha and beta diversity were carried out using the rarefied table at 10,000 sequences per sample; these analyses were computed in R (v 4.0.2) unless otherwise stated. We first perform Kruskal–Wallis (KW) and post hoc Wilcoxon ranks sum test were used to determine differences in alpha diversity (Shannon, Faith's Phylogenetic Diversity (PD) and ASV richness) between sample types (metamorphic, pre-metamorphic, sediment, and water. In addition we perform KW and post hoc Wilcoxon ranks sum test to evaluate the influence of seasonality, sampling location and Bd infection status over the skin microbiota of metamorphic and pre-metamorphic axolotls individually.

Beta diversity was evaluated using a weighted UniFrac distance matrix generated using the rarefied table at 10,000 sequences per sample to determine differences in bacterial community structure across sample types. In addition, we generated two independent weighted UniFrac distance matrices for metamorphic and pre-metamorphic axolotls to evaluate the influence of seasonality, sampling location and Bd infection status. Statistical comparisons were conducted with permutational multivariate analyses (PERMANOVA) using the q2-diversity plugin in Qiime2 (v 2020.2). Beta diversity dispersion was calculated from the each weighted UniFrac distance matrix using the function betadisper in the vegan package [94], and then we applied PERMUTEST based on 999 permutations to identify significant differences for dispersion, specifically we evaluate dispersion between sample types, as well between seasonality and sampling locations for the metamorphic and pre-metamorphic distance matrices.

ANCOM [95] was used to identify bacterial families that were differentially abundant between metamorphic and pre-metamorphic salamanders and between samples from consecutive seasons (summer-autumn, autumn–winter, winter-spring). Prior to analysis low abundant ASVs (< 50 reads) were filtered out and then we collapsed all ASVs at family level, ANCOM was performed using the q2-composition plugin in Qiime2. Briefly, ANCOM applies a centered log ratio transformation on the relative abundance of each bacterial family and tests the null hypothesis that mean log absolute abundance of each family does not differ between sample types. An internal statistic (W) is calculated each time a taxon rejects this null hypothesis, then ANCOM generates an empirical distribution using W values in order to test which taxon in this case which bacterial families are differentially abundant between samples. ANCOM between consecutive seasons was only applied if PERMANOVA results showed significant differences between consecutive seasons (winter-spring for metamorphic salamanders and autumn–winter and winter-spring for pre-metamorphic salamanders).

Core microbiome was calculated independently for metamorphic and pre-metamorphic axolotls using the feature-table plugin in Qiime2. In brief, we generated four feature tables that contain all the ASVs present in each sample type (metamorphic, pre-metamorphic, sediment and water samples). Then we identify all the ASVs present in ≥ 90% of the samples of each sample type, using the core-features function.

Additionally, correlations between the relative abundance of each ASV of the infected samples and Bd infection intensities were calculated with Kendall rank correlation coefficient correcting for multiple comparisons (Benjamini-Hochberg) using cor.test function of the stats package in R [96]. To generate graphics for all the results Qiime2 artifacts were imported to R using the package qiime2R [97], then figures were generated using packages ggplot2 [98, 99], Fantaxtic [100] and UpSetR [101], color pallet of the figures are colorblind friendly and were selected from the MetBrewer package in R [102].

### Biotic and abiotic factors influencing the skin microbial structure

In order to explore the specific influence of biotic (developmental stage, weight, tail length, snout vent length, Bd presence and Bd infection intensity) and abiotic factors (pH, conductivity, dissolved oxygen, mean season temperature, delta season temperature, and elevation) on skin bacterial community composition, we applied a distance-based redundancy analysis (dbRDA) on the weighted UniFrac distance matrix using the capscale function of the vegan package [94]. dbRDA is a canonical ordination method that applies multiple linear regression to a distance matrix and then computes a principal component analysis (PCA) [103]. Prior to analyses noncategorical biotic and abiotic variables were z-scored to control for differences in magnitudes between factors. The ordistep function of the vegan package [94] was used for model selection in both directions with 999 permutations to select the best regression model. Once the dbRDa was obtained anova.cca function was used to perform an ANOVA like permutation test to evaluate the significance of each calculated canonical axis (anova.cca, by = axix) and the specific significance of each factor in the regression model (anova.cca, by = terms).

#### Abbreviations

ANCOM: Analysis of composition of microbiomes; ASV: Amplicon sequence variant; Bd: *Batrachochytrium dendrobatidis*; KW: Kruskal–Wallis; dbRDA: Distance based redundancy analysis; MANOVA: Multivariate analysis of variance; PD: Phylogenetic diversity; PERMANOVA: Permutational multivariate analysis of variance; PERMUTEST: Permutation-based test of multivariate homogeneity of group dispersions.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-022-00215-7.

**Additional file 1.** Supplementary Figures.

**Additional file 2.** Supplementary Tables.

#### Acknowledgements

Emanuel. Martinez-Ugalde is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM), and has received a Consejo Nacional de Ciencia y Tecnología (CONACyT) fellowship (781100). We thank Paola Palacios, Juan Pablo Pérez, Honoria Rueda-González, Inés Rosas-Chávez, Luis Zárate, and Bienes Comunales of Santiago Tlazala for the field assistance and Dr. Rafael Peña Miller for the assistance in the preparation of field data loggers housing. We thank Molly Bletz and Doug Woodhams for providing barcoded primers used during library construction.

#### Author contributions

ER conceptualized and designed the study. ER and VAA secured the funding. All authors contributed to sample collection. EM-U performed the molecular work and bioinformatic analysis. EM-U and ER wrote the manuscript. All authors participated in the improvement of the manuscript.

#### Funding

This work was supported by PAPIIT/UNAM Grant No. IA201419 granted to ER.

#### Availability of data and materials

All 16 s rRNA gene raw data in this study are publicly available at the NCBI SRA under BioProject PRJNA819099. Sample metadata, output data of DADA2, R and Qiime2 scripts for analysis and figures included in this manuscript are available at https://github.com/EmanuelMartinez-Ugalde/A.-altamirani-16S-skin-microbiota.

## Declarations

#### Ethics approval and consent to participate

Our research was approved by the ethical standards of Universidad Nacional Autónoma de México, additionally capture and sampling of ***A. altamirani*** was approved by Subsecretaría de Gestión para la Protección Ambiental under the permit number: SGPA/DGVS/5673/19.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

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Received: 12 April 2022 Accepted: 16 October 2022
Published online: 12 December 2022

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## Publisher's Note

**Supplementary Figures:**

# The skin microbiota of the axolotl *Ambystoma altamirani* is highly influenced by metamorphosis and seasonality but not by pathogen infection.

**Emanuel Martínez-Ugalde[1], Víctor D. Ávila-Akerberg[2], Tanya M. González Martínez[3], Montserrat Vázquez Trejo[3], Dalia Zavala Hernández[3], Sara Anaya Morales[1], Eria A. Rebollar[1].**

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**Figure S1.** Taxonomic composition of *A. altamirani* skin samples and environmental samples: Metamorphic (M), pre-metamorphic (PM), sediment (S) and water samples (W). Stacked bar plots show the average relative abundances of the ten most abundant bacterial families. Sample size is shown below each bar.

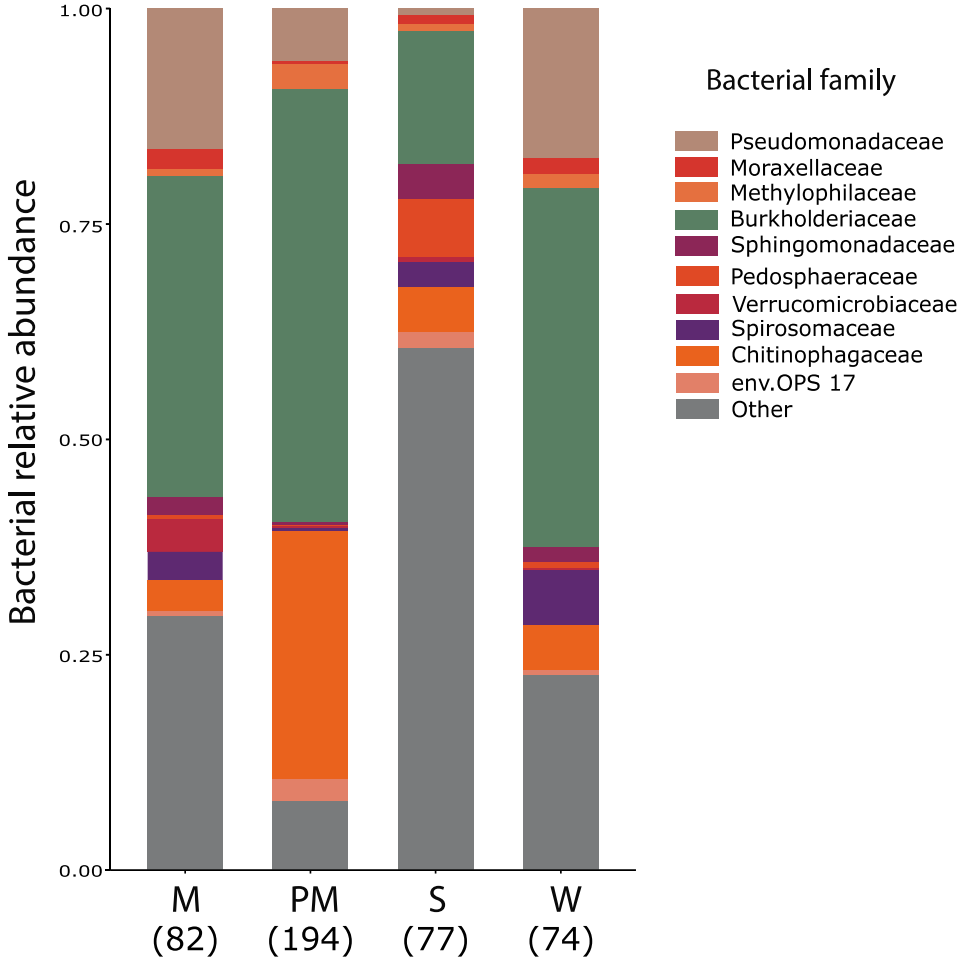


**Figure S2.** *A. altamirani* skin bacterial alpha and beta diversity across sampling locations. A) Phylogenetic diversity (PD) in metamorphic samples. B) Principal coordinate analysis (PCoA) based on weighted Unifrac distances for metamorphic samples C) PD for pre-metamorphic samples. D) Principal coordinate analysis (PCoA) based on weighted Unifrac distances for pre-metamorphic samples. Circles are color-coded by sampling site.

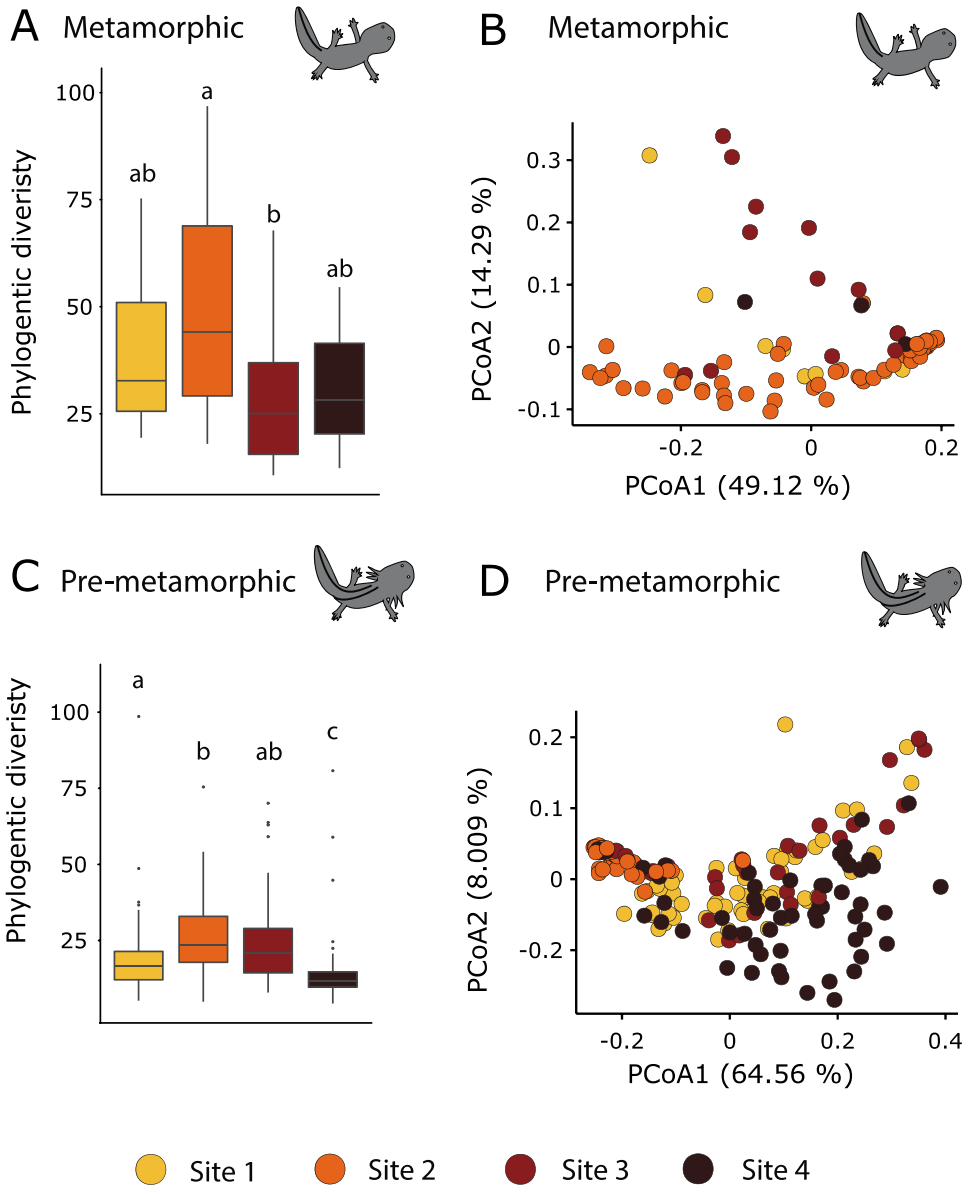
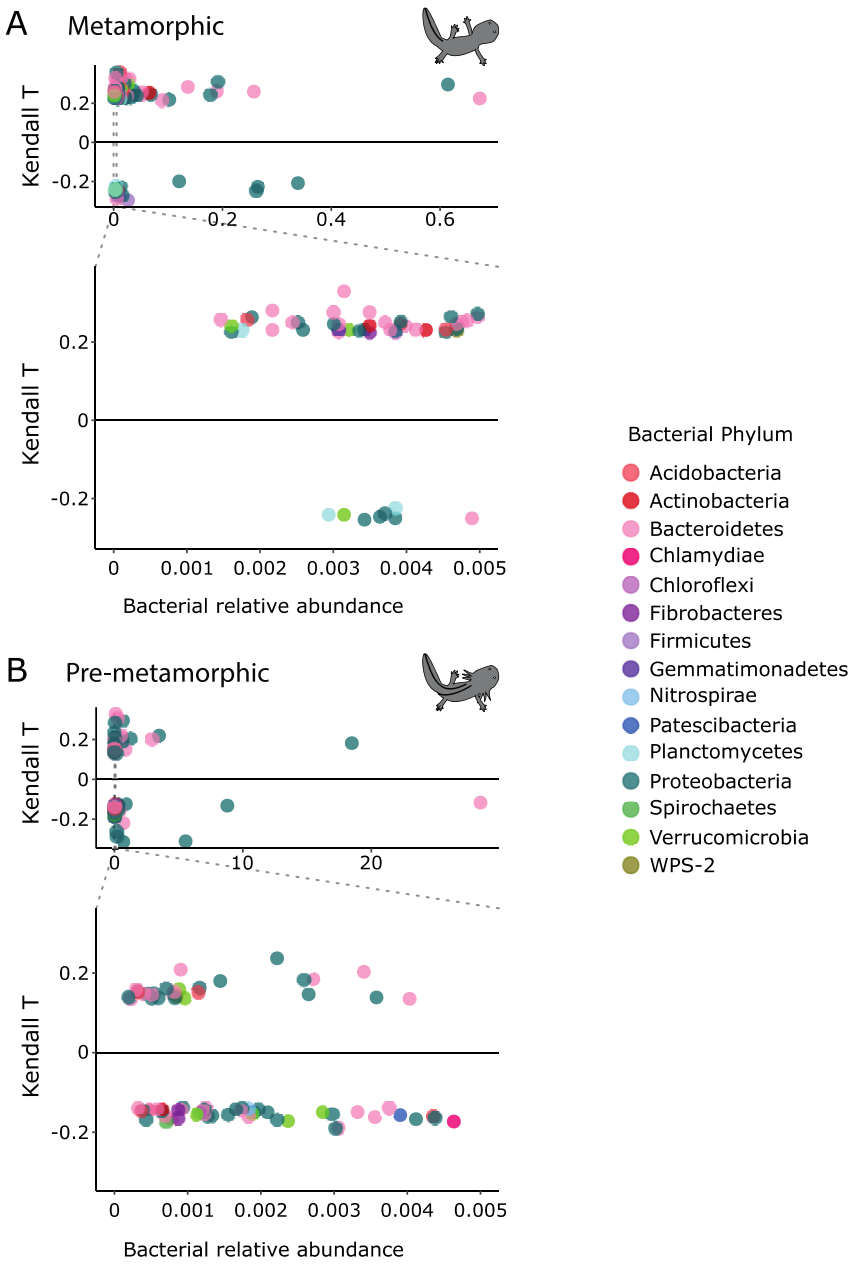


**Figure S3.** Kendall significant correlations between Bd infection loads and bacterial relative abundance in infected samples. A) Correlated ASVs on metamorphic samples. B) Correlated ASVs on pre-metamorphic samples. Y axis shows Kendall τ values for significant correlations, X axis shows the relative abundance of each ASV. Circles are color-coded by bacterial phylum



**Supplementary Tables**

# The skin microbiota of the axolotl *Ambystoma altamirani* is highly influenced by metamorphosis and seasonality but not by pathogen infection.

**Emanuel Martínez-Ugalde[1], Víctor D. Ávila-Akerberg[2], Tanya M. González Martínez[3], Montserrat Vázquez Trejo[3], Dalia Zavala Hernández[3], Sara Anaya Morales[1], Eria A. Rebollar[1].**

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**Table S1.** *Post-hoc* pairwise Wilcoxon tests for Shannon, observed ASV and Phylogenetic diversity (PD). Numbers in bold indicate significant p-values of paired comparisons. Metamorphic (M), pre-metamorphic (PM), sediment (S), water (W).

| | Shannon | Observed ASVs | PD |
|---|---|---|---|
| M-PM | **$7.20e^{-21}$** | **$9.44e^{-20}$** | **$4.95e^{-19}$** |
| M-S | **$6.80e^{-25}$** | **$2.20e^{-23}$** | **$8.32e^{-26}$** |
| M-W | 0.66 | 0.48 | **0.011** |
| PM-S | **$1.10e^{-36}$** | **$2.52e^{-36}$** | **$2.06e^{-36}$** |
| PM-W | **$1.00e^{-20}$** | **$3.29e^{-24}$** | **$2.92e^{-26}$** |
| S-W | **$3.30e^{-25}$** | **$8.00e^{-25}$** | **$6.80e^{-25}$** |

**Table S2.** Pairwise PERMANOVA comparisons between sample types. Numbers in **bold** indicate statistically significant p-values of each comparison.

| Group 1 | Group 2 | Sample size | Permutations | pseudo-F | p-value | q-value |
|---|---|---|---|---|---|---|
| Metamorphic | Pre-metamorphic | 272 | 999 | 50.20 | **0.001** | **0.0012** |
| Metamorphic | Sediment | 159 | 999 | 87.20 | **0.001** | **0.0012** |
| Metamorphic | Water | 156 | 999 | 4.32 | **0.002** | **0.002** |
| Pre-metamorphic | Sediment | 267 | 999 | 110.51 | **0.001** | **0.0012** |
| Pre-metamorphic | Water | 264 | 999 | 42.40 | **0.001** | **0.0012** |
| Sediment | Water | 151 | 999 | 111.25 | **0.001** | **0.0012** |

**Table S3.** Analysis of multivariate homogeneity of group dispersions between sample types. Numbers above the diagonal represent the permuted p-values while numbers bellow the diagonal represent observed p-values. Numbers in **bold** indicate significant p-values.

| | Metamorphic | Pre-metamorphic | Sediment | Water |
|---|---|---|---|---|
| Metamorphic | | **$1.00e^{-03}$** | **$1.00e^{-03}$** | 0.269 |
| Pre-metamorphic | **$8.96e^{-06}$** | | **$1.00e^{-03}$** | **0.001** |
| Sediment | **1.07e-$^{06}$** | **$5.33e^{-18}$** | | **0.002** |
| Water | 0.24 | **$9.05e^{-08}$** | **$1.07e^{-03}$** | |

**Table S4.** Amplicon sequence variants (ASVs) defining the bacterial core of sediment and water samples. ASVs that are shared with the metamorphic bacterial core are highlighted in **bold**.

| | ASV ID | Taxonomy at family level | Relative abundance | Persistence |
|---|---|---|---|---|
| **Sediment** | **6d0c9d0395e6a2a7667eb0b07c17a275** | **Burkholderiaceae** | **2.32** | **100.00** |
| | 5092297d795976b29921946d1d7aff2f | Spirosomaceae | 0.68 | 97.40 |
| | 8fd9eab61a0f63db6cbb201ce66b484b | Burkholderiaceae | 0.78 | 100.00 |
| | 4eece736dad7d5a26e82e2d2247d3a9b | Burkholderiaceae | 0.67 | 98.70 |
| | f5944006a2164242b3d35b6f35269be2 | Burkholderiaceae | 0.60 | 97.40 |
| | 6725ac4b1fde87ad69cedaf457fd2376 | Burkholderiaceae | 0.55 | 96.10 |
| | 3b20703d6681b21538748d73a9b83dc0 | Pedosphaeraceae | 0.57 | 94.81 |
| | 0124b03431ca1882134f36a8cb86c9b9 | Pedosphaeraceae | 0.55 | 98.70 |
| | ac415ad279cb27c6caecc356aef37309 | Pedosphaeraceae | 0.45 | 96.10 |
| | 6edfdf296fc994a55f9981dda43d242a | Rhodobacteraceae | 0.44 | 97.40 |
| | 282ecd235de20ec5393575e4e27582f7 | Xanthobacteraceae | 0.44 | 94.81 |
| | 5cb5f60773d589f49624fb3e212416d0 | Pedosphaeraceae | 0.38 | 98.70 |
| | dc3ea98362f99244fce2ece1cc293384 | Geobacteraceae | 0.42 | 98.70 |
| | 74aa1a6159865a8305e5fd7e98901154 | Burkholderiaceae | 0.36 | 93.51 |
| | 91291567e47efece2472186165552086 | Nitrosomonadaceae | 0.34 | 98.70 |
| | 89e24b9cf9c0922557315bebd4765fca | Chthoniobacteraceae | 0.27 | 100.00 |
| | 8da392b60860c28c114f4d1696bcb918 | Microscillaceae | 0.27 | 93.51 |
| | 69be5205c4f39f10d94806fd7abd891c | Microscillaceae | 0.21 | 93.51 |
| | 926a77485ba849b0dcb6f0b0fa54864b | TRA3-20 | 0.24 | 92.21 |
| | 841890b0612bcb02e31dab6907df8d1d | Cellvibrionaceae | 0.26 | 97.40 |
| | 64f6423765becb7998eb54f0bb4a514f | uncultured proteobacterium | 0.18 | 90.91 |
| | e2022b2ee4476a7c92a637dcd9887deb | env.OPS 17 | 0.18 | 100.00 |

| | | | | |
|---|---|---|---|---|
| | 852faa226ea1900e5befddd8f336e71e | Chitinophagaceae | 0.17 | 92.21 |
| | 8462829dec031a181482fc4c0fb1481c | Opitutaceae | 0.16 | 92.21 |
| | d07e75050a4e4029595a126ae8683d50 | Burkholderiaceae | 0.16 | 98.70 |
| | 0ccbe75e33f75230126df77dfaf8f511 | Devosiaceae | 0.15 | 94.81 |
| | 8813fd04fb20de3070defbb994e31ebb | metagenome | 0.13 | 90.91 |
| | 1e74e07ca37faaf31b1c59378c54d654 | Chitinophagaceae | 0.10 | 90.91 |
| | 4150e5d078d1f5e24dd501083242f7bf | uncultured Holophaga sp. | 0.10 | 92.21 |
| | 19373c69bd87f24161bd8fb593a988ef | P3OB-42 | 0.07 | 96.10 |
| **Water** | **9936daae333af6e517a9deb4b9e18ffa** | **Pseudomonadaceae** | **17.19** | **100.00** |
| | **be8eb25874b4202cf98050dbadeeb7ce** | **Burkholderiaceae** | **0.31** | **94.59** |

**Table S5.** Two-way Multivariate Analysis of Variance evaluating physicochemical variation across seasons and sampling locations. Mean temp (mean temperature), Max temp (maximum temperature), Min temp (minimal temperature), Delta temp (difference between max and min temp), DO (dissolved oxygen), Cond (conductivity). Numbers in bold indicate significant p-values.

| | Season | | | | | Locality | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Df | Sum Sq | Mean Sq | F-value | p-value | Df | Sum Sq | Mean Sq | F-value | p-value |
| Mean temp | 3 | 71.37 | 23.79 | 103.85 | **$2.20e^{-16}$** | 3 | 48 | 15.99 | 69.83 | **$3.68e^{-16}$** |
| Max temp | 3 | 68.61 | 22.87 | 24.24 | **$3.47e^{-09}$** | 3 | 159.54 | 53.18 | 56.38 | **$1.32e^{-14}$** |
| Min temp | 3 | 35.31 | 11.77 | 12.40 | **$6.53e^{-06}$** | 3 | 81.69 | 27.23 | 28.69 | **$3.66e^{-10}$** |
| Delta temp | 3 | 134.94 | 44.979 | 14.68 | **$1.22e^{-06}$** | 3 | 466.09 | 155.36 | 50.7 | **$7.41e^{-14}$** |
| pH | 3 | 2.55 | 0.85 | 14.85 | **$1.08e^{-06}$** | 3 | 3.005 | 1.001 | 17.48 | **$1.83e^{-07}$** |
| DO | 3 | 5429 | 1809.65 | 66.16 | **$9.20e^{-16}$** | 3 | 83.8 | 27.92 | 1.02 | 0.3933 |
| Cond | 3 | 1315.2 | 438.4 | 3.538 | **0.022** | 3 | 24675.9 | 8225.3 | 66.38 | **$8.69e^{-16}$** |

**Table S6.** *Post-hoc* pairwise Wilcoxon tests of Phylogenetic diversity (PD) for metamorphic and pre-metamorphic axolotls across seasons. Numbers in bold indicate significant p-values of paired comparisons between consecutive seasons. Summer (S), autumn (Aut), winter (Win), spring (Spr).

| | Metamorphic | Pre-metamorphic |
|---|---|---|
| Sum-Aut | 0.801 | 0.93 |
| Sum-Win | 0.451 | 0.93 |
| Sum-Spr | 0.009 | 0.93 |
| Aut-Win | 0.382 | 0.93 |
| Aut-Spr | 0.019 | 0.93 |
| Win-Spr | **0.005** | 0.93 |

**Table S7.** Pairwise PERMANOVA pairwise comparisons between seasons for metamorphic samples. Numbers in **bold** represent statistically significant p-values between consecutive seasons.

| Group 1 | Group 2 | Sample size | Permutations | pseudo-F | p-value | q-value |
|---|---|---|---|---|---|---|
| Autumn | Spring | 48 | 999 | 22.4070207 | 0.001 | 0.002 |
| Autumn | Summer | 51 | 999 | 1.2168406 | 0.302 | 0.302 |
| Autumn | Winter | 35 | 999 | 1.39273164 | 0.217 | 0.302 |
| Spring | Summer | 47 | 999 | 21.4223451 | 0.001 | 0.002 |
| Spring | Winter | 31 | 999 | 14.9815017 | **0.001** | **0.002** |
| Summer | Winter | 34 | 999 | 1.2181662 | 0.292 | 0.302 |

**Table S8.** Analysis of multivariate homogeneity of group dispersions between seasons for metamorphic axolotls. Numbers above the diagonal represent the permuted p-values while numbers bellow the diagonal represent observed p-values. Numbers in **bold** indicate significant p-values.

| | **Summer** | **Autumn** | **Winter** | **Spring** |
|---|---|---|---|---|
| **Summer** | | 0.979 | 0.993 | 0.066 |
| **Autumn** | 0.980268 | | 0.999 | 0.044 |
| **Winter** | 0.987862 | 0.999101 | | 0.137 |
| **Spring** | 0.065539 | 0.037628 | 0.122359 | |

**Table S9.** Pairwise PERMANOVA pairwise comparisons between seasons for pre-metamorphic samples. Numbers in **bold** represent statistically significant p-values between consecutive seasons.

| Group 1 | Group 2 | Sample size | Permutations | pseudo-F | p-value | q-value |
|---|---|---|---|---|---|---|

| | | | | | | |
|---|---|---|---|---|---|---|
| Autumn | Spring | 86 | 999 | 24.0061607 | 0.001 | 0.0012 |
| Autumn | Summer | 70 | 999 | 0.51792264 | 0.58 | 0.58 |
| Autumn | Winter | 92 | 999 | 13.4781367 | **0.001** | **0.0012** |
| Spring | Summer | 98 | 999 | 24.1275248 | 0.001 | 0.0012 |
| Spring | Winter | 120 | 999 | 12.6138699 | **0.001** | **0.0012** |
| Summer | Winter | 104 | 999 | 11.2951067 | 0.001 | 0.0012 |

**Table S10.** Analysis of multivariate homogeneity of group dispersions between seasons for pre-metamorphic axolotls. Numbers above the diagonal represent the permuted p-values while numbers bellow the diagonal represent observed p-values. Numbers in **bold** indicate significant p-values.

| | **Summer** | **Autumn** | **Winter** | **Spring** |
|---|---|---|---|---|
| **Summer** | | 0.544 | 0.095 | 0.063 |
| **Autumn** | 0.550837 | | 0.043 | **0.027** |
| **Winter** | 0.093814 | **0.036321** | | 0.608 |
| **Spring** | 0.057611 | **0.029374** | 0.600254 | |

**Table S11.** *Post-hoc* pairwise Wilcoxon tests for Phylogenetic diversity (PD) for metamorphic and pre-metamorphic axolotls between sampling locations. Numbers in bold indicate significant p-values of paired comparisons. Sampling location 1 (S1), sampling location 2 (S2), sampling location 3 (S3), sampling location 4 (S4).

| | Metamorphic | Pre-metamorphic |
|---|---|---|
| S1-S2 | 0.312 | **0.00098** |
| S1-S3 | 0.312 | 0.07816 |
| S1-S4 | 0.666 | **0.00062** |
| S2-S3 | **0.032** | 0.36012 |
| S2-S4 | 0.312 | **1.70E-07** |
| S3-S4 | 0.945 | **6.70E-05** |

**Table S12.** Pairwise PERMANOVA pairwise comparisons between sampling locations for metamorphic samples. Numbers in **bold** represent statistically significant p-values.

| | | Sample size | Permutations | pseudo-F | p-value | q-value |
|---|---|---|---|---|---|---|
| Group 1 | Group 2 | | | | | |
| Site 1 | Site 2 | 67 | 999 | 1.61 | 0.157 | 0.2355 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Site 3 | 23 | 999 | 2.43 | 0.039 | 0.117 |
| | Site 4 | 14 | 999 | 0.81 | 0.504 | 0.504 |
| Site 2 | Site 3 | 68 | 999 | 5.73 | **0.002** | **0.012** |
| | Site 4 | 59 | 999 | 1.38 | 0.255 | 0.306 |
| Site 3 | Site 4 | 15 | 999 | 1.75 | 0.121 | 0.2355 |

**Table S13.** Pairwise PERMANOVA pairwise comparisons between sampling locations for pre-metamorphic samples. Numbers in bold represent statistically significant p-values.

| | | Sample size | Permutations | pseudo-F | p-value | q-value |
|---|---|---|---|---|---|---|
| Group 1 | Group 2 | | | | | |
| Site 1 | Site 2 | 101 | 999 | 57.7 | **0.001** | **0.0015** |
| | Site 3 | 89 | 999 | 3.26 | **0.041** | **0.041** |
| | Site 4 | 118 | 999 | 9.2 | **0.001** | **0.0015** |
| Site 2 | Site 3 | 72 | 999 | 60.44 | **0.001** | **0.0015** |
| | Site 4 | 101 | 999 | 88.37 | **0.001** | **0.0015** |
| Site 3 | Site 4 | 89 | 999 | 4.97 | **0.005** | **0.006** |

**Table S14.** Analysis of multivariate homogeneity of group dispersions between sampling locations for metamorphic axolotls. Numbers above the diagonal represent the permuted p-values while numbers bellow the diagonal represent observed p-values. Numbers in **bold** indicate significant p-values.

| | **S1** | **S2** | **S3** | **S4** |
|---|---|---|---|---|
| **S1** | | 0.45 | 0.411 | 0.988 |
| **S2** | 0.46433 | | 0.902 | 0.661 |
| **S3** | 0.42941 | 0.88271 | | 0.244 |
| **S4** | 0.98528 | 0.65149 | 0.24319 | |

**Table S15.** Analysis of multivariate homogeneity of group dispersions between sampling locations for pre-metamorphic axolotls. Numbers above the diagonal represent the permuted p-values while numbers bellow the diagonal represent observed p-values. Numbers in **bold** indicate significant p-values.

| | **S1** | **S2** | **S3** | **S4** |
|---|---|---|---|---|
| **S1** | | 0.08 | **6.00E-03** | 0.095 |
| **S2** | 0.09 | | **1.00E-03** | **0.003** |

| | | | | |
|---|---|---|---|---|
| **S3** | **6.54E-03** | **6.72E-05** | | 0.134 |
| **S4** | 0.11 | **1.47E-03** | 0.12 | |

**Table S16**. Variance explained of each canonical axis calculated by dbRDA. Columns indicate: F statistic, p-values, variance explained by each canonical axis, and the cumulative variance calculated by the Permutational like ANOVA. Numbers in **bold** indicate significant p-values and the cumulative variance for each statistically significant canonical axis.

| | F | p-value | Variance explained | Cumulative variance |
|---|---|---|---|---|
| CAP1 | 63.3875 | **0.001** | **0.173** | **0.173** |
| CAP2 | 18.4679 | **0.001** | **0.050** | **0.223** |
| CAP3 | 9.8065 | **0.001** | **0.026** | **0.250** |
| CAP4 | 5.1977 | **0.006** | **0.014** | **0.264** |
| CAP5 | 2.5146 | 0.123 | 0.006 | 0.271 |
| CAP6 | 1.5675 | 0.387 | 0.004 | 0.275 |
| CAP7 | 1.1402 | 0.563 | 0.003 | 0.279 |
| CAP8 | 0.7233 | 0.764 | 0.001 | 0.281 |

**Table S17.** ASVs significantly correlated with Bd infection intensity and that are present in both metamorphic and pre-metamorphic samples. ASVs which differ in the type of correlation (positive or negative) between sample types (metamorphic/pre-metamorphic) are highlighted in **bold**.

| OTU | Metamorphic p-value | Metamorphic Kendall τ | Pre-metamorphic p-value | Pre-metamorphic Kendall τ | Best hit |
|---|---|---|---|---|---|
| 6b9a5f7e85b05d17e49402afedb5d09d | 0.0062 | 0.30667 | 0.0025 | 0.2083 | Sphingobacteriaceae (F) |
| 4a05a9e45659d8b2597fd39eccebcc69 | 0.0150 | 0.2602 | 0.0011 | 0.2068 | Flavobacteriaceae (F) |
| d42988b9162532f845c1fb5f283e0e36 | 0.04237 | 0.2309 | 0.0213 | 0.1594 | Opitutaceae (F) |
| **f5343248f66d395a4a3f81f0ec2e6139** | 0.02698 | **-0.2504** | 0.0251 | **0.1553** | **Acetobacteraceae (F)** |
| bc1aac689cb18f362a259d258c62d6c3 | 0.00383 | 0.3214 | 0.0296 | 0.1498 | Saprospiraceae (F) |
| 5e9e03d7bb698e92ce2c344e381f05cc | 0.04237 | 0.2309 | 0.0314 | 0.1492 | Nitrosomonadaceae (F) |
| 0db5c0f9a31014f597a82c40a97e5047 | 0.03373 | 0.2415 | 0.0482 | 0.1370 | AKYH767 (F) |
| **3c28f0caf9183357de05d1882a943f8e** | 0.02110 | **0.2242** | 0.0403 | **-0.1170** | **Chitinophagaceae (F)** |
| **d2a68e0d41ffc5747e42a888d8314c92** | 0.00895 | **0.2786** | 0.0488 | **-0.1342** | **Gemmataceae (F)** |

| | | | | | |
|---|---|---|---|---|---|
| **ee3f91c192699d4bc52b1c12179e2b00** | 0.01548 | **0.2690** | 0.0202 | **-0.1590** | **Acidobacteria (P)** |
| **ec7d28b8c8ab3ddb201d48b2ad5ee90a** | 0.03004 | **0.2422** | 0.0169 | **-0.1651** | **Flavobacteriaceae (F)** |
| **2ebb0a4d8c3eb5dfc6e277e14e5701e7** | 0.01223 | **0.2798** | 0.006 | **-0.1881** | **Chitinophagaceae (F)** |

**Table S18.** List of axolotl samples collected at each sampling location. Number of samples from the skin of *A. altamirani* individuals: metamorphic (M) and pre-metamorphic (PM).

| | **Summer** | | **Autumn** | | **Winter** | | **Spring** | | |
|---|---|---|---|---|---|---|---|---|---|
| | PM | M | PM | M | PM | M | PM | M | **Total** |
| **Site 1** | 13 | 3 | 15 | 6 | 17 | 0 | 15 | 3 | 72 |
| **Site 2** | 12 | 15 | 8 | 17 | 13 | 9 | 10 | 17 | 101 |
| **Site 3** | 3 | 6 | 2 | 4 | 13 | 0 | 14 | 2 | 44 |
| **Site 4** | 13 | 1 | 4 | 1 | 23 | 0 | 19 | 1 | 62 |
| **Total** | 41 | 25 | 29 | 28 | 66 | 9 | 58 | 23 | 279 |

## Capítulo 5. La diversidad genómica funcional del microbioma de la piel de *Ambystoma altamirani* varia en el tiempo y el espacio y contiene múltiples genomas bacterianos con potencial actividad antifúngica.

### 5.1 Resumen

En este trabajo se describió la diversidad funcional del microbioma de *A. altamirani* mediante la secuenciación de 40 muestras de ajolotes pre-metamórficos (con branquias), y se evaluaron cambios en los perfiles funcionales del microbioma a lo largo de un gradiente estacional (verano – otoño – invierno – primavera) y un gradiente altitudinal (3,087 – 3,177 – 3,447 metros sobre el nivel del mar (msnm)). Además, se evaluaron cambios funcionales entre ajolotes infectados y no-infectados con Bd.

Mediante el análisis de un catálogo de genes se observó que las funciones generales del microbioma varían a lo largo de los dos gradientes ambientales que se evaluaron en este trabajo. Específicamente, los resultados mostraron que, la diversidad funcional varia de manera significativa entre otoño – invierno e invierno – primavera. Por su parte, se observó que, la diversidad funcional difiere de manera significativa entre altitudes, siendo los microbiomas de la piel de ajolotes de altitudes bajas (3,087 msnm) claramente diferentes a los microbiomas de ajolotes de altitudes medias (3,177 msnm) y altas (3,447 msnm).

Adicionalmente se identificaron 5,196 genes asociados a mecanismos de comunicación bacteriana (formación de biopelículas, percepción de quórum y sistemas de secreción) y posibles funciones antifúngicas (síntesis de metabolitos secundarios y degradación de quitina). Estos genes provienen de 25 diferentes fila de organismos de acuerdo con la anotación taxonómica del catálogo de

genes, sin embargo, el 91 % (4,774) fueron clasificados como genes de los fila Proteobacteria (3,070) y Bacteroidetes (1,704). Interesantemente, estos genes mostraron patrones de variación similares a los de las funciones generales a lo largo de ambos gradientes ambientales.

Con el fin de evaluar la contribución especifica de ciertos grupos de bacterias se recuperó un set de 50 genomas ensamblados de metagenomas (MAGs por sus siglas en inglés) únicos de la piel de *A. altamirani*. La clasificación taxonómica de estos genomas reveló que estos pertenecen a 10 distintos ordenes bacterianos siendo los Burkholderiales (28) y Chitiniphagales (13) los MAGs más abundantes. La anotación funcional de estos genomas reveló la presencia de genes implicados en la formación de biopelículas, comunicación celular y sistemas de secreción en los 50 MAGs recuperados del microbioma de la piel. Por su parte, genes asociados con la degradación de quitina fueron identificados principalmente en los MAGs clasificados como Burkholderiales y Chitinophagales. Adicionalmente, se predijo la presencia de clústers biosintéticos (BGCs por sus siglas en inglés) asociados a la síntesis de diversos metabolitos secundarios siendo los terpenos y los péptidos ribosomales modificados postraduccionalmente (RIPP) los BGCs más comunes, notablemente estos fueron predichos principalmente en MAGs clasificados como Burkholderiales y Chitinophagales.

En conjunto, los resultados de este capítulo contribuyen a entender la relación que existe entre la variación ambiental y la diversidad funcional del microbioma, lo que es especialmente importante en modelos de anfibios en vida libre que están constantemente expuestos fluctuaciones ambientales. Adicionalmente, los resultados de este trabajo amplían el entendimiento de la contribución que tienen

los microbiomas de la piel de los anfibios en la defensa contra patógenos. Estos resultados pueden ser la base para futuros estudios en donde se analicen estrategias de inhibición contra Bd que han sido poco exploradas hasta el momento como pueden ser la acción de enzimas quitinolíticas o la síntesis de terpenos.

# Title: Functional genomic diversity of the skin microbiome of *Ambysotma altamirani* varies in time and space and includes bacterial genomes with potential antifungal activity

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Keywords: Amphibian skin microbiome, Seasonality, Elevation, Functional genomic diversity, *Ambystoma altamirani*.

## Abstract

Amphibian skin microbiomes can play a critical role in host survival against emerging diseases by protecting their host against pathogens. While a plethora of biotic and abiotic factors have been shown to influence the taxonomic diversity of amphibian skin microbiomes it remains unclear whether functional genomic diversity varies in response to temporal and environmental factors. Here we applied a metagenomic approach to evaluate whether seasonality, distinct elevations, and pathogen presence influence the functional genomic diversity of the *A. altamirani* skin microbiome. We obtained a gene catalog with 92,107 nonredundant annotated genes and a set of 50 unique metagenome assembled genomes (MAGs). Our analysis showed that genes linked to general and potential antifungal traits significantly differed across seasons and sampling locations at different elevations. Moreover, we found that the functional genomic diversity of *A. altamirani* skin microbiome differed between Bd infected and not infected axolotls only during winter, suggesting an interaction between seasonality and pathogen infection. In addition, we identified the presence of genes and biosynthetic gene clusters (BGCs) linked to potential antifungal functions such as biofilm formation, quorum sensing, secretion systems, secondary metabolite biosynthesis, and chitin degradation. Interestingly genes linked to these potential antifungal traits were mainly identified in Burkholderiales and Chitinophagles MAGs. Overall, our results identified functional traits linked to potential antifungal functions in the *A. altamirani* skin microbiome regardless of variation in the functional diversity across seasons, elevations, and pathogen presence. Our results highlight the importance of further exploring the antifungal function of Burkholderiales and

Chitinophagales as bacterial classes that could be key in host tolerance against chytridiomycosis.

## Data summary

Sequencing data have been deposited in the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA) within the BioProject PRJNA1010953. Metagenome assembled genomes were also deposited in the NCBI WGS under the BioProject PRJNA1010953, BioSample accessions for each MAG are included in Supplementary Table 9. Bioinformatic workflow is available at https://github.com/EmanuelMartinez-Ugalde/A.-altamirani-skin-microbiome-Gene-catalog-and-MAG-recovery

## Impact statement

The amphibian skin microbiome plays an essential role in protecting hosts against the lethal infectious disease called chytridiomycosis, caused by *Batrachochytrium dendrobatidis*. Here we describe the functional genomic diversity of the skin microbiome of the endangered axolotl *Ambystoma altamirani* and determine its variation across seasons, elevations, and the presence of the pathogen. Our work identified bacterial genomes that are prevalent across time and space and include a wide repertory of potential antifungal genes. Our study contributes to a better understanding of the skin microbiome contribution to protection against emerging diseases like chytridiomycosis.

## Introduction

The recent emergence of infectious fungal diseases that threaten various wildlife species [1–5] has led to the development of conservation strategies to

preserve and ameliorate the impact of these diseases on susceptible species [6–10]. Due to their contribution to host development, nutrition, and health [11, 12], microbiomes have emerged as a promising field to aid in the conservation of endangered species [13]. It has been shown that host-associated microbiomes can contribute to immune system maturation [14–17], or disease resistance against pathogens [18–22]. For example, bacteria present in amphibian and bat microbiomes can inhibit the growth of fungal pathogens that have caused severe population declines in both groups of vertebrates [3, 5, 23, 24].

Amphibians nowadays are considered the most vulnerable group of vertebrates due to habitat reduction, introduction of invasive species, and the emergence of infectious diseases known as chytridiomycosis [3, 25–27]. This disease is caused by the fungal pathogens *Batrachochytrium dendrobatidis* (Bd) [28] and *B. salamandrivorans* (Bsal) [29], and it has been linked to population declines of more than 500 amphibian species worldwide [3]. Nonetheless, it has been shown that amphibians can control these pathogens through various immune responses [30–34], some of them mediated by members of the skin microbiome which can inhibit the growth of Bd and Bsal [35–39].

Disease resistance mediated by microbiomes can occur by direct and indirect defense mechanisms [18, 19]. Direct defense mechanisms take place between members of the microbiome and the invading pathogens and involve functional traits linked to i) competition for space and nutrients (e.g. biofilm formation [40], ii) quorum sensing [41, 42]), iii) active antagonism (e.g. contact-dependent inhibition by secretion systems [43, 44]), iv) biosynthesis of inhibitory metabolites (e.g. secondary metabolites [21, 45] or cell wall degrading enzymes

[46, 47]). Indirect defense mechanisms refer to host responses triggered by microbiome members and these include i) modification of the chemical properties of mucus barriers [48, 49] or ii) synthesis and release of immune effectors (cytokines, antimicrobial peptides, or immunoglobulins [18, 50]).

In the case of amphibians, it has been shown that some members of the skin microbiomes can contribute to disease resistance against Bd and Bsal through direct defense such as biofilm formation [40, 51] or secondary metabolite biosynthesis [21, 45, 52]. However, until this day only a handful of anti-Bd secondary metabolites derived from amphibians have been fully characterized, and these include violacein [21], 2,4 DAPG [45], prodigiosin [53], tryptophol [54], and viscosine-like lipopeptides [55]. In addition, even when the anti-Bd potential of amphibian skin microbiomes has been described in numerous amphibian species through *in vitro* assays [36, 37, 40, 51, 53, 56, 57], there is little information about other microbial functional traits linked to host protection against chytridiomycosis [22, 58].

A plethora of host-related [59–62], microhabitat related [63–67], and biogeographically related [68–71] factors influencing the taxonomic diversity and structure of amphibian skin microbiomes have been described [23, 72]. In some cases the taxonomic variation of skin microbiomes has been associated with different health-disease outcomes against chytridiomycosis [63, 73, 74], however, it remains unclear whether microbiome taxonomic variation is reflected in changes in functional diversity.

Previously, we showed that skin bacterial diversity of the endangered axolotl *Ambystoma altamirani* varied across a seasonal gradient and between sampling locations [75]. In addition, despite high Bd prevalence across seasons

and sampling locations [76], the skin bacterial diversity of the *A. altamirani* microbiome did not vary between Bd infected and not infected axolotls [75]. In this study, we applied gene-level metagenomics and genome-level metagenome-assembled genomes (MAGs) to describe the functional diversity of *A. altamirani* skin microbiome and to evaluate whether seasonality, elevation, and Bd presence are related to variation in the general and potential antifungal traits of the *A. altamirani* skin microbiome.

We hypothesize that general functional traits of the *A. altamirani* skin microbiome would vary between consecutive seasons (summer-autumn, autumn-winter, and winter-spring) and among sampling locations with distinct elevations (high-medium-low) in line with changes in the taxonomic diversity [75]. In addition, considering that *A. altamirani* rarely shows symptoms of chytridiomycosis despite the presence of the pathogen [76], we also hypothesize that potential antifungal traits of skin microbiome should remain constant despite temporal and spatial variation or in the presence of Bd.

## Methods

### Sample selection and sequencing procedures

In a previous study [75], skin swabs from metamorphic and pre-metamorphic (gilled) *A. altamirani* axolotls were obtained across the four seasons of one year (from July 2019 to March 2020) from four different streams distributed along an altitudinal gradient from 3,087 meters above sea level (m.a.s.l) to 3,447 m.a.s.l. These samples were previously used for 16S metabarcoding [75] and Bd detection [76]. In this study, forty samples from pre-metamorphic axolotls were selected for shotgun metagenome sequencing since

pre-metamorphic axolotl bacterial communities were more influenced by seasonality and elevation. Specifically, ten skin microbiome samples were selected for each season, five of them were infected with Bd, and the other five were not infected [76] (Supplementary Table 1). Due to limited amounts of DNA in samples collected during autumn, eight Bd-infected samples and two not infected samples were selected for sequencing for this specific season. Of the forty selected samples 21 (3,447 masl), 7 (3,177 masl), and 12 (3,087 masl) were collected from streams located in high, medium, and low elevations respectively (Supplementary Table 1). The DNA from the selected samples was sent to CD Genomics (Shirley, NY, USA) for library construction and shotgun sequencing using the Illumina HiSeq 2x150 technology, at an average read depth of 20 million pair-end reads per sample.

**Sequence processing and construction of an *A. altamirani* skin microbiome gene catalog (AaSMGC)**

Trim Galore [77] (V 0.6.2) was used for quality filtering and adapter trimming. Briefly, sequencing adapters were removed, and low-quality bases (Phred < 20) were trimmed from each read. Then, host derived sequences and potential human contaminant sequences were removed from the whole data set by aligning sequenced reads against an index of the *A. mexicanum* (AmbMex60DD) and *Homo sapiens* (GRCh38) genome assemblies using Bowtie2 [78] (V 2.3.4.1, parameter: -sensitive). *A. mexicanum* (AmbMex60DD) genome was used as a reference to remove host contamination because to date is the only available genome for any species of the genus *Ambystoma*.

Microbiome derived reads were recovered using Samtools [79] (V 1.9) and these were assembled using MegaHit [80] (V 1.1.3, parameter: --presets

meta-sensitive -t 16 -m 0.5). Then, the BBmap package [81] was used to recover contigs with a minimum length of ≥ 500 bp. Open reading frames (ORFs) were predicted for each contig using Prodigal [82] (V 2.6.3, parameter: -p meta). After discarding ORFs with a length < 100 bp, the remaining ones were clustered using CD-HIT [83] (V 4.7, parameter: -c 0.95 -n 8 -G 0 -aS 0.9 -g 1 -d 0) to generate a dereplicated gene catalog referred here as *Ambystoma altamirani* Skin Microbiome Gene Catalog (AaSMGC).

**Functional and taxonomic profiling of the AaSMGC**

AaSMGC was functionally and taxonomically profiled using eggnog-mapper [84] (emapper-V 2.1.8, parameter: --cpu 40 -m diamond --itype CDS –translate –evalue 0.00001 --sensmode very-sensitive) and the eggnog Orthologous Groups database (V 5.0.2). Results from eggnog-mapper were used to calculate the relative abundance of each gene in the AaSMGC. For this, quality filtered sequences for each sample were mapped against the AaSMGC using Bowtie2 (V 2.3.4.1, parameter ---very-sensitive), and then the reads that mapped to any gene entry in the AaSMGC were quantified using featureCounts [85] (V 2.0.1, parameter: -O -p -C -t CDS). Relative abundance of each gene were calculated following Xie et al. (2021) [86] by transforming the recovered counts to transcripts per million (TPM) correcting for sampling depth and gene length in R (V 4.2 .3).

**Differential abundance analysis of the general and potential antifungal traits in the AaSMGC**

Gene abundance profiles of the A. *altamirani* skin microbiome were compared across seasons, different elevations, and between Bd infected and

not infected axolotls. First, gene entries in the AaSMGC with no functional annotation were filtered out, and the rest were used to generate a Bray-Curtis distance matrix using the TPM counts of each annotated gene entry. A principal coordinate analysis (PCoA) was performed based on the previously generated Bray-Curtis distance matrix, followed by a Permutational analysis of variance (PERMANOVA) using the Vegan [87] (V 2.6-4) and ecole [88] (V 4.2.3) packages to evaluate the influence and significance of seasonality, elevation, and Bd presence over the functional diversity of the AaSMGC. P-values for the permanova_pairwise (parameter: permutations = 10000, padj = "BH") test were adjusted for multiple comparisons using the Benjamini-Hochberg method [88].

DESeq2 [89] was used to identify differentially enriched genes in AaSMGC across seasons, elevations, and between Bd infected and not infected axolotls. Before running DESeq2, gene entries in AaSMGC with < 10 read counts among all samples were filtered out [89, 90], resulting in 42,281 AaSMGC gene entries. Specifically, the presence of differential genes was evaluated between i) consecutive seasons (e.g., summer-autumn), ii) distinct elevation ranges, and iii) Bd infected and not infected axolotls within each season. Genes with FDR ≤ 0.01 and LogFold change ≥ 2 were identified as differentially enriched. FDR values for each gene were calculated using the ashr package [91] implemented in DESeq2.

To evaluate if potential antifungal functions vary across seasons, elevations, and Bd presence on the *A. altamirani* skin microbiome, a subset of genes linked to biofilm formation, quorum sensing, secretion systems, secondary metabolite and chitinolytic enzyme biosynthesis referred henceforth as bacterial communication and competition traits (BCC, biofilm formation,

quorum sensing and secretion systems) and potential antifungal functions (AF, secondary metabolite and chitinolytic enzyme biosynthesis), was used to compare their abundance profiles using PERMANOVA and differential abundance analysis.

**Reconstruction of metagenome assembled genomes (MAGs)**

A binning approach was used to reconstruct metagenome assembled genomes (MAGs) from the forty individual assemblies. To recover the maximum amount of MAGs, samples obtained within each season under the same Bd infection status were co-assembled (e.g., summer samples from not infected axolotls). Briefly, assembled contigs were binned using the MetaWRAP [92] bin module (V 1.3.0, parameter: -t 16 -m 16 -l 1500 --maxbin2 --metabat2 –concoct). Then, all the recovered bins were refined using the MetaWRAP bin_refinement module (parameters: -t 30 -c 50 -x 10), Completeness and contamination for each MAG were calculated using CheckM [93] (V 1.0.12), and only MAGs with completeness ≥ 50% and ≤ 10% contamination were retained. dRep [94] (V 3.4.0, parameter: -p 80 -pa 0.90 -sa 0.99 -nc 0.30 -cm larger --ignoreGenomeQuality) was used to obtain a set of not redundant MAGs with an ANI cutoff of 99%, which resulted in the recovery of 50 not redundant MAGs.

Taxonomy was assigned to each not redundant MAG using GTDB-Tk [95] (V 2.1.1, parameter: classify_wf --extension fa --cpus 20). A maximum-likelihood tree was inferred using IQ-TREE [96] (V 2.1.4, parameter: -m LG+R4 -T 30 -B 1000) using a nucleotide substitution model that was selected with IQ-TREE ModelFinder (parameter: -m MF -T 30) based on the multiple sequence alignment of GTDB-Tk BAC120 marker set. The prevalence and abundance of

each recovered MAG on each sample were calculated as genome copies per million reads (GCPMR) using the quaint_bins module of MetaWrap.

### Inference of the potential antifungal functions on *A. altamirani* MAGs

MAGs were annotated to evaluate the presence of genes linked to BCC and AF functional traits that could be linked to host protection against fungal pathogens. First ORFs were predicted from each MAGs using Prokka (V 1.14.6, parameters: default) followed by functional annotation using eggnog-mapper (emapper-V 2.1.8, parameter: --cpu 40 -m diamond --evalue 0.00001). The presence of BCC and AF functional traits was considered if at least one gene linked to these functions was present in the MAGs. In addition, to better understand the potential contribution to host defense against fungal pathogens, biosynthetic gene clusters (BGCs) were predicted for each MAG using antiSMASH [97] (V 6.1.1, parameters: --genefinding-tool none --fullhmmer --tigrfam --cc-mibig --cb-general --cb-knownclusters --cb-subclusters --asf --rre --pfam2go --smcog-trees). The identity of BGCs was evaluated according to ClustBlast [97] and MiBiG [98] repositories.

## Results

### Taxonomy and functional features present on the *A. altamirani* skin microbiome

A total of 839.9 million pair-end reads were obtained from the forty *A. altamirani* skin microbiome samples. After quality filtering and host contamination removal, an average of 2 million quality pair-end reads were retained per sample ranging from 0.53 to 10.4 million reads (Supplementary Table 1). Then, quality-filtered reads were assembled into 494,430 contigs

(Supplementary Table 2), and 1,098,389 open reading frames (ORFs) were predicted from the assembled contigs.

After de-replication 190, 932 unique ORFs were retained and, from these 92,107 ORFs (48.24 %) were functionally and taxonomically annotated. The annotated genes comprehend the *A. altamirani* skin microbiome gene catalog (AaSMGC) that was used for all further analyses. Gene entries of the AaSMGC were mostly derived from Bacteria (73.4 %) followed by Eukaryota (26.33 %), Viruse (0.158 %), and Archaea (0.0786 %) (Figure 1A), Eukaryotes include protists and fungi among other groups but were mainly represented by the phylum Ciliophora (11.5 %) (Figure 1A). Noteworthy, our results showed that 94.5 % of bacteria genes in the AaSMGC were derived from the Proteobacteria (66.2 %) and Bacteroidetes (28.3 %) phyla (Figure 1B).

Functional annotation of AaSMGC showed 24 Clusters of Orthologous Genes (COG), being S (function unknown 23.5%), L (replication, recombination, and repair 11.8 %), E (amino acid transport and metabolism 7.12 %), M (cell wall or envelop biogenesis (6.4 %), and C (energy production and conversion (5.5 %) the most abundant COGs (Figure 1C).

**General functions of the *A. altamirani* skin microbiome vary in time and space**

We found that the functional diversity of the *A. altamirani* skin microbiome varied across seasons (PERMANOVA, F = 3.01, p-value = 9.9e-5, Figure 2A) and different site elevations (PERMANOVA, F = 3.25, p-value = 9e-4, Figure 2B). Except for the summer-autumn comparison (paired PERMANOVA, F = 0.89, p-value = 0.52), significant differences were found

between all comparisons of consecutive seasons (Supplementary Table 3) and PERMANOVA, F = 0.89, p-value = 0.52), significant differences were found between all comparisons of consecutive seasons (Supplementary Table 3) and between all distinct elevations (Supplementary Table 4). No functional differences were found between Bd infected and not infected axolotls (PERMANOVA, F = 1.44, p-value = 0.128) (Figure 2C).

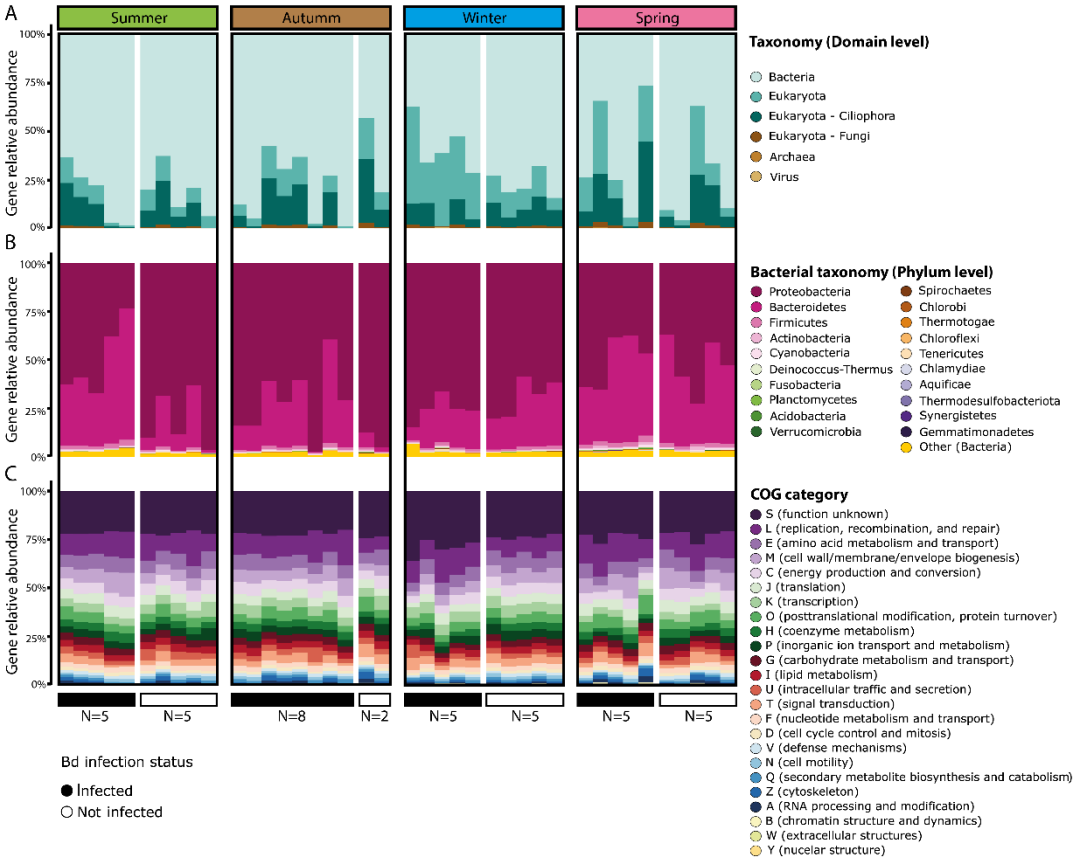


Figure 1. Relative abundance of gene entries in the AaSMGC. A) Taxonomic relative abundance at the domain level. B) Taxonomic relative abundance at the phylum level only for bacterially derived gene entries. C) Functional relative abundance of gene entries based on COG categories. Each bar corresponds to a single sample and is grouped based on sampling season and Bd infection status.

To further identify genes that were explaining differences across seasons and elevations we used DESeq2. In the case of seasonal comparisons, we found a total of 34, 789 and 4,063 differentially enriched genes between

summer-autumn, autumn-winter, and winter-spring seasons respectively. For the summer-autumn comparison, 85.3% (29 out of 34) differential genes were significantly enriched during summer, while the rest were enriched in autumn (Figure 3A).

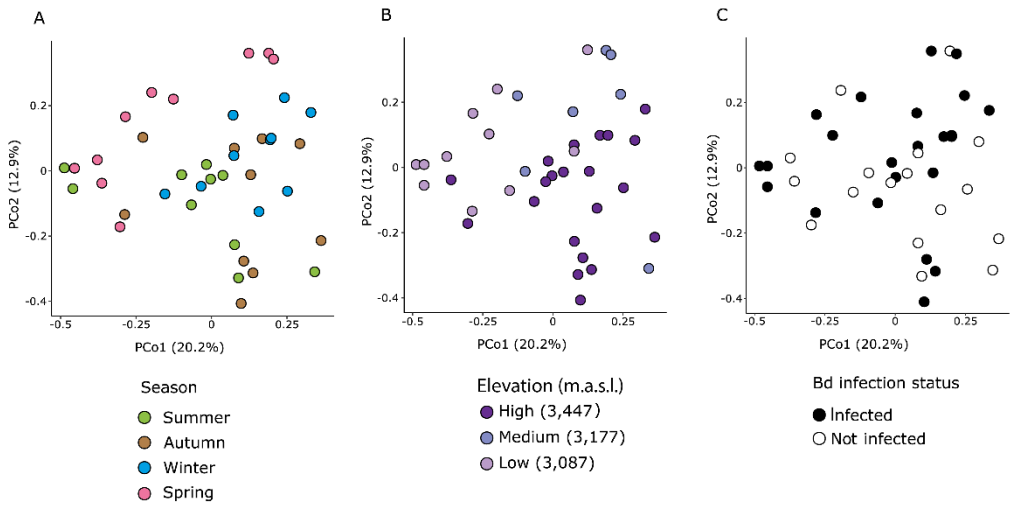


Figure 2. Principal coordinate analyses (PCoA) comparing the functional genomic diversity of A. altamirani skin microbiome based on Bray-Curtis distances. A) Ordination comparing functional genomic diversity across seasons. B) Ordination comparing functional genomic diversity across site elevations. C) Ordination comparing functional genomic diversity between Bd infected and not infected axolots.

For the autumn-winter comparison, 95.3% (752 out of 789) of the genes were significantly enriched in autumn, while the rest were enriched in winter (Figure 3B). For the winter-spring comparison, 84.4% (3,428 out of 4,063) of the genes were significantly enriched during winter, and the rest were significantly enriched in spring (Figure 3C). Differentially enriched genes found across seasonal comparisons were mainly linked to the COG categories S (function unknown), L (replication, recombination, and repair), E (amino acid transport

and metabolism), M (cell wall/membrane/envelope biogenesis) and C (energy production and conversion).

When comparing axolotl metagenomes from distinct elevations, we found that 432 genes were differentially enriched between the high-medium comparison, 84% (363 out of 432) them were significantly enriched in high elevation sites, while the rest were significantly enriched in medium elevation sites (Figure 3D). A total of 2,753 and 2,395 genes were differentially enriched in high-low (Figure 3E) and medium-low (Figure 3F) comparisons respectively. Noteworthy, 95% (2,628 out of 2,753) and 99.3% (2,379 out of 2,395) of high-low and medium-low comparisons were enriched in low elevations samples respectively. Differentially enriched genes found along the elevation gradient were mainly linked to the COG categories S (function unknown), L (replication, recombination, and repair), and M (cell wall/membrane/envelope biogenesis).

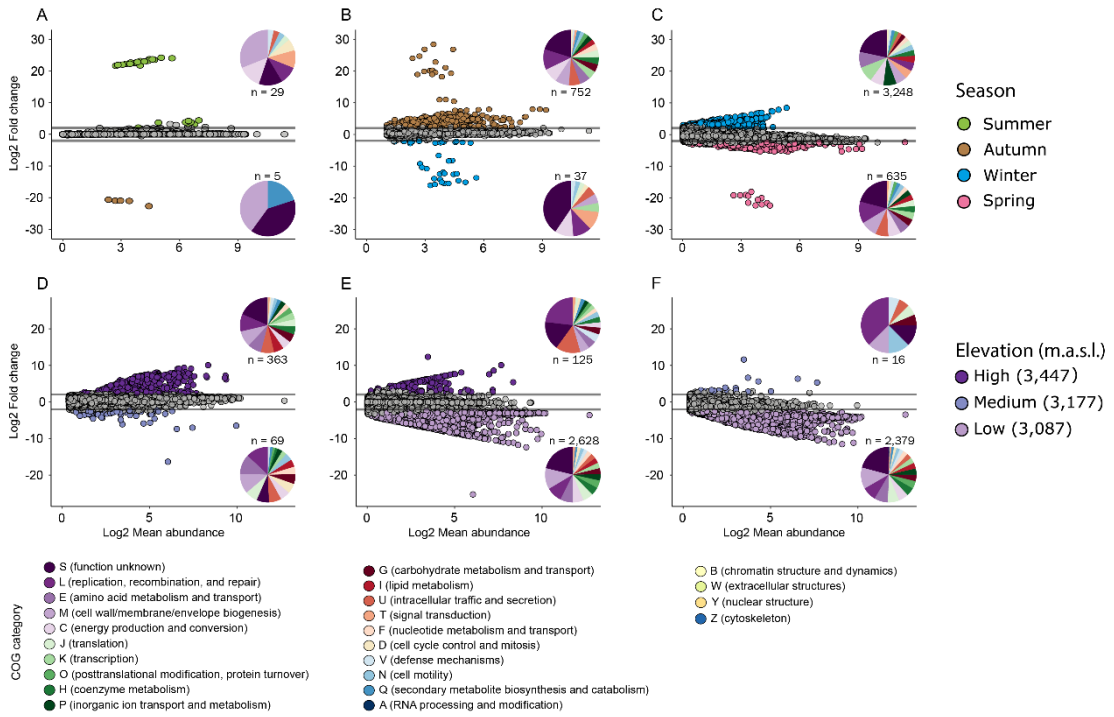

Figure 3. Differentially enriched genes between seasonal and site elevation pairwise comparisons identified with DESeq2 and shown in MA plots. A) Summer-autumn comparison. B) Autumn-winter comparison. C) Winter-spring comparison. D) High-medium elevation comparison. E) High-low elevation comparison. F) Medium-low elevation comparison. Solid black lines represent a 2 Log Fold change threshold. Pie plots inside each panel depict the functional identity of the differentially enriched genes based on COG categories.

We assessed whether the presence of Bd influenced the functional genomic diversity within each season and elevation. Our results showed that only during winter genes differ significantly between Bd infected and not infected axolotls (PERMANOVA, F = 2.62, p-value = 0.035) (Supplementary Figure 1A-D), Supplementary Table 5). A total of 1,091 genes were identified between Bd infected and not infected axolotls within the winter season and 99.9% of them were enriched in not infected axolotls. These genes were mainly classified under the S (function unknown), M (cell wall/membrane/envelope biogenesis), E (amino acid metabolism and transport), and L (replication, recombination, and repair) COG categories (Supplementary Figure 1C). No significant differences were identified between Bd infected and not infected axolotls across different elevations (Supplementary Table 5).

## Potential antifungal functions on the *A. altamirani* skin microbiome vary in time, space, and Bd infection status

A total of 5,196 (out of 42,281) genes of the AaSMGC were linked to BCC and AF. Our results showed that 72.8% of these genes were linked to secondary metabolism, followed by quorum sensing (15.95%), biofilm formation (8.8%), secretion systems (1.67%) and chitinolytic enzyme biosynthesis (0.71%) Changes in the abundance profiles of BCC and AF genes showed that these genes varied across seasons (Figure 4A) (PERMANOVA, F = 1.98, p-value = 0.002). However, pairwise comparisons showed that genes significantly varied only between autumn and winter seasons (PERMANOVA, F = 2.52, p-

value = 0.034) (Supplementary Table 6) and 95.7% of them were enriched in autumn and mainly belonged to secondary metabolism.

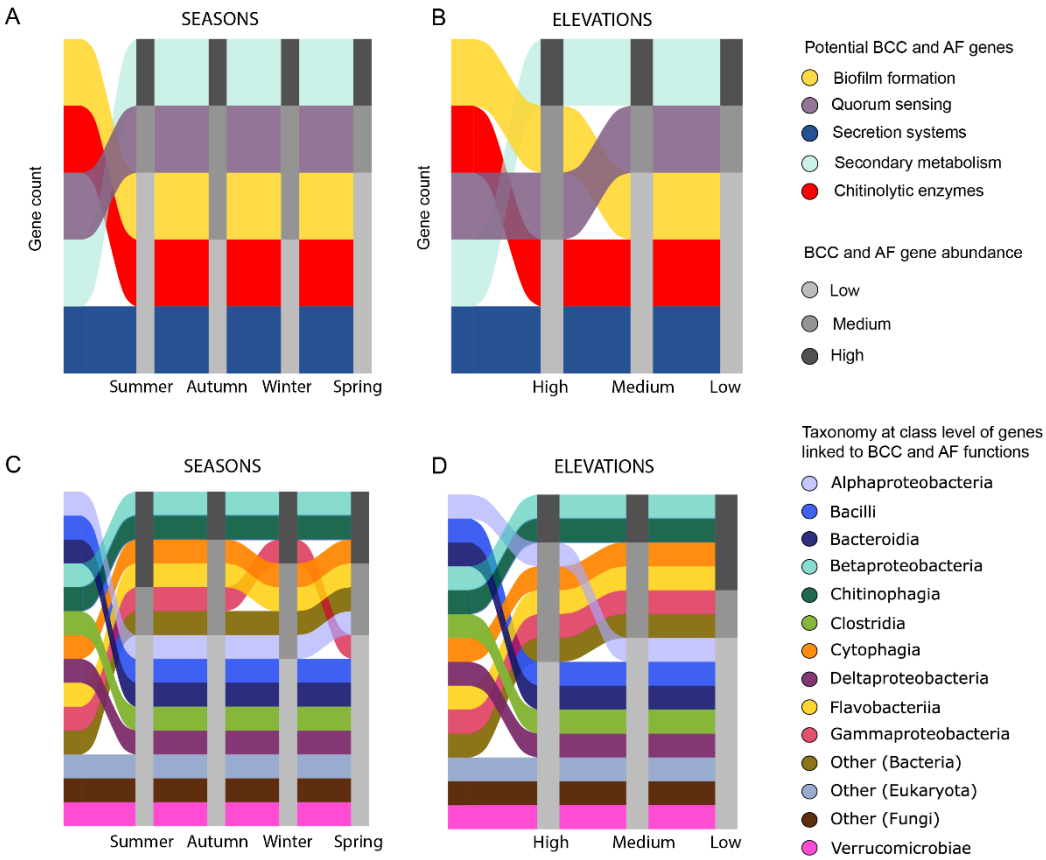


Figure 4. Changes in gene abundances linked to BCC and AF functions across seasons and elevations. A) Alluvial plot showing gene count variation of BCC and AF genes across seasons. B) Alluvial plot showing gene count variation for each gene linked to BCC and AF functions across elevation sites. C) Alluvia plot showing taxonomic identity variation at class level for the BCC and AF genes between consecutive seasons, D) Alluvial plot showing taxonomic identity variation at class level for the BCC and AF genes across site elevation. Bars in the alluvial plots colored black-gray scale depict abundance levels for genes within each season or elevation range.

In addition, our results showed that BCC and AF genes varied significantly among elevations (PERMANOVA, F = 2.63, p-value = 0.006) including all pairwise comparisons (Figure 4B) (Supplementary Table 7). Most genes showing significant changes in abundance across elevations were linked to secondary metabolism (Figure 4D). Noteworthy, 52.2% of the BCC and AF genes were classified at the class level as Betaproteobacteria, followed by

Gammaproteobacteria (20.9%), and Alphaproteobacteria (11.2%) derived genes (Figure 4C and D).

Moreover, the results showed that BCC and AF genes significantly varied between infected and not infected axolotls only during the winter season (PERMANOVA, F = 3.53, p-value = 0.03 between Bd) (Supplementary Table 8), and 72.2% (112 out of 155) of these significantly enriched genes were linked to the secondary metabolite biosynthesis.

## MAGs from the *A. altamirani* skin microbiome are taxonomically diverse and prevalent across seasons and elevations

To better understand the contribution of specific bacterial groups and their contribution to pathogen protection, we obtained metagenome-assembled genomes (MAGs) from the *A. altamirani* skin microbiome. After binning and MAG refinement, we recovered 151 MAGs with ≥ 50% completeness and ≤ 10% contamination. After dereplication, the initial set of 151 MAGs was reduced to 50 not redundant MAGs. These ranged from 0.76 Mb to 6.8 Mb, with N50 values ranging from 2.1 Kb to 271.5 Kb (Supplementary Table 9).

The 50 MAGs recovered from the *A. altamirani* skin microbiome were classified into four different bacterial phyla (Bacteroidetes, Bdellovibrionota, Patescibacteria, and Proteobacteria) and nine bacterial orders (Figure 5A). According to GTDB-Tk classification criteria based on the relative evolutionary divergence (RED) of MAGs, 16 of these MAGs were identified as taxonomic novelties. Novel MAGs were classified at order level as Chitinophagales (8 MAGs), AKYH767 (1 MAG, within the phylum Bacteroidetes), Burkholderiales (5

MAGs), Rickettsiales (1 MAG), and BD1-5 (1 MAG, within the phylum Patesibacteria) (Figure 5A).

A total of 43,286 genome copies per million reads (GCPMR) from the 50 not redundant MAGs were found across the 40 skin microbiome samples. Burkholderiales (23,904 GCPMR) and Chitinophagales (13,319 GCPMR) MAGs were the most abundant accounting for 85% of the relative abundance of MAGs across seasons and elevations (Figure 5B, C, Supplementary Table 9). Moreover, the 50 MAGs were prevalent across seasons (Figure 5B, Supplementary Table 10) and elevations (Figure 5C, Supplementary Table 11), with a few exceptions (Figure 5B and C).

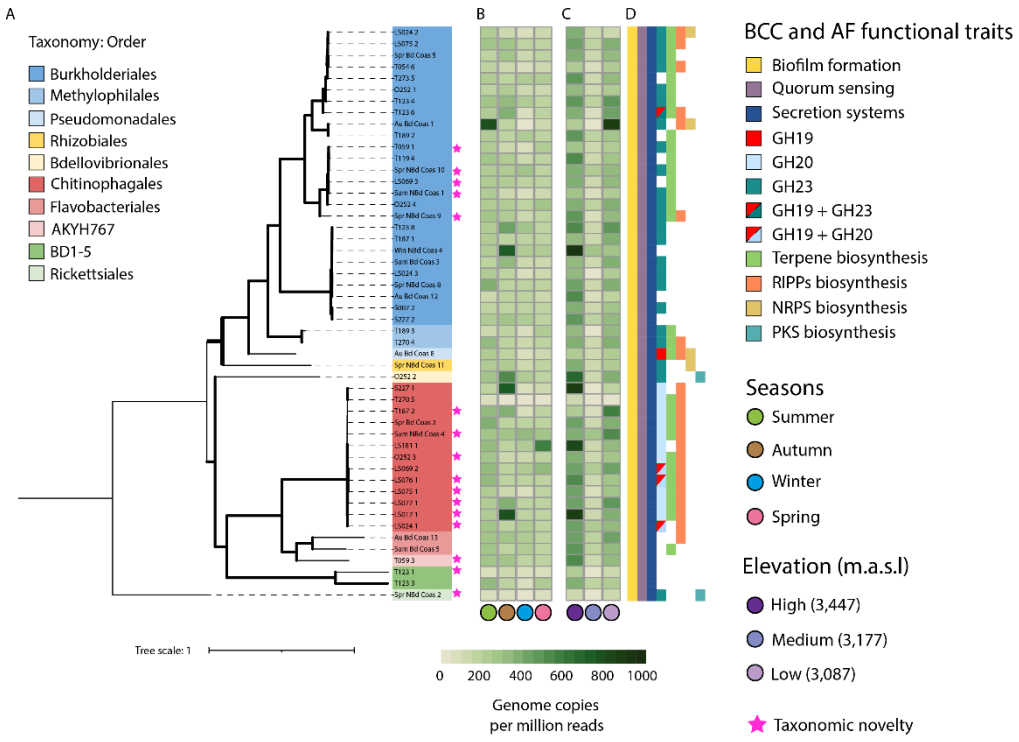


Figure 5. MAG taxonomy, abundance, and antifungal potential. A) Maximum likelihood tree of the 50 *A. altamirani* skin microbiome derived MAGs based on GTDB-Tk taxonomic classification. Pink starts aside tree tips depict MAGs classified as taxonomic novelties by GTDB-Tk. B) Summarized MAG abundance as GCPMR between samples within seasons. C) Summarized MAG abundance as genome copies per million reads (GCPMR) between samples within elevations. D) Potential antifungal functions predicted for each MAG. Bold lines in the maximin likelihood tree represent branches with bootstrap support higher than 70.

## BCC and AF genes are a common functional trait in *A. altamirani* derived MAGs

We found that genes linked to BCC were present at least once in all the 50 recovered MAGs (Figure 5D). In addition, MAG annotation revealed the presence of genes linked to chitinolytic enzyme biosynthesis in 38 MAGs, which were mainly from the orders Burkholderiales and Chitinophagales. Chitinolytic enzyme coding genes were annotated as glycoside hydrolases from the GH19, GH20, and GH23 families. The families GH19 and GH23 are recognized as chitinolytic enzymes, while GH20 act as N-acetylglucosaminidases.

Biosynthetic gene clusters (BGCs) were predicted for each MAG using antiSMASH (Supplementary Table 12). Our results showed that BGCs were present in 40 of the 50 MAGs recovered from the *A. altamirani* skin microbiome (Figure 5D). Specifically, we identified 79 BGCs (Supplementary Table 12), most of them linked to terpene biosynthesis (34 out of 79) and ribosomally synthesized and post-translationally modified peptides (RiPP, 27 out of 78) (Figure 5D). Noteworthy 63 (out of 79) of the predicted BGCs were identified in Burkholderiales (36 BGCs), and Chitinophagales (27 BGCs) MAGs (Figure 5D).

## Discussion

In this work, we described the functional genomic diversity of the *A. altamirani* skin microbiome and evaluated whether general and potential antifungal traits varied across time (seasonal), space (elevations), and in relation to Bd presence. We compiled a gene catalog (AaSMGC) composed of unique annotated genes to describe functional genomic variation across

seasons, elevations, and between Bd infected and not infected axolotls. Then we tested whether genes linked to BCC and AF traits varied in response to the same variables. Lastly, we evaluated the potential contribution of the most abundant bacterial groups in host protection against Bd through the analysis of MAG taxonomic and functional traits.

It has been shown that amphibian skin microbiome taxonomic diversity is influenced by several biotic and abiotic factors [64, 72, 74, 99], however, it remains unclear whether taxonomic diversity variation is reflected in microbiome functional genomic diversity. We previously reported that bacterial communities of *A. altamirani* skin varied across seasons and sampling locations (located at distinct elevations) [75]. Here we demonstrated that *A. altamirani* skin microbiome genomic functions varied significantly across seasons and elevations and that genes linked to functional traits related to i) replication, recombination, and repair, ii) amino acid metabolism and biosynthesis, iii) cell wall/membrane/envelop biogenesis and iv) energy production and conversion, were those that exhibited the greatest variation in time and space.

Previous evidence suggests that Bd presence has contrasting effects on amphibian skin microbiome. In some amphibian species skin, microbial diversity significantly differs between Bd infected and not infected hosts [63, 64, 66, 69, 73], but in other species including *A. altamirani*, no changes in the microbial diversity are detected [75, 100, 101].

Here we showed that the functional genomic diversity of *A. altamirani* skin microbiome differs between Bd infected and not infected axolotls only during one season (winter). This result suggests possible interactions between the pathogen and season specific factors that would require further

explorations. This is particularly interesting considering that increased prevalence [102, 103] and mortality [104, 105] due to Bd infection have been reported in other amphibian species during winter. In addition, our group previously showed that Bd infection intensity and prevalence were higher during winter in the same *A. altamirani* populations analyzed in this work [76].

The antifungal potential of amphibian skin microbiomes has been studied in various amphibian species [38, 52, 56, 57, 73, 106] but little is known about the genetic mechanisms behind these functional traits. Specifically, to date, it has been shown that microbial functional traits linked to BCC (biofilm formation [40], quorum sensing [21, 107], and secretion systems [58]) as well AF (biosynthesis of secondary metabolites [53, 58, 108] and chitin degradation [22]) could be responsible to host protection against Bd.

We found that genes linked to BCC and AF were identified in all *A. altamirani* skin metagenomes regardless of changes in gene abundance across seasons and elevations. Noteworthy BCC and AF genes were mainly derived from the Proteobacteria and Bacteroidetes phyla, suggesting that these taxonomic groups play an important role in host protection against pathogens as seen in *in vitro* assays [45, 51, 52, 56, 57].

To evaluate the potential contribution of specific bacterial groups in host protection against pathogens, we recovered a set of 50 unique MAGs from A. altamirani skin and described the presence of genes linked to BCC and AF. Given that BCC genes (biofilm formation, quorum sensing, and secretion systems) could play a critical role in bacterial responses to environmental cues [41, 42, 44, 109] we expected to find them in *A. altamirani* MAGs and our results showed that these genes were present in all the recovered MAGs.

Considering that only a handful of metabolites with anti-Bd properties derived from bacteria present in amphibian skin microbiomes have been fully characterized [21, 45, 53–55], the use of genomic approaches has emerged as a promising to tool identify novel potential compounds with anti-Bd activity [22, 58, 108]. Previous studies have identified chitinolytic enzymes [22], ribosomal and not ribosomal peptides (RiPP and NRPS), aryl-polyenes, polyketides, or bacteriocins [108, 110, 111] with potential anti-Bd activity.

Here we identified genes linked to chitinolytic enzyme biosynthesis and several BGCs in MAGs recovered from *A. altamirani skin* microbiome, and most of these genes were identified in Burkholderiales (Proteobacteria) and Chitinophagales (Bacteroidetes) MAGs. We previously showed that Burkholderiales and Chitinophagales were highly abundant on *A. altamirani* skin especially in not metamorphic axolotls [75], and it has been shown that these bacteria from these orders can inhibit the growth of Bd in experimental assays [56, 57, 108, 112].

Chitinolytic enzyme biosynthesis has been recognized as a defense mechanism [46, 113–115] employed by bacteria, plants, animals, and fungi [116–118] to inhibit the growth of fungi. In the case of amphibians, it has been shown that amphibians exposed to Bd exhibit an increased abundance of a bacterial-derived chitin deacetylase [22], an enzyme that contributes to chitin degradation [119]. Our analysis revealed the presence of genes linked to glycoside hydrolases from the families GH19 [120], GH20 [121, 122], and GH23 [113, 123, 124] which are recognized as chitinolytic enzymes. Noteworthy GH23 enzymes were mainly identified in Burkholderiales MAGs, while GH20 enzymes

518 were identified only in Chitinophagales MAGs suggesting that bacteria from
519 these orders may use different strategies to degrade chitin.

520 BGCs identified in Burkholderiales and Chitinophagles MAGs were
521 mainly associated with terpene and RiPP biosynthesis. Specifically, terpenes
522 are a vast group of chemical compounds produced by a wide range of
523 organisms from bacteria to plants [125–127], and these metabolites have
524 diverse ecological functions related to environmental stress responses [128],
525 host-microbial communication [129, 130], and microbial-microbial competition
526 [116, 126, 131]. Recent studies suggest that bacterial derived terpenes
527 metabolites could act as sex specific scents in amphibians [132], and genes
528 linked to terpene biosynthesis have been identified in the skin microbiomes of
529 various frog species [58, 133] and in the genomes of bacteria isolated from the
530 amphibian skin [134]. However, it remains unclear if terpenes produced by
531 amphibian skin bacteria can inhibit the growth of Bd.

532 RiPPs are a wide group of peptides mainly implicated in microbial-
533 microbial interactions [135, 136], However, nearly half of the RiPP clusters
534 predicted in this study were linked to bacteriocin biosynthesis, thus they likely
535 play a role in bacteria-bacteria interactions [137–139] within the skin bacterial
536 community of A. altamirani.

537 **Conclusions**

538 Overall, our results indicate that the functional genomic diversity of the *A.*
539 *altamirani* microbiome varies across time and space (seasons and elevations),
540 suggesting that taxonomic variation [75] is directly linked to functional variation
541 in this system. Moreover, we identified genes linked to BCC and AF functions in

all the recovered MAGs, suggesting that potential antifungal functions are a prevalent trait in the skin microbiome of *A. altamirani* likely contributing to host tolerance against Bd infection. Some of these genes code for terpenes and chitinolytic enzymes that deserve future explorations with respect to their potential antifungal activity and protective role against chytridiomycosis.

## Author contributions

EAR conceptualized the study and secured funding for the project. EM-U and EAR, TG-M and VA-A performed sample collection and processing. EM-U performed all bioinformatic analyses. EM-U and EAR interpreted the data and wrote the manuscript.

## Conflicts of interest

Authors declare no conflicts of interest.


## Funding information

This work was supported by UNAM-PAPIIT IA200921 granted to EAR.


## Ethical approval

Our research was approved by the ethical standards of Universidad Nacional Autónoma de México, additionally capture and sampling of *A. altamirani* was approved by Subsecretaría de Gestión para la Protección Ambiental under the permit number: SGPA/DGVS/5673/19.


## Acknowledgments

Emanuel Martínez-Ugalde is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México

(UNAM), and has received a Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCyT) fellowship (781100).

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## Capítulo 6. Discusión

Allan Konopka planteó en su ensayo “What is microbial community ecology?” tres preguntas principales que deberíamos abordar al estudiar comunidades de microrganismos que son: i) ¿Cuál es la estructura de estas comunidades?, ii) ¿Cuál es la función de estas comunidades?, y iii) ¿Cómo varia la estructura y la función de estas comunidades a través del tiempo y el espacio? A continuación, se presentarán una serie de conclusiones basadas en los datos generados en este trabajo con la intención de abordar cómo los resultados obtenidos nos permiten responder en parte a estas preguntas.

¿Cuál es la estructura de las comunidades de microorganismos de la piel de *A. altamirani*?

Los resultados presentados en el capítulo 3 de este trabajo muestran que la estructura y diversidad bacteriana de la microbiota de la piel de *A. altamirani* piel difiere significativamente entre ajolotes metamórficos (sin branquias) y pre-metamórficos (con branquias). De acuerdo con la abundancia y clasificación de los amplicones del 16S rRNA obtenidos de ambos morfos, se describo que las familias de bacterias Burkholoderiaceae, Chitinophagaceae y Pseudomonadaceae son las más abundantes sobre la piel de ajolotes metamórficos y pre-metamórficos. El hecho de que estas tres familias bacterianas sean altamente abundantes sobre la piel de ambos morfos se puede explicar si consideramos que ajolotes metamórficos y pre-metamórficos coexisten en el mismo ambiente y por ende están expuestos a la misma fuente de microorganismos ambientales.

A pesar de que, a nivel de familia las comunidades bacterianas de la piel de ajolotes metamórficos y pre-metamórficos son dominadas por las familias Burkholoderiaceae, Chitinophagaceae y Pseudomonadaceae. Las comparaciones de las métricas de diversidad alfa entre ambos morfos (índice de Shannon y diversidad filogenética de Faith) mostraron que la microbiota de la piel de ajolotes metamórficos presenta una mayor diversidad bacteriana.

Por su parte, la comparación de las distancias UniFrac de la microbiota de la piel entre ajolotes metamórficos y pre-metamórficos mostró que la estructura de las comunidades bacterianas de piel difiere de manera significativa entre ambos morfos. Con base en estas comparaciones se puede decir que las comunidades bacterianas de la piel de ajolotes metamórficos son más estables a lo largo del tiempo a diferencia de las comunidades bacterianas de la piel de ajolotes pre-metamórficos que mostraron mayor variación entre estaciones y localidades de muestreo.

Estos resultados resaltan la influencia de la metamorfosis sobre la microbiota de la piel de *A. altamirani*, y permiten sugerir que posiblemente los mecanismos relacionados con el reclutamiento de miembros de la microbiota de la piel como, los cambios en la estructura química de las mucosas o un sistema inmune estable podrían explicar los patrones de diversidad que se observaron entre ajolotes metamórficos y pre-metamórficos [164].

Diferencias ecológicas asociadas a la preferencia de hábitat entre individuos metamórficos y pre-metamórficos podrían explicar en cierto grado los patrones de diversidad registrados entre los dos morfos de *A. altamirani*. Sin embargo, todos los ajolotes que se analizaron en este trabajo fueron capturados dentro de

cuerpos de agua, y la literatura indica que los individuos metamórficos de esta especie no abandonan los cuerpos de agua [229,230], pero debido a la falta de estudios ecológicos en esta y otras especies de *Ambystoma*, no podemos descartar que los ajolotes metamórficos visiten hábitats terrestres esporádicamente y por ende estén expuestos a grupos de microgramos que no están presentes dentro de su hábitat acuático.

Los resultados presentados en el Capítulo 4 confirmaron que las familias bacterianas Burkholderiaceae y Chitinophagaceae son altamente abundantes sobre la piel de *A. altamirani*. Adicionalmente, el trabajo realizado para la segunda parte de este trabajo (capitulo 4) reveló la presencia de arqueas, hongos, eucariotas microscópicos (e.g. algas y ciliados) y partículas virales sobre la piel de *A. altamirani*. Sin embargo, de acuerdo con los resultados obtenidos estos grupos de microorganismos presentan abundancias relativas bajas comparadas con el componente bacteriano del microbioma de la piel de esta especie de ajolote.

ii) ¿Cuál es la función de las comunidades microbianas de la piel de *A. altamirani*?

En las primeras secciones de este trabajo se discutió el papel que juegan los microorganismos de la piel de los anfibios en la defensa contra los hongos causantes de la quitridiomicosis. Para el capítulo 4 de este trabajo se describió la diversidad funcional del microbioma de la piel de *A. altamirani*, y se evalúo la influencia de la estacionalidad, elevación y presencia de Bd sobre las diversidad funcional del microbioma de la piel.

Debido a la importancia que los microbiomas de la piel de los anfibios tienen en la defensa contra patógenos, los análisis del capítulo 4 se centraron en describir la presencia de genes relacionados con posibles funciones antifúngicas en el microbioma de la piel de *A. altamirani*. Específicamente se evalúo la presencia de genes implicados en: formación de biopelículas, comunicación bacteriana, sistemas de secreción, síntesis de metabolitos secundarios y degradación de quitina.

Mediante la anotación funcional de un catálogo de genes se confirmó la presencia de genes asociados a posibles funciones antifúngicas. Los resultados revelaron que, genes relacionados con formación de biopelículas, comunicación bacteriana y síntesis de metabolitos secundarios son prevalentes a lo largo de los gradientes ambientales que se analizaron; sin embargo, estos genes difieren en abundancia de manera significativa entre estaciones y elevaciones.

Por su parte, se predijo la presencia de clústers biosintéticos y genes asociados a la síntesis de enzimas quitinolíticas en los MAGs recuperados del microbioma de la piel de *A. altamirani*. Interesantemente estos genes fueron predichos con mayor frecuencia en MAGs de las familias Burkholderiales y Chitinophagales que son altamente abundantes sobre la piel de esta especie de ajolote, específicamente se predijo la presencia de clústers biosintéticos asociados con la síntesis de terpenos y de glicosil hidrolasas de las familias GH20 y GH23. Sin embargo, hasta el momento no existen suficientes reportes sobre la actividad inhibitoria de estas enzimas contra Bd o Bsal en ensayos *in vitro*.

Es importante mencionar que los resultados del capítulo 4 deben ser tomados con cautela, ya que la descripción funcional del catálogo de genes y los MAGs esta sesgada a la información disponible en las bases de datos. Esto implica que los resultados presentados podrían estar subestimando la diversidad de posibles mecanismos de inhibición contra Bd. Ejemplo de esto, es el hecho de que únicamente el 48.24% de los genes presentes en el catálogo del microbioma de la piel de *A. altamirani* se lograron anotar de manera funcional.

Adicionalmente, la caracterización funcional que se realizó en este trabajo se enfocó únicamente en describir la presencia e identidad de posibles mecanismos de inhibición contra patógenos fúngicos, sin considerar otras funciones que podrían ser importantes en la interacción microbioma-hospedero o interacciones inter-especie o intra-especie entre los integrantes de las comunidades de microorganismos. Sin embargo, vale la pena resaltar que los resultados de los capítulos 3 y 4 de este trabajo siguieren que el microbioma de la piel de *A. altamirani* juega un papel importante en la defensa contra Bd, mediante las síntesis de diversos compuestos antifúngicos. Además, la alta abundancia de las familias Burkholderiales y Chitinophagales aunado a la presencia de genes relacionados con posibles funciones antifúngicas, sugieren que estos grupos de bacterias podrían tener un papel esencial en el estado de salud de estos ajolotes frente a la quitridiomicosis.

iii) ¿Cómo varia la estructura y la función de las comunidades de microrganismo de la piel a través del tiempo y el espacio?

En los capítulos 3 y 4 de este trabajo se evaluó la influencia de una serie de factores bióticos y abióticos como: i) el estado metamórfico de los ajolotes, ii) la

estacionalidad, iii) la presencia de Bd, y iv) la localidad de muestreo sobre el microbioma de la piel de *A. altamirani*. Los resultados presentados en estos capítulos demostraron que las comunidades de microorganismos de la piel de *A. altamirani* varían significativamente con relación al tiempo (e.g., gradiente estacional) y el espacio (e.g., localidad de muestreo).

Específicamente, los resultados mostraron que el estado metamórfico, la localidad de muestreo y a la estacionalidad, influyen de manera importante sobre la diversidad taxonómica de las comunidades de bacterias presentes en la piel de *A. altamirani*. Por su parte, la diversidad funcional del microbioma varió de manera significativa entre las localidades de muestreo que están ubicadas en distintos rangos de elevación. Notablemente, la diversidad funcional mostró patrones de variación entre estaciones similares a los reportados para la diversidad taxonómica lo que sugiere que la diversidad funcional depende de la estructura y diversidad de las comunidades de microorganismos de la piel.

En cuanto a la influencia que tiene Bd sobre la diversidad taxonómica y funcional del microbioma de la piel de *A. altamirani*, los resultados mostraron que la presencia del patógeno no influye sobre la diversidad del microbioma. Sin embargo, la interacción de otros factores como las estacionalidad y la intensidad de la infección tienen un efecto sobre los patrones de diversidad taxonómicos y funcionales del microbioma de la piel.

La variación estacional y geográfica de factores fisicoquímicos y biológicos influyen de manera importante sobre el microbioma de la piel de *A. altamirani*. Sin embargo, es importante mencionar que factores como, la variación genética entre las poblaciones de ajolotes que se analizaron en este trabajo [240,241],

también expliquen en cierta media los patrones de diversidad taxonómica y funcional del microbioma de la piel de *A. altamirani*.

Finalmente, vale la pena mencionar que el diseño experimental planteado para el capítulo 4 de este trabajo estuvo sesgado debido a la disponibilidad de material genético para secuenciar las muestras de ajolotes metamórficos. Sin embargo, los resultados mostrados en los capítulos 3 y 4 permiten sugerir que la diversidad funcional del microbioma de la piel de ajolotes metamórficos varia de manera significativa en respuesta a la variación estacional. Adicionalmente, los datos presentados en el capítulo 3 permiten sugerir que la diversas funcional del microbioma de ajolotes metamórficos difiere significativamente de individuos pre-metamórficos, en futuros trabajos sería interesante evaluar las diferencias funcionales del microbioma asociadas al estado metamórfico del hospedero.

## Capítulo 7. Conclusiones

En este trabajo se describió la influencia de diversos factores bióticos y abióticos sobre la diversidad taxonómica y funcional del microbioma de la piel del ajolote de arroyo de montaña (*A. altamirani*).

Específicamente, los resultados presentados en los capítulos 3 y 4 de este trabajo demuestran que:

i) Las comunidades bacterianas asociadas a la piel de *A. altamirani* difieren de manera significativa de las comunidades ambientales del ambiente donde estos ajolotes habitan. Estas observaciones coinciden con observaciones previas que sugieren que los anfibios seleccionan que microorganismos pueden colonizar su piel [171].

ii) El estado metamórfico del hospedero es el factor biótico que influye en mayor medida sobre la diversidad taxonómica del microbioma de la piel de A. altamirani. Además, la influencia de factores abióticos como la estacionalidad y la ubicación geográfica depende del estado metamórfico de los hospederos. Específicamente los resultados demostraron que las comunidades bacterianas de la piel de individuos metamórficas presentan menor variación cuando se compara con las comunidades de los individuos pre-metamórficos.

iii) Las diferencias observadas en la diversidad y estructura de las comunidades bacterianas entre ajolotes metamórficos y pre-metamórficos están explicadas por cambios en la abundancia de bacterias de las familias Chitinophagales, Burkholderiaceae o Verrucomicrobiaceae.

iv) La variación en la diversidad genómica funcional del microbioma de ajolotes pre-metamórficos entre estaciones y elevaciones, es un reflejo de los cambios en la diversidad taxonómica de las comunidades bacterianas a causa de estos mismos factores abióticos.

v) El microbioma de la piel de A. altamirani cuenta con una serie de genes asociados a posibles funciones antifúngicas que varían entre estaciones y elevaciones, estos genes se derivan principalmente de bacterias de los órdenes Burkholderiales o Chitinophagales.

vi) A pesar de la alta prevalencia de Bd en las muestras analizadas en este trabajo, la presencia del patógeno no tiene un efecto significativo en la diversidad taxonómica y funcional del microbioma de *A. altamirani*. Por lo que, el estatus de tolerancia ante la quitridiomicosis reportado para A. altamirani podría estar explicado por la alta abundancia de grupos bacterianos que cuentan con genes asociados a posibles funciones antifúngicas.

## Capítulo 8. Perspectivas

Los resultados generados en este trabajo contribuyen a entender los factores que influyen sobre los patrones de diversidad taxonómica y funcional del microbioma de la piel de los ajolotes, un grupo de salamandras que hasta el momento se encuentra subrepresentado entre los diversos estudios que han caracterizado el microbioma de la piel de los anfibios. Considerando algunos de los hallazgos principales de este trabajo se presentan a continuación algunos puntos que considero importantes para entender mejor la relación que existe entre *A. altamirani* y las comunidades microbianas presentes sobre la piel de este ajolote.

1. Se requiere de una descripción más amplia de la diversidad taxonómica y funcional del microbioma de la piel de anfibios, en donde no solo se considere la porción bacteriana de la microbiota. En este sentido, la descripción de las comunidades fúngicas, de micro eucariotas y partículas virales mediante la secuenciación de librerías de amplicones de regiones como los ITS, el gen 18S rRNA o análisis metagenómicos permitiría un mejor entendimiento de la diversidad y función de estos grupos sobre la piel de los anfibios. Sin embargo, la implementación de estrategias de culturómica acopladas a análisis genómicos, metagenómicos o transcriptómicos permitiría evaluar de mejor manera la contribución de determinados grupos de bacterias sobre la función inhibitoria contra patógenos y explorar las interacciones inter-especie que ocurren entre los integrantes del microbioma de la piel.
2. Es necesario explorar los mecanismos de control que los ajolotes ejercen sobre las comunidades microbianas mediante aproximaciones

experimentales. Los resultados del capítulo 3 sugieren que los cambios fisiológicos que ocurren durante la metamorfosis influyen de manera importante sobre la diversidad bacteriana entre ajolotes metamórficos y pre-metamórficos. Considerando que se ha reportado que los AMP's pueden promover el crecimiento de ciertos grupos de bacterias simbiontes de la piel [182]. Sería interesante evaluar si: 1) este mismo efecto ocurre entre *A. altamirani* y las comunidades de microorganismos presentes sobre su piel; 2) de ser el caso, ¿este efecto es diferencial entre grupos bactrianos específicos? (e.g. grupos presentes en abundancias diferenciales); además, 3) explorar los mecanismos implicados en la interacción entre *A. altamirani* y su microbioma, por ejemplo, mecanismos de resistencia hacia AMP's sintetizados por el hospedero.

3. Por último, los resultados de este trabajo revelaron que la variación de diversos factores fisicoquímicos entre estaciones influye sobre las comunidades microbianas de la piel de *A. altamirani*. Sin embargo, se sabe muy poco sobre el efecto de la variación de factores específicos como la temperatura o pH sobre la fisiología de los miembros del microbioma. Por esta razón, realizar ensayos controlados en donde los miembros del microbioma sean expuestos a gradientes de temperatura o pH, nos permitirían entender mejor el impacto que la variación ambiental tiene sobre las comunidades microbianas de la piel de los anfibios y especialmente sobre su capacidad inhibitoria.

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## Capítulo 9. Apéndices

Durante mi estancia en el doctorado tuve la oportunidad de participar como coautor y colaborar en el desarrollo de otros trabajos, los artículos publicados como parte de mi colaboración en esos proyectos se incluyen en esta sección.

Específicamente participe en la escritura de un artículo de revisión, en donde se discute el rol que de los microbiomas de la piel de anfibios en la defensa contra los patógenos causantes de la quitridiomicosis. Adicionalmente, el trabajo que realice en campo durante el primer año del doctorado, contribuyo a la descripción de la presencia de Bd y dinámicas de infección por este patógeno en las poblaciones en cuatro poblaciones de *A. altamirani*. Adicionalmente, se publicó una nota de divulgación en donde se reporta la presencia de Bd en individuos muertos de *A. altamirani* que presentaban altos grados de infección por este patógeno.

# The Amphibian Skin Microbiome and Its Protective Role Against Chytridiomycosis

Authors: Rebollar, Eria A., Martínez-Ugalde, Emanuel, and Orta, Alberto H.

Source: Herpetologica, 76(2) : 167-177

Published By: The Herpetologists' League

URL: https://doi.org/10.1655/0018-0831-76.2.167

*Herpetologica*, 76(2), 2020, 167–177


# The Amphibian Skin Microbiome and Its Protective Role Against Chytridiomycosis

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Abstract: Here we review the knowledge about skin microbiomes in amphibians accumulated over the last two decades and the evidence regarding the protective role of skin bacteria. Amphibians all over the world are declining because of several factors, including chytridiomycosis disease caused by the fungal pathogens *Batrachochytrium dendrobatidis* and *B. salamandrivorans*. In this context, the antifungal capacities of many bacteria living symbiotically on amphibian skin, which have been described both in vitro and in vivo, are important in disease prevention. We discuss the major factors influencing amphibian skin bacterial communities, the fungal component of the amphibian skin microbiome, and the potential use of antifungal bacteria as probiotics. The structure of amphibian skin microbial communities is influenced by host-specific microhabitat, biogeographic, and climatic factors, but the functional aspects of these microbiomes and how these nested factors modulate skin microbial functions remains largely unexplored. However, the field has grown considerably, and recent technologies have prompted the exploration of exciting new questions aimed at providing more detailed knowledge about the ecology of amphibian–microbial symbioses and the precise role of the skin microbiome in protecting host amphibians against emerging diseases.

**Key words:** Antifungal bacteria; *Batrachochytrium*; Probiotics

Symbiotic relationships between microbes and multicellular organisms are ubiquitous in nature. In the last two decades, a great body of evidence has shown that microbes associated with animals and plants play several functions that are relevant for host health and survival (Berg 2009; Berendsen et al. 2012; Cho and Blaser 2012; Ross et al. 2019). These microbes, and their genetic repertoire, are known collectively as microbiomes. Even though most microbiome research has focused on humans (Turnbaugh et al. 2007; Spor et al. 2011; Balter et al. 2012), several recent studies have contributed knowledge about the role of microbiomes in a large variety of animal groups, including cnidarians, sponges, corals, insects, amphibians, birds, and mammals (McFall-Ngai et al. 2013; Fraune et al. 2014; Kwong et al. 2017; Clayton et al. 2018; Grond et al. 2018; Dunphy et al. 2019). The study of microbiomes has also opened new possibilities for finding successful conservation strategies for endangered species such as amphibians (Redford et al. 2012; Trevelline et al. 2019; West et al. 2019).

Chytridiomycosis has been known for some time to be a threat to amphibians (Lips et al. 2006; Briggs et al. 2010; Scheele et al. 2019). This disease, caused by *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998) and *B. salamandrivorans* (*Bsal*; Martel et al. 2013), has been implicated in population declines of more than 500 amphibian species and extinctions of at least 90 of them (Scheele et al. 2019). There is evidence that certain symbiotic bacterial species present on amphibian skin play an important role in protecting the host against chytridiomycosis (Harris et al. 2006; Becker et al. 2011; Kueneman et al. 2016a). Although we will focus on the role of symbiotic bacterial communities in host protection against chytridiomycosis, amphibians are also threatened by viral, bacterial, and parasitic diseases (Pessier 2014), and recent studies have started identifying correlations between viral and parasitic infections with changes in skin microbial diversity (Federici et al. 2015; Campbell et al. 2019; Harrison et al. 2019).

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The knowledge accumulated so far indicates that the skin microbiome is a line of defense against disease for amphibians in addition to, or in synergy with, the innate and adaptive immune system (Rollins-Smith 2020). To integrate and summarize the research on the amphibian skin microbiome, we compiled a database of all publications that we could find on this topic published from 2006 through 2019. We searched for publications using PubMed and ISI Web of Knowledge using the key words amphibians, microbiome, microbiota, frogs, toads, salamanders, newts, bacterial communities, skin microbiome, and skin microbiota. For each publication we documented the following features: year of publication, use of culture-dependent or independent methods, amphibian species studied (temperate or tropical), field survey or experimental approaches, and whether the study included associations, interactions or correlations with *Bd* or *Bsal*.

To our knowledge, 153 scientific papers on this topic were published between 2016 and 2019. As the number of publications increased, the number of studied species also increased, to 331, covering approximately 4.9% of the total amphibian species diversity (Fig. 1A). Still, many groups are underrepresented. Only four tropical species from the order Caudata were surveyed and no studies included members of the Gymnophiona (caecilians). In many cases, the studied species were only surveyed once. The majority of the studied amphibian species (67.4%) were ranked as Least Concern (LC) according to IUCN (International Union for Conservation of Nature), emphasizing the need to study more species that are considered to be threatened (Fig. 1A). Of all the publications analyzed here, 37.25% used culture-dependent approaches, 54.25% used culture independent approaches, and 8.5% used both. These studies not only have increased in number since the first findings in 2006, but have also transitioned from studies relying on bacterial culturing to metagenomic approaches (Fig. 1B), which have been boosted by significant advances in next-generation sequencing technologies (NGS) and bioinformatic tools.

The majority of the publications (65.4%) conducted field surveys of wild amphibian populations and addressed the

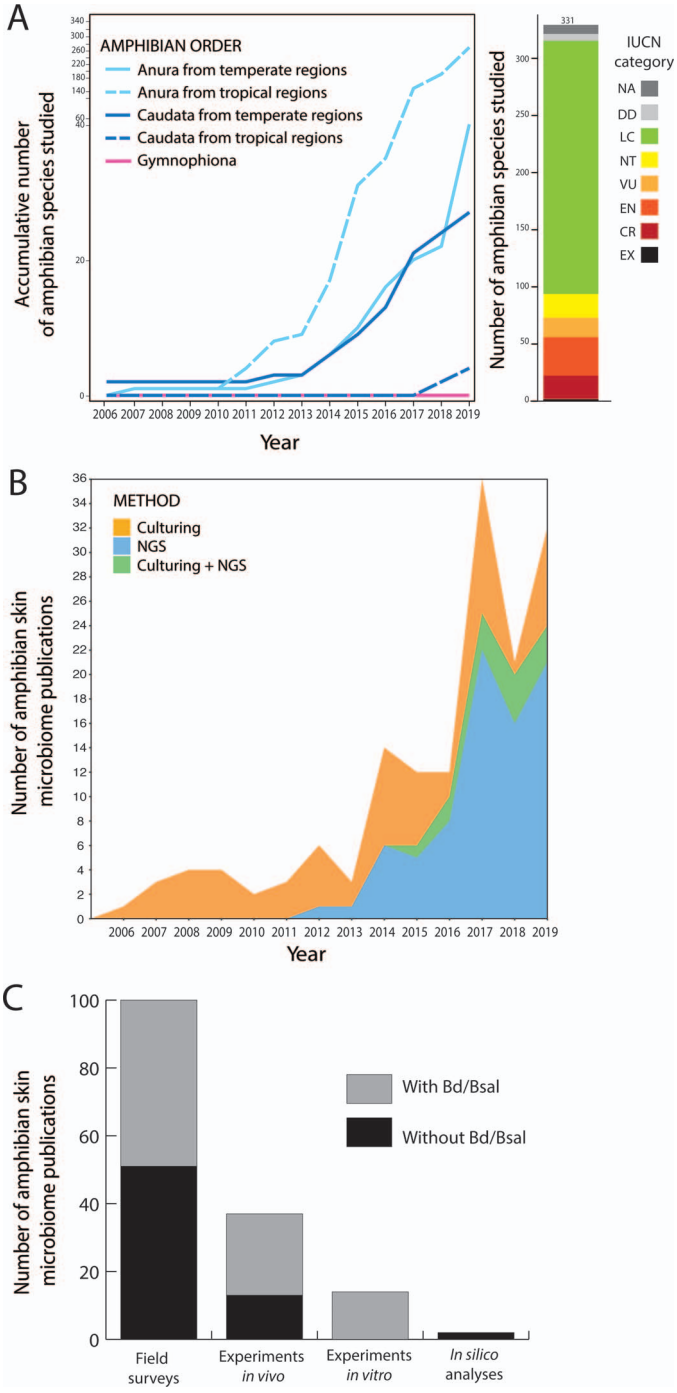


FIG. 1.—(A) Number of amphibian species for which the skin microbiome has been described, from 2006 to 2019. The left panel shows the accumulative number of studied species separated by order (Anura, Caudata, or Gymnophiona) and climate (temperate or tropical). The right panel indicates the proportion of species assessed as extinct (EX), critically endangered (CR), endangered (EN), vulnerable (VU), near threatened (NT), least concern (LC), or data deficient (DD) according to IUCN red list categories, or for which no data are available (NA). (B) Number of publications about the amphibian skin microbiome from 2016 through 2019, excluding reviews. Articles are divided into those that only used bacterial culturing techniques, those that only used next-generation sequencing (NGS) techniques and those that used both methods. (C) Number of publications about the amphibian skin microbiome from 2016 through 2019, classified according to methodology: field surveys, experimental trials in vivo, experimental trials in vitro and in silico analyses.

factors shaping skin microbiomes (e.g., McKenzie et al. 2012; Kueneman et al. 2014). In many of these studies, bacterial strains were isolated and tested in vitro for their antifungal capacities (e.g., Becker et al. 2015; Bletz et al. 2017a). Experimental trials on amphibians in vivo comprised 24.2% of the publications and included studies documenting changes in skin microbiota in response to factors like pathogen presence, probiotic inoculation, temperature variation, and host susceptibility (e.g., Harris et al. 2009a; Longo and Zamudio 2017a). Experimental trials of amphibian skin bacteria in vitro made up 9.2% of publications, including characterization of antifungal molecules produced by bacteria or evaluation of antifungal traits of bacterial synthetic communities (e.g., Brucker et al. 2008a; Antwis and Harrison 2018). Only 1.3% of publications conducted analyses in silico, including meta-analyses with available published data and genome sequencing of skin bacteria (Bletz et al. 2019; Kueneman et al. 2019). Independent of approach (field surveys or experimental), more than half of all publications (56.9%) correlated the presence and infection intensity of *Bd* or *Bsal*, or experimentally tested the growth inhibition capacity of bacteria or fungi against *Bd* or *Bsal* (Fig. 1C).

It is clear that the field of amphibian microbial ecology has expanded and has led to important findings. However, more research is needed. Field surveys are fundamental for describing the skin microbiota of many underrepresented species in consideration of their risk status and geographical distribution. Moreover, longitudinal analyses in the wild are scarce but very important to tease apart abiotic from biotic factors shaping these microbial communities. In addition, experimental trials are key to understanding fully the major ecological principles shaping amphibian skin microbiomes and the interactions occurring between hosts, pathogens, and symbiotic microbes.

## The Discovery of Skin Antifungal Bacteria in Amphibians

In one of the first publications on antifungal bacteria isolated from amphibian skin, Harris et al. (2006) isolated bacteria from the skin of Eastern Red-backed Salamanders, *Plethodon cinereus*, and Four-toed Salamanders, *Hemidactylium scutatum*, from the Appalachian Mountains. These bacterial strains belonged to eight genera and were able to inhibit the growth of several fungi, including *Bd*. Lauer et al. (2007) observed bacteria on the skin of *P. cinereus* using scanning electron microscopy and described the bacterial community using fingerprinting techniques that identified several dominant, nonculturable bacterial taxa. One of these dominant bacterial species from *P. cinereus* skin was the Betaproteobacterium, *Janthinobacterium lividum*, which was isolated in culture and proven to have a strong antifungal activity against *Bd* using in vitro challenge assays (Lauer et al. 2007). These findings were particularly relevant, as *P. cinereus* has shown resistance to *Bd* infection in experimental trials (Becker and Harris 2010; Venesky et al. 2015) and *Bd* prevalence in wild *P. cinereus* is historically very low or absent (Muletz et al. 2014). The antifungal activity of *J. lividum* and other skin bacteria was later explained by chemical analyses showing that these bacterial symbionts are able to secrete secondary metabolites such as violacein, 2,4-diacetylphloroglucinol, and indol-3-carboxal-

dehyde. These compounds inhibit *Bd* growth in vitro (Brucker et al. 2008a,b) and, specifically, violacein was found in high concentration in *P. cinereus* skin (Brucker et al. 2008b).

Several other antifungal molecules also have been identified in bacteria isolated from temperate and tropical amphibian species. For instance, tryptophol is produced by a co-culture of *Bacillus* sp. and *Chitinophaga arvensicola* isolated from *P. cinereus* skin (Loudon et al. 2014a). Prodigiosin and volatile compounds are produced by isolates from the genus *Serratia*, and these were isolated from *Atelopus zeteki*, *Mantella aurantiaca*, and *Alytes obstetricans* (Woodhams et al. 2017). Not only small organic molecules but also viscosin-like lipopeptides were found to be secreted by bacteria isolated from seven tropical frog species (Martin et al. 2019). Overall, bacterial residents of amphibian skin are able to produce a wide repertoire of antifungal molecules that are likely produced to compete with other microbes for space and/or resources and, as a side effect, are able to protect their host against deadly diseases such as chytridiomycosis.

Differential amphibian susceptibility to chytrid fungi might, in part, be explained by specific skin bacterial communities (Rebollar et al. 2016a) and the proportion of anti-*Bd* isolates present on different host species (Woodhams et al. 2007a,b; Lam 2010; Burkart et al. 2017). Becker and Harris (2010) showed that reduction of the bacterial community in *P. cinereus* caused an incremental change in chytridiomycosis symptoms. Similar results were obtained with Southern Leopard Frog, *Lithobates sphenocephalus*, juveniles, indicating that the skin microbiome may be a key defense player in organisms that do not have a fully mature immune system (Holden et al. 2015). In addition, elimination of skin and gut bacterial in Cuban Treefrog, *Osteopilus septentrionalis*, tadpoles increased infections of parasite worms later in life as adults, along with a reduction in skin and gut bacterial diversity, suggesting an important priming effect of bacteria at early-life stages (Knutie et al. 2017).

Discovery of the protective role of skin bacteria in amphibians stimulated the search for antifungal bacteria in many amphibian species all over the world. Initially, studies screened bacterial strains using classical competition assays in Petri dishes (Harris et al. 2006; Woodhams et al. 2007b; Lauer et al. 2008). Currently, though, *Bd*-challenge assays are based on measuring *Bd* growth in the presence of bacterial supernatant using absorbance units (Bell et al. 2013). This strategy is more accurate, quantifiable, and reproducible, and allows for a larger number of bacterial strains to be tested simultaneously. To date, thousands of antifungal bacterial strains have been isolated from amphibian species worldwide (Flechas et al. 2012; Becker et al. 2015; Woodhams et al. 2015; Medina et al. 2017; Rebollar et al. 2019). Sequencing of the 16S rRNA gene of most of these isolates showed that antifungal capacity occurs in all culturable bacterial phyla (Becker et al. 2015; Bletz et al. 2017a). However, some bacterial classes, such as Gammaproteobacteria, Betaproteobacteria, and Bacilli, had higher proportions of anti-*Bd* strains (Bletz et al. 2017a; Catenazzi et al. 2018; Rebollar et al. 2019).

## Bacterial Therapy as a Chytridiomycosis Mitigation Strategy

Some antifungal bacteria have been used in laboratory trials and shown to have a protective effect against *Bd* infections in vivo in some amphibian species (Harris et al. 2009a,b; Becker et al. 2014; Kueneman et al. 2016a). The evidence obtained from these studies led to the possibility of using antifungal skin bacteria as probiotics to mitigate the effects of chytridiomycosis in susceptible amphibians (Harris et al. 2009b). Probiotics (microbial therapy), can be defined as the bioaugmentation (increase in concentration) of one or several locally occurring microbial taxa with the aim of mitigating host diseases (Haas and Défago 2005; Becker and Harris 2010; Gerritsen et al. 2011). Probiotics have been successfully used in aquaculture, agriculture, and humans (Babalola 2010; Gerritsen et al. 2011; Kesarcodi-Watson et al. 2012; Papadimitriou et al. 2015) and have been proposed as a strategy to mitigate emerging wildlife diseases, including white nose syndrome in bats (Hoyt et al. 2015, 2019) and chytridiomycosis in amphibians (Bletz et al. 2013; McKenzie et al. 2018). Effective probiotics may be identified through the integration of multiple molecular and bioinformatic strategies (Rebollar et al. 2016b; Song et al. 2019).

Bacterial therapy has been proposed as a feasible strategy for the mitigation of amphibian chytridiomycosis (Bletz et al. 2013; Woodhams et al. 2016). In geographic regions that have not yet been impacted by this disease, a bank of potential probiotics could be developed that could be implemented once the pathogen is present (Bletz et al. 2017a). Reintroduction programs might benefit from applying probiotics to increase the chance of survival and could also help keep species in captivity by avoiding fungal infections and maintaining host health. However, none of the above strategies have been implemented yet.

In amphibians, clear evidence for the protective function of skin bacteria was observed with the first probiotic inoculation trials. Addition of the anti-*Bd* bacterium, *Pseudomonas reactans*, ameliorated the negative effects of chytridiomycosis, for example, body mass loss, in *P. cinereus* (Harris et al. 2009a). Also in *P. cinereus*, adding *J. lividum* significantly increased violacein concentration on the skin and consequently caused an increase in survival in individuals infected with *Bd* (Becker et al. 2009). Adding *J. lividum* prevented *Bd*-induced morbidity and mortality in Mountain Yellow-legged Frogs, *Rana muscosa*, from the Sierra Nevada Mountains of California, a species that is highly susceptible to *Bd* (Harris et al. 2009b).

Following these first laboratory trials, though, additional trials with probiotics and amphibians have had mixed success. On one hand, inoculation of *J. lividum* to *Bd*-infected *R. muscosa* adults increased survival up to 40% relative to *Bd*-infected individuals that did not receive the probiotic (Kueneman et al. 2016a). On the other hand, adding this same bacterium to Panamanian Golden Frogs, *Atelopus zeteki*, did not protect them against *Bd*, and *J. lividum* levels decreased gradually across the experiment (Becker et al. 2011, 2014). The loss of probiotic bacteria in laboratory trials has been observed in other studies also. Inoculation of Green Frog, *Lithobates clamitans*, tadpoles with *J. lividum* did not dramatically modify the skin community structure, and the probiotic bacterium decreased

in concentration over time until it reached its original level (Rebollar et al. 2016c). Likewise, the inoculation of *Lysinibacillus fusiformis* to Panama Rocket Frogs, *Colostethus panamensis*, did not modify the skin community structure but, instead, triggered the production of antimicrobial peptides (AMPs) in the host (Küng et al. 2014). Contrarily, inoculating Midwife Toad, *Alytes obstetricans,* tadpoles with *Pseudomonas fluorescens* and *Flavobacterium johnsoniae* dramatically modified the skin microbial community structure and function (Davis et al. 2017). These results indicate that the bioaugmentation outcome will differ between amphibian species that likely have distinct symbiotic communities and particular host immune responses. Moreover, different bacterial species can exert variable effects in the skin microbial community.

Even though thousands of anti-*Bd* bacterial strains have been isolated and characterized, only a small subset of bacterial strains inhibited a wide variety of *Bd* strains in vitro (Antwis et al. 2015; Antwis and Harrison 2018). The inhibitory capacity of bacteria varied depending on pathogen genotype (*Bsal* and *Bd* strains) and temperature conditions (Muletz-Wolz et al. 2017; Robak and Richards-Zawacki 2018). Thus, probiotic therapy should seek those bacterial strains with broad-spectrum inhibition capacities under variable conditions.

Considering that the beneficial effects of skin microbiota likely result from interactions within the microbial community, a more realistic scenario for probiotic therapy would be to employ bacterial consortia instead of single isolates. Antwis and Harrison (2018) showed that the antifungal function of bacterial consortia was stronger than in single isolates, particularly in the case of consortia composed by multiple bacterial genera with greater genetic distance among them. In addition, in vitro synthetic communities showed that biofilms that included a greater number of bacterial species exhibited a greater reduction in *Bd* proliferation (Piovia-Scott et al. 2017), suggesting that *Bd* inhibitory capacities are mainly driven by a combination of dominance and complementarity effects occurring within the synthetic communities.

Even though probiotic bacterial therapy is a promising mitigation strategy, an effective probiotic should conform to a series of principles. First, it should be able to colonize and maintain itself in the skin community at optimal levels (Fig. 2A). Second, once inserted into the community, the bacterium should be able to produce and secrete the antifungal molecules onto the skin to be able to protect the host against *Bd* or *Bsal* (Fig. 2B,C). Third, the probiotic bacterium should ideally be propagated through vertical, horizontal, and/or environmental transmission (Fig. 2D). Overall, to be able to select an effective probiotic, we need to have a deeper understanding of the mechanisms driving colonization, establishment. and propagation of skin bacteria (Loudon et al. 2016), as well as the ecological interactions occurring between skin microbiomes and their hosts.

## The Skin Microbiome is Influenced by Factors Acting at Different Scales

Host-associated microbiomes have been widely recognized as dynamic microbial communities that are influenced by multiple factors. To be able to understand how amphibian microbiomes contribute to the host's health, a large amount of work has focused on identifying the factors that drive the structure and function of these communities (Jiménez and Sommer 2016; Rebollar et al. 2016b; Walke et al. 2017). Based on this work and the understanding that skin microbiomes constantly interact with their hosts and the environment, we propose that groups of abiotic and biotic factors act at different scales. Current knowledge indicates that three layers of factors influence skin microbiomes in amphibians (Fig. 3).

### Host-Specific Factors

Amphibian skin microbial communities vary across species that coexist in the same environment (McKenzie et al. 2012; Kueneman et al. 2014; Rebollar et al. 2016a; Abarca et al. 2018a). Likewise, the skin microbiome changes in diversity and composition through development, suggesting that host immune system maturation may influence microbial skin composition (Kueneman et al. 2014; Griffiths et al. 2018; Prest et al. 2018). On the other hand, a site-specific effect appears when analyzing distinct populations of the same species (Belden et al. 2015; Rebollar et al. 2016a; Abarca et al. 2018b; Albecker et al. 2018). The results suggest that host-specific traits may somehow modulate skin microbiome structure and function.

Amphibian skin is a complex organ involved in gas exchange and osmoregulation, while at the same time functioning as a selective barrier to the external environment (Varga et al. 2019). The mucus on the outermost part of the skin is a niche for many microbes and is composed of heavily glycosylated mucins and mucopolysaccharides that function to maintain the moisture of the skin. In addition, the mucus contains a wide variety of defensive molecules such as AMPs, alkaloids, lysozymes, and antibodies. Thus, mucus composition may provide different conditions for particular microbes to colonize the skin that could protect hosts against pathogens (Conlon 2011; Rollins-Smith et al. 2011; Varga et al. 2019).

The diversity and quantity of AMPs produced by host amphibian species is highly variable and can act synergistically with skin antifungal bacteria (Woodhams et al. 2007a,b; Myers et al. 2012). Bacterial communities and their hosts can thus interact via the production of AMPs in vivo. For example, on one hand the inoculation of specific bacterial strains on the skin of Sierra Yellow-legged Frogs, *Rana sierra*e, induced the reduction of certain AMPs, while on the other hand, those AMPs promoted the growth of anti-*Bd* bacterial species, such *J. lividum*, on the host skin (Woodhams et al. 2020).

Another host-associated factor that can influence skin microbial composition is genetic structuring among host populations, which is likely linked to immune genetic variation. In tadpoles of Phofung River Frogs, *Amietia hymenopus*, of Lesotho, host genetic distance, evaluated with microsatellites, showed a significant correlation with microbial community dissimilarity when controlling for geographical distances (Griffiths et al. 2018). However, there are still very few studies addressing the effect of the host genetic background on skin microbiome composition (Hernández-Gómez et al. 2017; Jani and Briggs 2018). The effect of phylogenetic distance among taxa has recently started to be evaluated, but so far, other factors such as

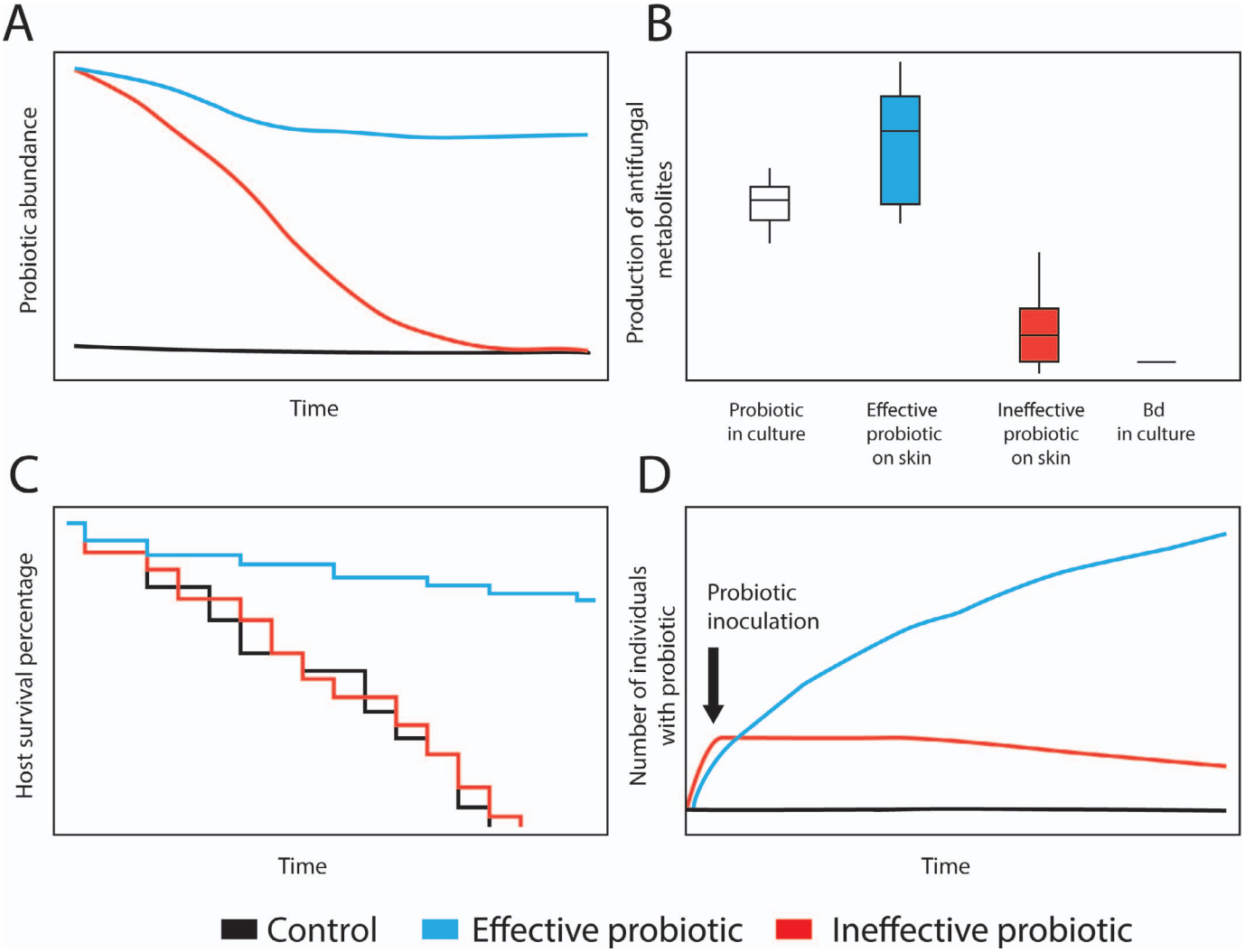


Fig. 2.—Diagrammatic illustrations of tests needed to determine if a microbe, or combination of microbes, works effectively as a probiotic. (A) Abundance of the probiotic on the skin through time. (B) Level of the antifungal metabolite produced by the probiotic. (C) Inoculation trial of the probiotic in *Bd* or *Bsal* infected individuals across time. (D) Number of individuals with the probiotic in a population or community. Probiotic inoculation is only performed on a subset of the individuals. A color version of this figure is available online.

climate or habitat conditions have shown a greater effect (Bletz et al. 2017b; Bird et al. 2018; Ellison et al. 2018; Kueneman et al. 2019). In summary, host genetic variation, specifically in those traits associated with immune response, should be evaluated to determine the degree to which amphibian hosts shape their microbiomes. Methods like RNA-seq (RNA sequencing), quantitative trait loci analyses (through SNP genotyping) or gene target enrichment may aid linking host genetic traits with skin microbiome traits.

## Microhabitat Factors

Amphibian microhabitats play fundamental roles shaping skin microbial diversity and composition. Specifically, recent evidence has identified strong patterns of microbial composition associated with microhabitat (riparian, terrestrial, or arboreal) in a high number of amphibian species (Rebollar et al. 2016a; Bletz et al. 2017b; Kueneman et al. 2019). This effect can be linked to differences in environmental reservoirs for microbes in soil, water, and plants (Rebollar et al. 2016a); local bacterial transmission, whether vertical or horizontal, among hosts (Walke et al. 2011; Muletz et al. 2012); variation in local environmental conditions (Kueneman et al. 2014; Abarca et al. 2018b); and the presence and/or abundance of pathogens (Familiar López et al. 2017; Jani et al. 2017; Bates et al. 2018).

Several studies have shown that environmental bacterial communities are significantly different from skin microbial communities (Fitzpatrick and Allison 2014; Sanchez et al. 2016; Albecker et al. 2018). The most abundant taxa in skin microbial communities are generally under-represented in environmental reservoirs (Walke et al. 2014; Rebollar et al. 2016a). However, the lack of environmental reservoirs in the case of captive amphibian colonies causes reductions in skin microbial diversity, indicating that the surrounding bacteria are a necessary source of diversity and are important for maintaining skin microbiome composition (Becker et al. 2014; Loudon et al. 2014b; Michaels et al. 2014).

There is indirect evidence of vertical transmission of skin bacteria in amphibian species with parental care behavior. In the salamander, *H. scutatum*, females showing parental care

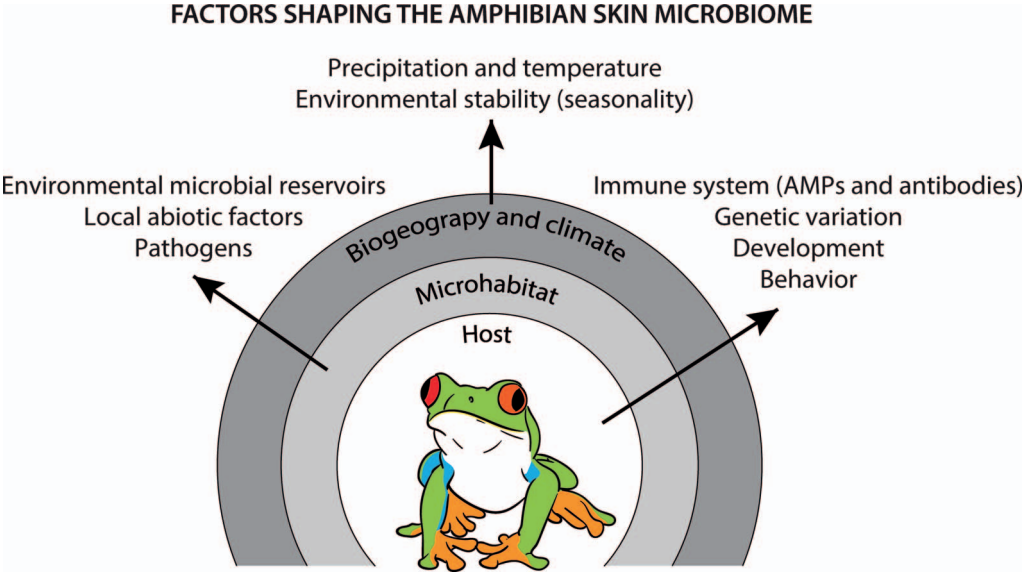


FIG. 3.—Nested biotic and abiotic factors that influence the structure and function of the amphibian skin microbiome. A color version of this figure is available online.

had a similar bacterial community as their embryos (Banning et al. 2008). Likewise, in male Plantation Glass Frogs, *Hyalinobatrachium colymbiphyllum*, which also exhibit parental care, paternal skin microbiota and AMPs were very similar to those of their egg masses (Walke et al. 2011). Both of these studies suggest that parents may transmit skin bacteria to their offspring via parental care. However, as another recent study on *H. colymbiphyllum* showed, egg masses can maintain a stable microbial community without parental care, indicating that both parent and offspring microbiomes have the same relationship with environmental sources (Hughey et al. 2017). Horizontal transmission of microbiota at different developmental stages in amphibians may occur either through direct contact or through indirect, environmental transmission (Muletz et al. 2012; Rebollar et al. 2016c; Becker et al. 2019). In tadpoles, body size and behavior appear to be major factors influencing bacterial transmission among hosts (Keiser et al. 2019).

Variations in pH (Kueneman et al. 2014; Varela et al. 2018), salinity (Albecker et al. 2018), temperature and moisture (Longo and Zamudio 2017b) are all correlated with changes in amphibian skin microbiome composition. In vitro studies have shown that the production of secondary metabolites by antifungal bacteria are determined by the temperature at which they are cultured (Woodhams et al. 2014). Thus, temperature variations at a local scale in an amphibian system may be expected to modulate functionality of the skin microbiome and, in turn, also modulate protective capacity against pathogens (Daskin et al. 2014).

As part of the microhabitat, pathogen presence and abundance can have an effect on skin microbial composition. Experimental trials on several amphibian species have shown that the skin microbiota is modified in response to *Bd* infections (Jani and Briggs 2014; Walke et al. 2015; Longo and Zamudio 2017a). Field studies have identified bacterial taxa enriched in *Bd*-positive sites in contrast to *Bd*-negative sites (Rebollar et al. 2016a; Familiar López et al. 2017). Moreover, populations that display enzootic and epizootic dynamics of *Bd* infection show clear differences in skin bacterial diversity (Jani et al. 2017; Bates et al. 2018). Finally, high *Bd* infection intensity in a highly susceptible species, such as *R. sierra*, is linked to a clear reduction in skin bacterial richness (Ellison et al. 2018).

### Biogeographical and Climatic Factors

The spatial distribution of microbes has become a hot topic over the past two decades. Biogeographical patterns have been found in free-living and host-associated bacteria from a wide variety of marine, freshwater, and terrestrial environments (Martiny et al. 2006; Fierer and Jackson 2006; Galand et al. 2009; Peay et al. 2016; Thompson et al. 2017). A recent meta-analysis has shown that diversity patterns of amphibian skin microbiomes across the globe are associated with distinct geographical areas with contrasting climatic conditions (Kueneman et al. 2019). Specifically, amphibians distributed in seasonal environments and regions with cold winters have higher skin microbial diversity than amphibians from more climatically stable regions.

Studies evaluating the influence of climatic factors on skin microbiota in amphibians have shown that changes in precipitation and temperature over time are correlated with changes in microbial diversity. These changes in skin microbial diversity and composition have been seen in field and experimental trials in both temperate and tropical species (Bletz et al. 2017c; Longo and Zamudio 2017a,b). Such changes in diversity may be directly or indirectly caused by climatic fluctuations. For example, seasonal variations can influence host body temperature and shedding rate, in turn causing changes in skin bacterial community structure and function (Meyer et al. 2012; Rowley and Alford 2013; Ohmer et al. 2014; Woodhams et al. 2014). Alternatively, seasonal environmental fluctuations may modify bacterial reservoirs in soil, leaf litter and water and thereby alter skin microbiome diversity. Overall, evidence so far indicates that skin microbiomes are influenced by large-scale biogeographical patterns and climatic fluctuations linked to seasonality.

## Beyond Bacteria There is the Skin Fungal Microbiome

Compared with the knowledge about fungal communities associated with plant hosts and those present in different soil and water environments, we know very little about the function and diversity of fungal communities associated with animal hosts (Peay et al. 2016). The few studies published show that, unlike plant-associated fungal communities, there is no clear evidence of beneficial interactions occurring between fungal microbial communities and their animal hosts and it has been proposed that in many cases the fungal microbiome can be considered a reservoir of pathogenic or opportunistic microbes (Huffnagle and Noverr 2013). However, such observations have only been made in humans.

In the case of the amphibian skin microbiome, studies of fungal skin microbial communities have shown that some of the same factors such as host species, pathogen load, developmental stage, and captivity that influence the bacterial microbiome also influence the diversity and composition of the fungal microbiome (Kueneman et al. 2016b; Kearns et al. 2017; Medina et al. 2019). As seen in skin bacterial microbiomes (McKenzie et al. 2012; Bletz et al. 2017d; Ellison et al. 2018; Rebollar et al. 2019), diversity of skin fungal microbiomes differs between host species that inhabit different climatic regions (Medina et al. 2019). Furthermore, the relative abundance of certain fungal taxa differs between captive and wild dart poison frogs, *Dendrobates* spp., and these differences are related to the frogs' ability to overcome *Bd* infections (Kearns et al. 2017).

Host development also influences the composition of the fungal skin microbiome. For example, fungal taxa are less abundant over the skin of Western Toads, *Anaxyrus boreas*, during early developmental stages, occupying only 3.3% of total micro-eukaryote (i.e., fungi, protists, and metazoans) diversity over the skin, whereas in subadult and adult stages, fungi attain up to 57% total skin micro-eukaryote diversity. These differences could be due to changes in microhabitat, especially in species where larval stages are aquatic and adults are terrestrial. In these cases, organisms are exposed to different microbial reservoirs across developmental stages (Prest et al. 2018). For example, several fungal taxa with mycorrhizal or lichenized lifestyles, which are characteristic of soil environments, become abundant in *A. boreas* adults, although these fungi are absent in tadpoles (Kueneman et al. 2016b).

Skin fungal communities of amphibians in the wild are composed of at least eight fungal phyla: Ascomycota, Basidiomycota, Chytridiomycota, Mortierellomycota, Glomeromycota, Rozellomycota, Zygomycota, and Neocallimastigomycota (Kueneman et al. 2016b; Medina et al. 2019). However, amphibians held in captivity harbor much less diverse skin fungal communities composed mainly of Ascomycota and Basidiomycota (Kearns et al. 2017). This observed loss of diversity in the fungal skin microbiota among captive amphibians may be due to the lack of environmental fungal reservoirs, as has been shown for skin bacterial microbiomes (Loudon et al. 2014b).

Although the identification of inhibitory bacterial taxa against *Bd* is a major focus of skin microbiome research in amphibians (Bletz et al. 2013; Woodhams et al. 2016), some fungal isolates from captive frogs also demonstrate anti-*Bd* abilities. Ascomycote fungi appear to have a greater ability to inhibit *Bd* compared to Basidiomycotes, which may actually facilitate *Bd* growth (Kearns et al. 2017). When used as probiotics in in vivo trials, *Penicillium expansum* induced a reduced amount of corticosterone release compared to the bacterial probiotics *J. lividum* and *F. johnsoniae*. Corticosterone interferes with immune processes like the growth of lymphocytes or the renewal of granular glands with AMPs (Rollins-Smith et al. 2011).

In summary, several biotic and abiotic factors influence the composition of the amphibian skin fungal microbiome, as is also the case for the bacterial microbiome. Moreover, fungal skin communities could play a defensive role against fungal pathogens. Thus, characterization of anti-*Bd* fungal isolates could lead to improved probiotic treatments against *Bd* and *Bsal*. Even though studies published to date report some general aspects of the ecology and function of the amphibian skin fungal communities, we still lack detailed information about the interactions occurring between fungi and bacteria on amphibian skin. To address this topic, the use of both in vitro co-culture assays and in silico ITS (internal transcribed spacer) amplicon sequencing and co-occurrence networks are fundamental.

## Conclusions and Future Directions

Microbes present in amphibian skin are now known to play a protective role against pathogens. Thousands of bacterial strains have been tested against *Bd* and *Bsal*, and specific metabolites with antifungal capacities have been identified. The evidence accumulated so far has inquiries focused on understanding the ecology of the amphibian–microbial symbiosis. We have learned that the structure of skin microbial communities is influenced by host-specific, microhabitat, biogeographic, and climatic factors. However, we still know very little about the functional aspects of these microbiomes and how these functions are modulated. Experimental trials integrating abiotic and biotic factors will allow their effects on skin microbiome structure and function to be teased apart. Future studies should aim to describe the microeukaryote (i.e., fungi, protists, and metazoans), archaean, and viral components of the microbiome to understand the ecological interactions and population dynamics of the particular microbes occurring within the skin microbiome. Moreover, it is important to continue exploring the effects that pathogens other than *Bd* and *Bsal* (e.g., *Ranavirus* and parasitic protists) have on the skin microbiome and on the amphibian host. This is particularly relevant considering that amphibians are prone to co-infections of pathogens that can synergistically alter skin microbial community structure and function and, in turn, compromise the host's health.

Determining the interactions that occur between the host immune system and the skin microbiome is also fundamental for understanding how pathogen protection functions in amphibians that are not susceptible to chytridiomycosis. It is essential to integrate culture-dependent and culture-independent methods, as well as multivariate statistical and bioinformatics tools, to investigate this. For instance, changes in host gene expression (RNA-seq) and AMP detection (through high-performance liquid chromatography and mass spectrometry) in response to specific bacterial strains should shed light on the molecular pathways involved

in bacteria–host interactions with or without the presence of co-occurring fungal pathogens. Moreover, electron microscopy, FISH (fluorescence in situ hybridization), and microbial quantification via real-time polymerase chain reaction should be used to better describe in situ patterns of microbial diversity on amphibian skin and the interactions between microbes, host skin cells, and the chemical components of the mucus. Explorations to look deeper into the ecological and molecular interactions occurring among skin microbes, hosts, and pathogens should eventually allow for the implementation of effective microbial therapies against chytridiomycosis and other amphibian diseases.

**Acknowledgments.**—We thank S. Ramírez-Barahona for his help with the figures. We also thank two anonymous reviewers who revised the manuscript and made crucial comments to improve it.

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**Accepted on** 9 March 2020
**Associate Editor:** David Lesbarrères

Animal Conservation

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Animal Conservation. Print ISSN 1367-9430

# Metamorphosis and seasonality are major determinants of chytrid infection in a paedomorphic salamander

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**Keywords**
chytridiomycosis; paedomorphic salamanders; amphibian diseases; *B. dendrobatidis*.

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Editor: John Ewen
Associate Editor: Robert Jehle

Received 13 December 2021; accepted 09 September 2022

doi:10.1111/acv.12824

## Abstract

Chytridiomycosis, an emerging disease caused mostly by the pathogen *Batrachochytrium dendrobatidis*, has caused massive amphibian population declines and extinctions worldwide. The ecology of this disease is mainly explained by the interaction of environmental factors, pathogen biology, and host traits including development. For paedomorphic salamanders, differences in *B. dendrobatidis* infection may be explained by metamorphosis and water physicochemical conditions. In this study, we aimed to determine the influence of environmental and host factors on *B. dendrobatidis* prevalence and infection intensity in the facultative paedomorphic salamander *Ambystoma altamirani*. We determined *B. dendrobatidis* prevalence and infection load in four populations of *A. altamirani* along 1 year (four seasons) and assessed their relationship with environmental factors and host metamorphic status (gilled or non-gilled). We found that *B. dendrobatidis* prevalence and infection load are largely explained by metamorphic status and environmental factors such as elevation, seasonality, water temperature, pH, conductivity, and dissolved oxygen. To our knowledge, this is the first study to empirically show the effect of metamorphosis on *B. dendrobatidis* infection status across locations and seasons. This information may be used to understand the temporal dynamics of *B. dendrobatidis*–host interactions and to identify potential disease outbreaks that may cause cryptic sublethal effects on salamander populations. Our results will help in the development of conservation strategies for paedomorphic salamanders that are already considered threatened by anthropogenic factors such as habitat loss and climate change.

## Introduction

Emerging diseases are a significant threat to global biodiversity and are responsible for species extinctions and population declines around the globe (Fisher *et al*., 2012). Chytridiomycosis is one of these emerging diseases and is caused by the fungal pathogens *Batrachochytrium dendrobatidis* and *B. salamandrivorans* (Longcore, Pessier, & Nichols, 1999; Martel *et al*., 2013). Specifically, *B. dendrobatidis* has caused mass die-offs and amphibian population declines worldwide (Scheele *et al*., 2019). This pathogen has an aquatic zoospore stage that enters the amphibian skin disrupting the epithelial structure and causing osmotic imbalances, often leading to cardiac arrest and death of susceptible individuals (Voyles *et al*., 2009). The incidence of *B. dendrobatidis* infection is driven by multiple factors, including environmental conditions as well as pathogen and host-associated traits. Variations in the environment, host species, populations, and amphibian life stages may influence both the pathogen and the host, and in turn may explain differences in infection outcome (Lips, Reeve, & Witters, 2003; Bielby *et al*., 2008; Olson *et al*., 2013; Kueneman *et al*., 2014, 2016; McMillan *et al*., 2020).

Temporal and geographic variation in temperature, humidity, and pH has been shown to influence *B. dendrobatidis* prevalence and infection intensity (Bielby *et al*., 2008; Olson *et al*., 2013; Chestnut *et al*., 2014; Lenker *et al*., 2014; Familiar López *et al*., 2017). Most *B. dendrobatidis* infections found in the field have occurred in cool regions and seasons (Berger *et al*., 2004; Retallick *et al*., 2004; Schlaepfer *et al*., 2007; Forrest & Schlaepfer, 2011; Ruggeri *et al*., 2020; Le Sage *et al*., 2021). For example, a seasonal

peak of *B. dendrobatidis* infections during the cooler months was found in Australia by Retallick *et al*. (2004), and similarly, high *B. dendrobatidis* prevalence in frogs of Arizona and Brazil occurred during the colder months (Schlaepfer *et al*., 2007; Forrest & Schlaepfer, 2011; Ruggeri *et al*., 2020). These results coincide with the thermal growth range of *B. dendrobatidis* in the laboratory, which is between 4°C and 25°C (Piotrowski, Annis, & Longcore, 2004), suggesting that specific environments may favor the pathogen and affect infections of the host.

Water bodies facilitate the contact between *B. dendrobatidis* and amphibians, thus providing opportunities for *B. dendrobatidis* dispersal. The zoospores actively swim and are assumed to be more concentrated in smaller water bodies, increasing the probability of infection in aquatic or semi-aquatic species (Piotrowski, Annis, & Longcore, 2004; Berger *et al*., 2005). Additionally, water pH has been suggested as a possible cofactor in the development of chytridiomycosis. For example, Kärvemo *et al*. (2018) found that the pH of ponds was positively associated with *B. dendrobatidis* infection prevalence in amphibians of Sweden. Similarly, Blooi *et al*. (2017) found that streams with pH values of 6–8 had an optimal growth of *B. dendrobatidis* in contrast to acidic microhabitats such as water held by bromeliads.

The impact of abiotic factors on *B. dendrobatidis* prevalence and infection intensity also depends on host-associated traits such as habitat preference and life history (Lips *et al*., 2003; Bielby *et al*., 2008; Rollins-Smith, 2017). These traits may influence the probability, degree and duration of infection, and the probability of disease-induced mortality (Lips *et al*., 2003; Bielby *et al*., 2008). Given that *B. dendrobatidis* is an aquatic fungus, species that spend a large proportion of their life in or near permanent water bodies tend to be more affected than terrestrial species (Laurance, McDonald, & Speare, 1996; Bielby *et al*., 2008). Moreover, *B. dendrobatidis* infection in anurans changes across developmental stages. The infection of tadpoles is restricted to their keratinized mouthparts, leading to a lower proliferation of *B. dendrobatidis* compared with metamorphic individuals (Fisher, Garner, & Walker, 2009). In the case of salamanders, *B. dendrobatidis* is present on the skin of both larval and metamorphic individuals, for example in *Taricha granulosa*, *Salamandra salamandra*, and *Ambystoma opacum* (Venesky, Parris, & Altig, 2010; Piovia-Scott *et al*., 2011; Medina *et al*., 2015). Generally, pre-metamorphic salamanders (including larvae and paedomorphic individuals) posses gills and a high tail fin in contrast to metamorphic individuals (Harris *et al*., 1990). During metamorphosis, they lose the gills and undergo physiological and biochemical changes that include transformations in the skin tissue (Wake, 1980; Duellman & Trueb, 1994). To our knowledge, differences in *B. dendrobatidis* infection status between pre-metamorphic (gilled) and metamorphic (non-gilled) salamanders across seasons have not been reported yet. Understanding how metamorphic status and environmental conditions may modify disease outcomes in salamanders will provide valuable insights into the dynamics of host–pathogen interactions.

Paedomorphosis is the phenomenon of reaching reproductive maturity while retaining larval external morphology (Duellman & Trueb, 1994). Some *Ambystoma* species are considered obligate paedomorphs which never go through metamorphosis, and others are considered facultative paedomorphs which can metamorphose under certain conditions to transform from aquatic larvae to terrestrial adults (Everson *et al*., 2021). *Ambystoma altamirani* is a facultative paedomorphic salamander with a distribution restricted to the high mountain streams of Central Mexico (Lemos-Espinal *et al*., 1999, 2016; Woolrich-Piña *et al*., 2017; IUCN, 2020). This species is considered threatened by Mexican law (NOM-059, SEMARNAT, 2015) and Endangered by the IUCN (IUCN, 2020). Populations of *A. altamirani* are subject to many threats such as urbanization, pollution, land-use change, the introduction of invasive fish, and diseases (Lemos-Espinal *et al*., 1999, 2016; Frías-Alvarez *et al*., 2008; Basanta *et al*., 2021). Because gilled and non-gilled individuals stay in or near aquatic habitats throughout their entire life (Lemos-Espinal *et al*., 1999; Camacho *et al*., 2020), the presence of aquatic pathogens such as *B. dendrobatidis* may pose a substantial risk to populations' health, and may differentially affect gilled and non-gilled individuals. Previous studies have found the presence of *B. dendrobatidis* in seemingly unaffected as well as dead individuals of *A. altamirani* (Frías-Alvarez *et al*., 2008; Basanta *et al*., 2021) but no studies have reported *B. dendrobatidis* prevalence and infection intensity at population levels.

In this study, we assessed *B. dendrobatidis* prevalence and infection intensity in four populations of *A. altamirani* spanning four seasons over the course of 1 year. We specifically addressed two questions: How do *B. dendrobatidis* prevalence and infection intensity vary across seasons and locations for gilled and non-gilled individuals? And which environmental factors are most likely influencing *B. dendrobatidis* prevalence and infection intensity? Since gilled and non-gilled salamanders differ in skin structure affecting their physiology (Wake, 1980; Duellman & Trueb, 1994), and environmental factors may influence *B. dendrobatidis* infection outcomes (Berger *et al*., 2004; Piotrowski, Annis, & Longcore, 2004; Bielby *et al*., 2008; Olson *et al*., 2013), we hypothesized that *B. dendrobatidis* infections would differ across seasons and between developmental stages. We predict that *B. dendrobatidis* infection (prevalence and intensity) will be higher in gilled individuals and in seasons with environmental conditions that are optimal for *B. dendrobatidis* growth.

## Material and methods

### Field sites, sample and data collection

We swabbed the skin of 279 *A. altamirani* individuals from four streams at Isidro Fabela municipality in Estado de México, México: location A (3400 m a.s.l.), location B (3360 m a.s.l.), location C (3210 m a.s.l.), and location D (3087 m a.s.l.) (Fig. 1). The locations are separated by mountains and the streams are unconnected. We sampled all

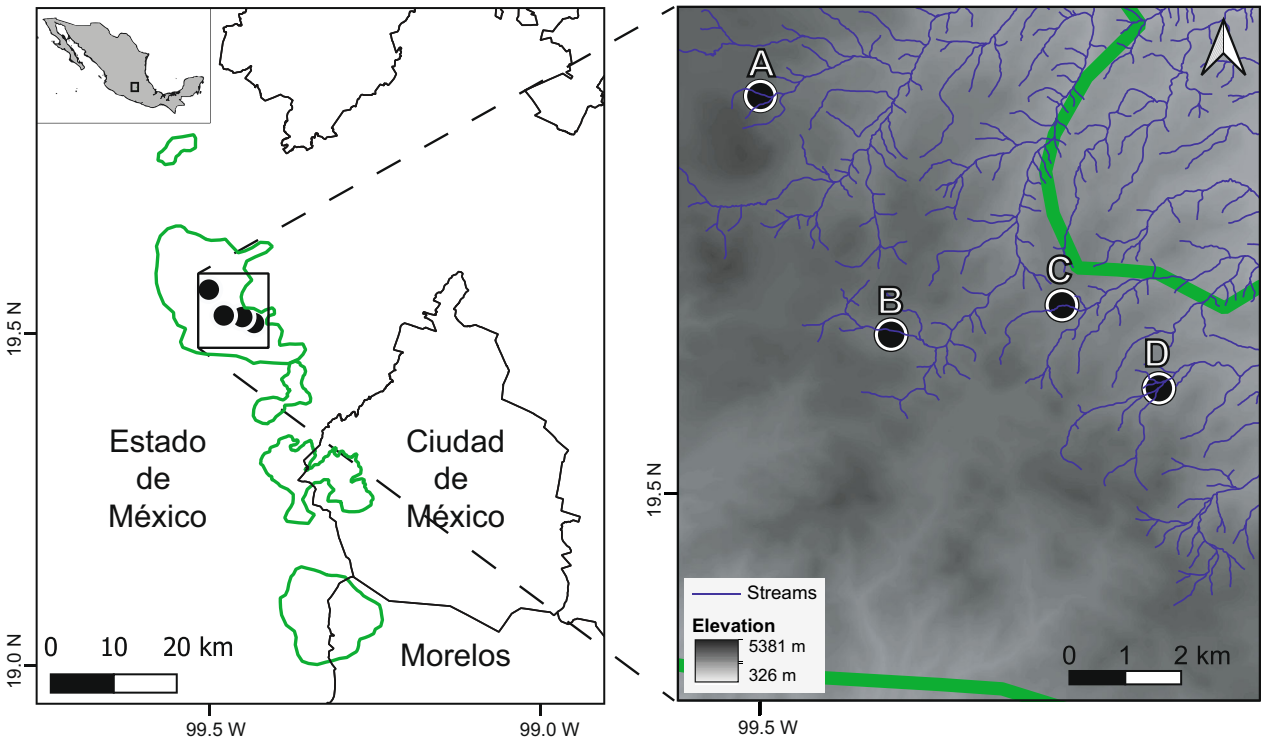


**Figure 1** Map representing the *Ambystoma altamirani* distribution (IUCN, 2020) in green lines, covering three states (left image). The four locations (A–D) where *A. altamirani* individuals were sampled for *B. dendrobatidis* detection (right image). Purple lines represent streams.

four localities during four consecutive seasons: summer (July 2019), fall (October 2019), winter (January 2020), and spring (April 2020). Because the individuals were not marked, we do not know whether some of them have been recaptured along seasons. For each sampling period, we recorded water temperature (mean temperature during sampling, °C), dissolved oxygen, conductivity, and pH using a calibrated electrical probe (Hanna instruments HI98194, Limena, Italia) at three points along each stream. Additionally, in order to record water temperature variation throughout the whole sampling period (minimum, maximum, and mean temperature values by season, °C), we measured water temperature every hour from July 2019 to April 2020 using one data logger (Hobo Onset, Bourne, MA, USA) at each of the four locations.

To capture *A. altamirani* individuals we used clean and disinfected nets, and each individual was manipulated with a fresh pair of nitrile gloves. For *B. dendrobatidis* sampling, skin swab samples were collected according to previously published procedures (Hyatt *et al.*, 2007; Rebollar *et al.*, 2014). Briefly, each individual was rinsed with 25 mL of sterile water before swabbing to ensure that the sample primarily included skin-associated microorganisms. Following rinsing, each individual was swabbed with a sterile swab (MW-113; Medical Wire and Equipment, Corsham, UK). Once sampled, all individuals were returned to their original location. Each skin swab was stored into a 1.5 mL microcentrifuge tube with 170 µL of DNA shield (Zymo Research, Irvine, CA, USA) and stored at 4°C until arrival at the laboratory where tubes were stored at −80°C until processing. For each individual, we measured the snout–vent length (SVL), weight and assessed the presence of clinical signs of chytridiomycosis such as lack of reflexes, stiffness, or extreme skin shedding. Finally, the metamorphic stage of each animal was categorized based on the presence or absence of gills. Animals with gills included pre-metamorphosed juveniles and paedomorphic adults (gilled). Animals without gills included metamorphosed adults (non-gilled). Collection permits for animal use at Isidro Fabela municipality in Estado de México, México were provided by the Mexican Government, Secretaría del Medio Ambiente y Recursos Naturales SGPA/DGVS/5673/19.

## *B. dendrobatidis* detection and quantification

The DNA from all samples was extracted using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol including a pretreatment with lysozyme (Rebollar *et al.*, 2019). DNA extracts were used for the detection of *B. dendrobatidis* using Taqman real-time PCR assay according to Boyle *et al.* (2004). We performed qPCRs with a final reaction volume of 15 µL. Each sample was assayed in triplicate including a negative control (sterile water). To quantify the pathogen load (intensity), we used six standards of DNA synthetic fragments (Longo *et al.*, 2013) to estimate the

number of *B. dendrobatidis* internal transcribed spacer (ITS) copies on each sample (1, 10, 100, 1000, 10 000, and 100 000 ITS copies). Samples were considered positive if at least two of three qPCRs revealed a positive result. Samples that showed a positive signal in only one well were run a second time in a single well. If the sample showed a positive signal in this second run, we classified it as positive. *B. dendrobatidis* infection intensity per sample was obtained by averaging the *B. dendrobatidis* ITS copies estimated by qPCR from the three well reactions, and values of infection intensity were log-transformed ($\log_{10}$ (*B. dendrobatidis* load + 1)) to approximate a normal distribution.

### Statistical analyses

All statistical analyses were performed using the software R v.3.6.1 (Core Team, 2019). To test for physicochemical differences among locations and seasons, we used Kruskal–Wallis tests. Prevalence was calculated as the proportion of infected individuals per location with 95% confidence intervals (CIs) using the *prop.test* function. Differences in *B. dendrobatidis* prevalence and infection intensity across locations, seasons, and metamorphosis stage (pre-metamorphic/gilled, metamorphic/non-gilled) were assessed using a Chi-square test and a Kruskal–Wallis test, respectively. Additionally, we analyzed *B. dendrobatidis* prevalence and infection intensity separately for gilled and non-gilled individuals across locations and seasons.

To test the influence of environmental variables on *B. dendrobatidis* prevalence and infection intensity, we fitted two generalized linear models (GLM): one for *B. dendrobatidis* presence and a second for *B. dendrobatidis* infection intensity. Environmental factors (mean water temperature of sampling day, maximum and minimum seasonal water temperature, mean pH, mean dissolved oxygen, mean conductivity) and host SVL were included as continuous variables. Location, season, and metamorphosis stage were included as categorical variables. Before GLM construction, we calculated the mean value of each environmental factor measured at each stream/location/season. Then, to eliminate potential multicollinearity among environmental and host-associated variables, we estimated Pearson correlations among variables and selected the following uncorrelated variables: mean water temperature of sampling day, minimum and maximum seasonal water temperature, mean pH, mean dissolved oxygen, mean conductivity, and SVL (Supporting Information Table S1).

We fitted a GLMs for *B. dendrobatidis* presence using binomially distributed errors and logit link function, in which infection status (*B. dendrobatidis* positive or negative) was used as the unit of analysis. Additionally, we fitted another GLM for *B. dendrobatidis* infection intensity using Poisson distributed errors and a logarithmic link function. In both GLMs (presence/infection intensity) both forward and backward stepwise variable selection procedures for two-way interaction terms were conducted to build a multivariable model that only contained the statistically significant factors associated with *B. dendrobatidis* (presence/infection intensity) as implemented in the *MASS* package (Venables & Ripley, 2002). Then, analyses of variance were used to compare Akaike Information Criterion (AIC) scores (Burnham & Anderson, 2002) and to select the most parsimonious model with the lowest AIC value for each case (*B. dendrobatidis* presence/infection intensity). Finally, we visualized how *B. dendrobatidis* presence and infection intensity were predicted by the significant variables with marginal means and 95% CIs using the *effects* package (Fox, 2003).

## Results

### *B. dendrobatidis* infection status in *A. altamirani*

Of the total number of sampled individuals ($N$ = 279), more than two-thirds were infected with *B. dendrobatidis* (70.3%; 95% binomial CI 64.6–75.3). Moreover, *B. dendrobatidis* intensity ranged from 58 to 15 258 766 *B. dendrobatidis* ITS copies.

*Batrachochytrium dendrobatidis* prevalence and infection intensity differed across locations ($\chi^2_3$ = 17.144, $P$ = 0.001; Kruskal–Wallis test, $\chi^2_3$ = 35.606, $P$ < 0.001; Fig. 2a). Location A (the location with the highest elevation) had the lowest prevalence (51.4%, CI 40–62; $P$ = 0.001, Supporting Information Table S2) and the lowest infection intensity (Wilcoxon test, all comparisons $P$ < 0.001, Supporting Information Table S2; Fig. 2b). *B. dendrobatidis* prevalence also differed across seasons ($\chi^2_3$ = 10.786, $P$ = 0.012), with summer being significantly different to winter and spring ($P$ < 0.050; Supporting Information Table S2; Fig. 2b). *B. dendrobatidis* infection intensity was similar across seasons (Kruskal–Wallis test, $P$ = 0.090, Supporting Information Table S2, Fig. 2b). Metamorphosis stage did not have a significant effect on *B. dendrobatidis* prevalence ($\chi^2_1$ = 2.6426, $P$ = 0.104) or infection intensity (Kruskal–Wallis test, $P$ = 0.089, Supporting Information Table S2).

### *B. dendrobatidis* infection in gilled and non-gilled individuals

Gilled individuals (pre-metamorphosed juveniles and paedomorphic adults) differed in *B. dendrobatidis* prevalence and *B. dendrobatidis* infection load across locations ($\chi^2_3$ = 15.476, $P$ = 0.001; Kruskal-Wallis test, $\chi^2_3$ = 40.858, $P$ < 0.001; Fig. 3a) and seasons ($\chi^2_3$ = 16.184, $P$ = 0.001; Kruskal–Wallis test, $\chi^2_3$ = 10.996, $P$ = 0.012; Fig. 3b). Location A had the lowest prevalence and infection intensity (all comparisons $P$ < 0.050, Supporting Information Table S3; Fig. 3a), and summer season showed the lowest prevalence and infection intensity (all comparisons, $P$ < 0.050; Supporting Information Table S3; Fig. 3b).

Non-gilled individuals (metamorphosed adults) showed significant differences only in *B. dendrobatidis* prevalence across locations ($\chi^2_3$ = 16.184, $P$ = 0.001; Fig. 3c), with location A showing a lower prevalence than location B ($P$ = 0.024, Supporting Information Table S4, Fig. 3c). *B. dendrobatidis* infection intensity between locations, and

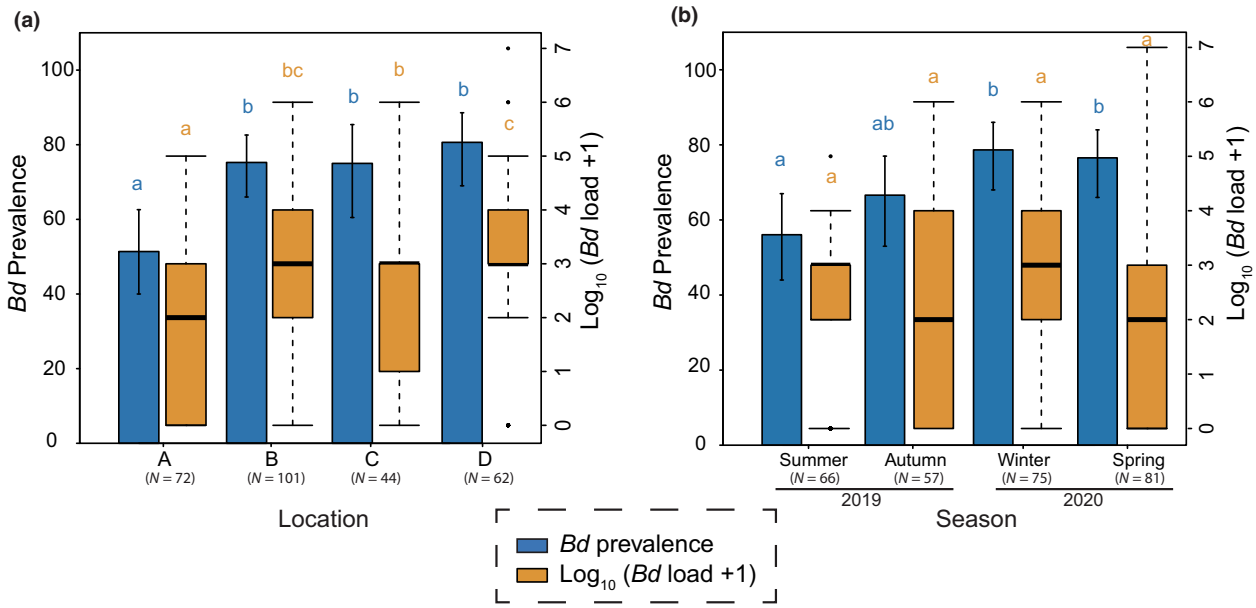


**Figure 2** *Batrachochytrium dendrobatidis* prevalence and infection intensity across (a) localities, and (b) seasons. Blue indicates *B. dendrobatidis* prevalence and orange indicates *B. dendrobatidis* load (ITS copies). Letters indicate significant differences obtained with post hoc Wilcoxon tests. Bar chart of *B. dendrobatidis* prevalence with 95% binomial confidence intervals. Boxplot of *B. dendrobatidis* infection intensity: boxes represent 25 and 75 percentiles, the horizontal line indicates that the median and whiskers are maximum and minimum values of infection intensity, and points represent outliers. Bd, *B. dendrobatidis*.

*B. dendrobatidis* prevalence and infection intensity between seasons were similar (Locations: $\chi^2_3 = 6.8621$, $P = 0.070$, Fig. 3c; Seasons: $\chi^2_3 = 4.95$, $P = 0.170$; Kruskal–Wallis test, $\chi^2_3 = 2.575$, $P = 0.460$; Fig. 3d).

## Water physicochemical differences across locations and seasons

We found significant differences across locations in mean water temperature of sampling day, maximum and minimum seasonal temperature, mean pH, and mean conductivity (Kruskal–Wallis tests: $\chi^2_3 = 10.713$, $P = 0.013$; $\chi^2_3 = 24.002$, $P < 0.001$; $\chi^2_3 = 30.9$, $P < 0.001$; $\chi^2_3 = 20.929$, $P < 0.001$; $\chi^2_3 = 37.773$, $P < 0.001$; respectively), but not in dissolved oxygen (Kruskal–Wallis test, $\chi^2_3 = 5.3465$, $P = 0.150$; Supporting Information Figure S1). Across seasons, we found significant differences in mean water temperature of sampling day, maximum and minimum seasonal temperature, pH, and dissolved oxygen (Kruskal–Wallis tests: $\chi^2_3 = 29.513$, $P < 0.001$; $\chi^2_3 = 8.53$, $P = 0.036$; $\chi^2_3 = 9.739$, $P = 0.020$; $\chi^2_3 = 15.443$, $P = 0.001$; $\chi^2_3 = 30.13$, $P < 0.001$; respectively), but not in mean conductivity (Kruskal–Wallis test, $\chi^2_3 = 2.5714$, $P = 0.500$) (Supporting Information Figure S1).

## Environmental factors and the presence of gills correlate with *B. dendrobatidis* infection

The best model for predicting *B. dendrobatidis* prevalence included physicochemical characteristics of the environment and host-associated traits: season ($P = 0.029$), mean water temperature of sampling day ($P < 0.001$), mean water pH ($P = 0.044$), and the interaction of gilled individuals with dissolved oxygen ($P = 0.005$) were the significant variables (Supporting Information Tables S5 and S6). The presence of *B. dendrobatidis* was positively correlated with summer, winter, and spring seasons, water temperature, and the interaction between the presence of gills and water dissolved oxygen, while water pH was negatively correlated (Supporting Information Table S6; Fig. 4).

The best-fitting GLM for predicting *B. dendrobatidis* infection intensity included physicochemical characteristics of the environment and host-associated traits: locations ($P < 0.001$), mean water temperature of sampling day ($P = 0.003$), mean water conductivity ($P = 0.003$), seasons in gilled individuals ($P = 0.001$), mean water temperature of sampling day in gilled individuals ($P = 0.011$), and maximum seasonal water temperature in gilled individuals ($P = 0.009$) were the significant variables (Supporting Information Tables S7 and S8). Infection intensity was positively correlated with location D and negatively correlated with locations B and C. Water temperature was positively correlated to *B. dendrobatidis* infection intensity. The interaction between the presence of gills with autumn, winter and spring seasons was positively correlated with *B. dendrobatidis* infection load. Additionally, the interaction between the presence of gills with mean water temperature of sampling day was positively correlated with *B. dendrobatidis* infection load, whereas seasonally maximum water temperature was negatively correlated (Supporting Information Table S8, Fig. 5).

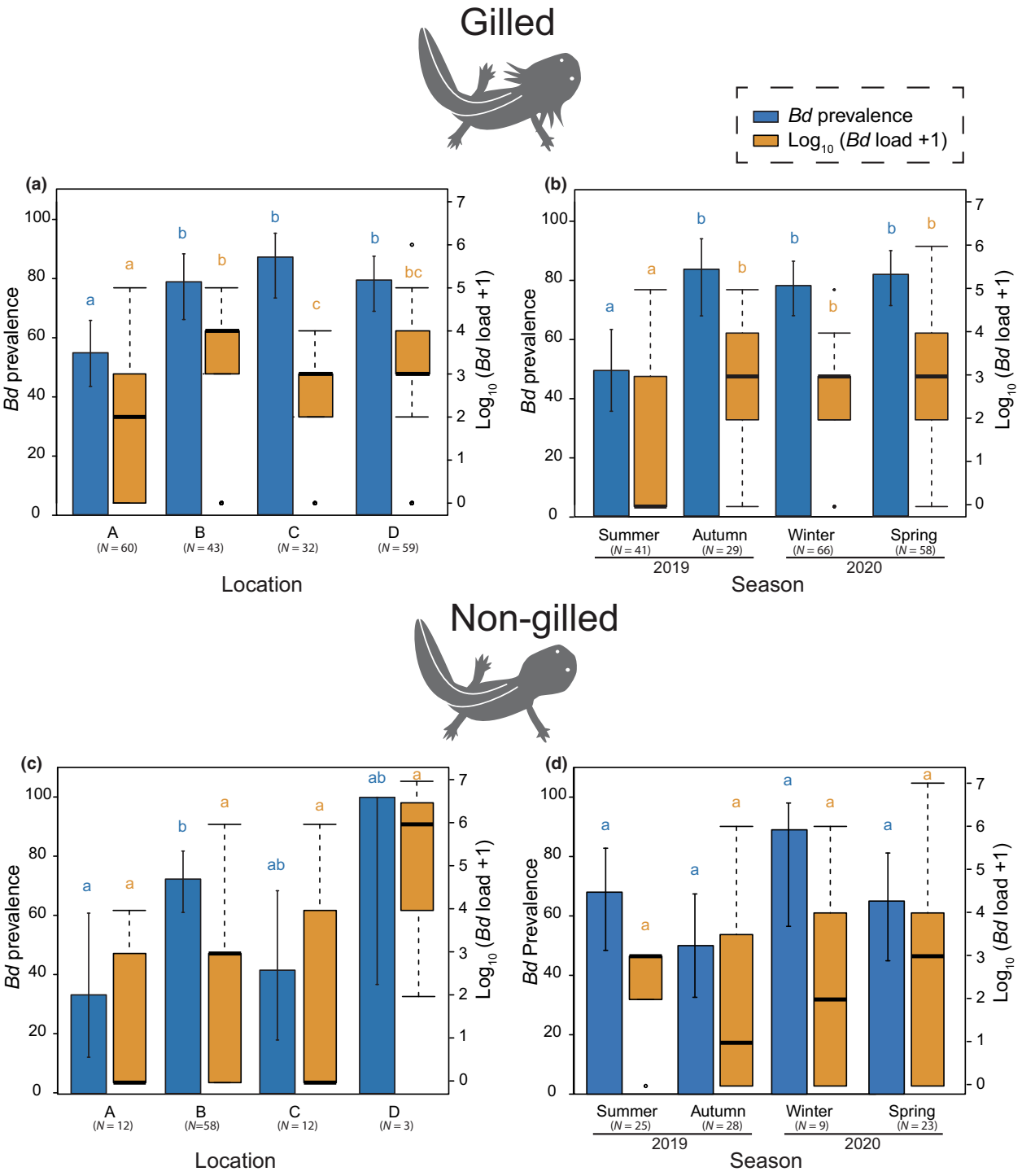


**Figure 3** *Batrachochytrium dendrobatidis* prevalence and infection load in gilled (top) and non-gilled (bottom) *Ambystoma altamirani* across sites (left panels) and seasons (right panels). (a) differences across locations in gilled individuals, (b) differences across seasons in gilled individuals, (c) differences across locations in non-gilled individuals, (d) differences across seasons in non-gilled individuals. Locations are ordered from higher to lower elevation A–D in panels (a) and (c). Seasons are ordered sequentially according to the sampling periods. Bar chart of *B. dendrobatidis* prevalence with 95% binomial confidence intervals. Boxplot of *B. dendrobatidis* infection intensity: boxes represent 25 and 75 percentiles, the horizontal line indicates that the median and whiskers are maximum and minimum values of infection intensity, and points represent outliers. Blue indicates *B. dendrobatidis* prevalence and orange indicates *B. dendrobatidis* load (ITS copies). Letters indicate significant differences obtained with post hoc Wilcoxon tests. Bd, *B. dendrobatidis*.

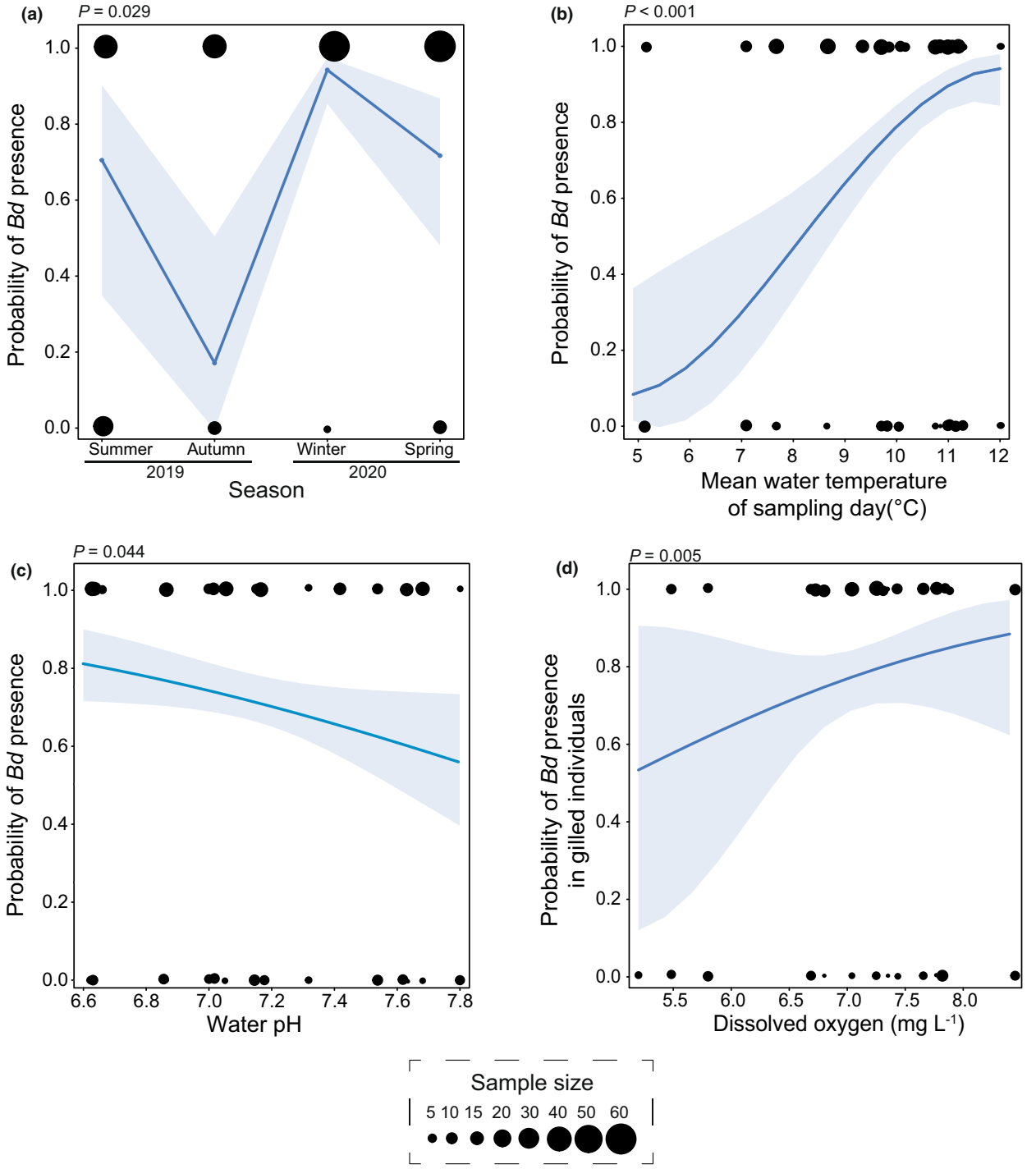


**Figure 4** Estimated response curves (logistic regression: probability presence) of generalized linear models (GLM) for predicted *Batrachochytrium dendrobatidis* presence. (a) Seasons, (b) mean water temperature of sampling day, (c) water pH, (d) dissolved oxygen in gilled individuals. The lighter shaded area represents the 95% confidence intervals. Black circles represent observed *B. dendrobatidis* presence data, and the circle size indicates the sample size. Bd, *B. dendrobatidis*.

## Discussion

In this study, we evaluated for the first time the effect of metamorphosis stage and specific environmental factors on *B. dendrobatidis* infection status across seasons in four populations of the facultative paedomorphic salamander *A. altamirani*. Overall, we found a high *B. dendrobatidis* prevalence and infection load in *A. altamirani* populations. Gilled

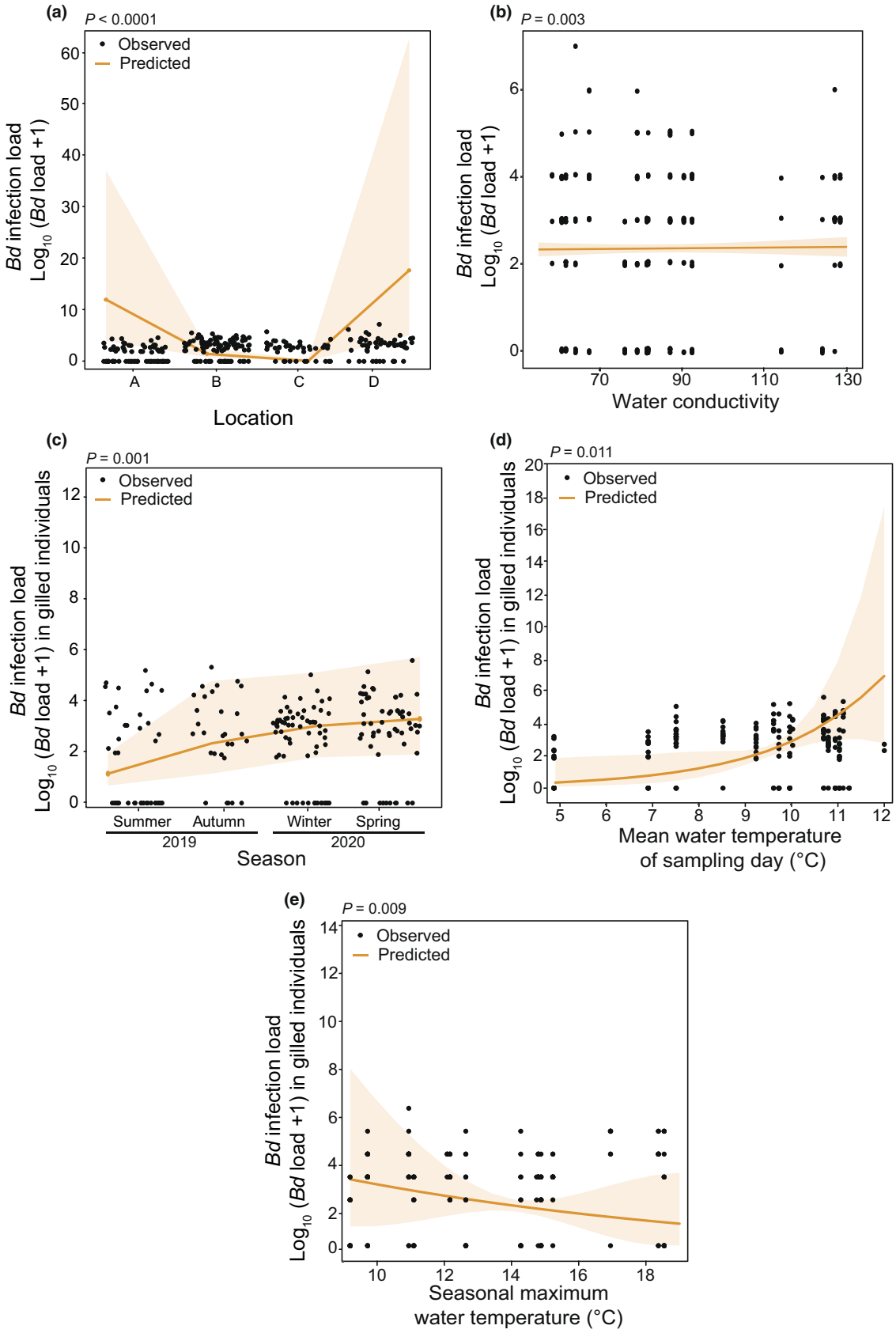

**Figure 5** Estimated response curves (logistic regression: *Batrachochytrium dendrobatidis* load) of generalized linear models (GLM) for predicted *B. dendrobatidis* infection load in *A. altamirani*. (a) Locations, (b) water conductivity, (c) seasons in gilled individuals, (d) mean water temperature of sampling day in gilled individuals, and (e) seasonal maximum water temperature in gilled individuals. The lighter shaded area represents the 95% confidence intervals. Black points represent observed *B. dendrobatidis* infection intensity data. Bd, *B. dendrobatidis*.

individuals had differences in *B. dendrobatidis* prevalence and infection load along the year while non-gilled individuals did not. Our results suggest that *B. dendrobatidis* prevalence and infection load can be greatly influenced by metamorphosis and environmental factors (water temperature, pH, and dissolved oxygen) that are associated with seasonal fluctuations and site-specific conditions (elevation).

## Metamorphosis status influences *B. dendrobatidis* prevalence and infection load

During metamorphosis, aquatic larval morphology is reconfigured for the terrestrial lifestyle, thus differential habitat use could be linked to variations in *B. dendrobatidis* infection. Unfortunately, little is known about the natural history and habitat use of gilled and non-gilled individuals of *A. altamirani*. Previously published information includes anecdotal observations of individuals under rocks near to streams (Lemos-Espinal *et al*., 1999), without any specification for the presence of gills. In our study, all individuals were found in the streams, and we speculate that differences between developmental stages could be related to differences in skin composition and structure and/or immune differences.

Previous studies have shown that the epidermis of gilled individuals has an interstitial layer of mucus secreting Leydig cells that may protect from bacteria and viruses (Jarial, 1989), while in non-gilled individuals Leydig cells disappear, gland maturation occurs and the epidermis is thinner (Fahrmann, 1971*a*, 1971*b*, 1971*c*; Brandon, 1976). These differences may be related to variations in skin antimicrobial peptides and skin microbiome that may inhibit *B. dendrobatidis* infection (Harris *et al*., 2009; Rollins-Smith, 2009). To date, few studies have examined the antifungal activity of skin antimicrobial peptides in salamander species (Sheafor *et al*., 2008; Pereira *et al*., 2018; Pereira & Woodley, 2021), and the differences between gilled and non-gilled salamanders have not been documented. For the skin microbiome, previous studies have documented that the community structure suffers substantial shifts before and after metamorphosis (Kueneman *et al*., 2014; Kueneman *et al*., 2016; Sanchez *et al*., 2017; Demircan *et al*., 2018) that may influence the abundance of antifungal bacteria and the outcome of *B. dendrobatidis* infection (Kueneman *et al*., 2014, 2016; Demircan *et al*., 2018). Future studies should test if differences in skin microbiome composition and/or antimicrobial secretions in paedomorphic salamanders are responsible for a differential *B. dendrobatidis* infection status between developmental stages.

During metamorphosis amphibians require a substantial investment of resources and undergo drastic changes in their immune system (Duellman & Trueb, 1994; Rollins-Smith, 1998; Rollins-Smith *et al*., 2011). There are no immunological studies on *A. altamirani*, but a previous study in *A. mexicanum* showed that metamorphic individuals have a humoral immune response (increase in circulating plasma cells) after repeated antigen challenge, whereas paedomorphic individuals do not (Ussing & Rosenkilde, 1995). Our results showed that gilled individuals had differences in *B. dendrobatidis* infection across seasons in contrast to non-gilled individuals, suggesting that gilled individuals likely have a reduced immune function that could increase their susceptibility to *B. dendrobatidis* in changing seasons.

## Environmental conditions influence *B. dendrobatidis* prevalence and infection load

Environmental conditions in aquatic environments can have a direct effect on *B. dendrobatidis* transmission rate and dynamics of infection by altering the density of viable zoospores (Schmeller *et al*., 2014). Here we found that *B. dendrobatidis* prevalence and infection load in gilled individuals were highest during winter, supporting previous evidence that cooler temperatures favor *B. dendrobatidis* infections in both temperate (Berger *et al*., 2004; Retallick *et al*., 2004; Schlaepfer *et al*., 2007; Forrest & Schlaepfer, 2011; Robak & Richards-Zawacki, 2018; Robak *et al*., 2019; Le Sage *et al*., 2021) and tropical amphibians (Ruggeri *et al*., 2020; Das Neves-da-Silva *et al*., 2021). *B. dendrobatidis* strains from temperate regions can attain high growth rates at temperatures as low as 2–3°C (Voyles *et al*., 2017), and the combination of temperature conditions and amphibian immunity may cause the increased virulence of *B. dendrobatidis* at cooler temperatures. For instance, the effectiveness of amphibian antimicrobial peptides and *B. dendrobatidis* inhibitory skin bacteria are temperature dependent, suggesting that cooler conditions decrease amphibian protection capacity (Matutte *et al*., 2000; Sheafor *et al*., 2008; Daskin *et al*., 2014; Longo & Zamudio, 2017; Robak *et al*., 2019).

Site-specific environmental conditions and seasonal changes modulate patterns in resource availability, host-associated traits (immune function, contact rates), and pathogen-associated factors such as abundance and growth rates (Rachowicz & Vredenburg, 2004; Rowley & Alford, 2007; Bielby *et al*., 2008; Chestnut *et al*., 2014; Lenker *et al*., 2014; Familiar López *et al*., 2017). Aside of the studied parameters, the region is also characterized by urban expansion which has resulted in exploitation and contamination of water bodies, legal and illegal logging, the presence of exotic invasive species (e.g., rainbow trout, *Oncorhynchus mykiss*), as well as livestock management (mainly cows and sheep), which largely depend on the water from springs and creeks in the area (Vega-Chávez

*et al*., 2022). We found that site A had the lowest *B. dendrobatidis* prevalence and infection intensity. This site has the highest elevation among all sites and also has the least human influence. We consider that both of these factors may influence our findings.

Despite the high prevalence of *B. dendrobatidis* during the winter season, we found that both *B. dendrobatidis* prevalence and infection load increased with temperature. The ability of *B. dendrobatidis* to persist and grow at 2–4°C would allow it to overwinter within hosts and, as temperatures rise, *B. dendrobatidis* could start reproducing rapidly during the spring and summer seasons (Piotrowski, Annis, & Longcore, 2004; Voyles *et al*., 2017). A previous study with four *Ambystoma* species in the United States has shown that body temperatures of ambystomatids may have a mean minimum of 5.8°C and a maximum of 26.5°C (Brattstrom, 1963). If *A. altamirani* have similar body temperatures, the observed decrease in water temperature during winter and spring could be related to changes in immune functions causing an increase in infection (Ellison *et al*., 2020).

Water availability and amphibian population density may influence infection dynamics (Rachowicz & Briggs, 2007; Longo, Burrowes, & Joglar, 2010; Adams *et al*., 2017). In the case of our study sites, the winter and spring seasons correspond to the drier months of the year (usually between October and May). Even though *A. altamirani* lives in streams with water all year-round, differences in flow regimes among locations and across seasons may influence both the density of amphibian hosts and *B. dendrobatidis* zoospores. Thus, drier and cooler months are a dangerous combination that would favor *B. dendrobatidis* transmission and infection when the density of *A. altamirani* individuals increases in the few places with water. Because environmental conditions could change between years affecting amphibian communities as well as disease dynamics, further studies should include more years of sampling to determine whether the observed patterns are still present.

Physicochemical factors, such as pH and dissolved oxygen, are also likely to fluctuate geographically and seasonally due to precipitation, oxidation of sulfide-containing sediments through the production of sulfuric acid, algal blooms, and released bases or acids from residues of fertilizers and pesticides (Kong *et al*., 2009). Variations in these factors have been previously associated with differences in *B. dendrobatidis* growth and infection status (Piotrowski, Annis, & Longcore, 2004; Gleason *et al*., 2008; Chestnut *et al*., 2014; Blooi *et al*., 2017). We found that the pH values recorded in the four locations fall within the range considered optimal for the growth of *B. dendrobatidis* (pH: 6–8; Piotrowski, Annis, & Longcore, 2004). However, in contrast with experimental and field observations of increased *B. dendrobatidis* growth rates with increasing pH (Piotrowski, Annis, & Longcore, 2004; Chestnut *et al*., 2014; Blooi *et al*., 2017), *B. dendrobatidis* prevalence was negatively correlated with pH. The pH is influenced by abiotic and biotic factors in any ecosystem, and its effect on *B. dendrobatidis* prevalence may be a reflection of other processes in this aquatic system that are important to *B. dendrobatidis* ecology.

Dissolved oxygen is an important factor for the respiration of both pathogens and aquatic hosts, and its variation along sites and seasons may imply differences in *B. dendrobatidis* infection. We found that dissolved oxygen is a significant predictor of *B. dendrobatidis* prevalence in gilled individuals: the more dissolved oxygen, the higher the *B. dendrobatidis* prevalence. For gilled individuals, oxygen availability is crucial for respiration (Whitford & Sherman, 1968), and chytrid fungi like *Batrachochytrium* are thought to be mostly obligate aerobes since their growth rates depend on dissolved oxygen concentrations (Gleason *et al*., 2008). Previous studies have found that individuals of *A. altamirani* prefer stream sites with small pools and high dissolved oxygen levels (Lemos-Espinal *et al*., 2016). This high preference for sites with high dissolved oxygen levels could increase the population density of gilled individuals, thus favoring *B. dendrobatidis* infection. Future studies should test if gilled population density is directly associated with dissolved oxygen and *B. dendrobatidis* infection across *A. altamirani* distribution.

Decreasing population trends have been reported for *A. altamirani* and this species has been categorized as Endangered by the IUCN (2021). This species lives only in high mountain streams in Estado de México, Morelos, and Mexico City (Woolrich-Piña *et al*., 2017), where habitat loss, pollution, and introduced predatory fish are their principal threats (IUCN, 2021; Vega-Chávez *et al*., 2022). During our sampling most individuals did not show any visible sign of chytridiomycosis, and previous studies have shown that several *Ambystoma* species seem to be tolerant and resistant to *B. dendrobatidis* and in some cases to *B. salamandrivorans* (Michaels *et al*., 2018; Basanta *et al*., 2019; Barnhart *et al*., 2020; Nava-González *et al*., 2020). However, clinical signs of the disease have been detected for *A. mexicanum* in captivity (Del Valle & Eisthen, 2019). Thus, the presence of *B. dendrobatidis* and the high infection loads detected in one moribund individual in our sampling in addition to other death individuals found in the region (Basanta *et al*., 2021) suggests that *B. dendrobatidis* could be a threat to *A. altamirani* under certain conditions. Additional stressors such as climate change and the arrival of other pathogens may affect *B. dendrobatidis* infection dynamics causing cryptic sublethal effects on population health and, in turn, lead to long-term population declines. We suggest that action is urgently needed to better understand the conservation risk that pathogens pose to these populations.

Our study highlights the influence of metamorphosis, seasonality and environmental conditions on *B. dendrobatidis* infection dynamics. Considering that *A. altamirani* and most *Ambystoma* salamanders in Mexico are considered at risk of extinction or endangered, metamorphosis status and environmental factors should be considered in future studies to estimate chytridiomycosis risk in their populations and to elaborate effective conservation and management plans.

## Acknowledgments

MDB thanks the Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), CONACyT for a

postdoctoral fellowship (Project CF-2019/373914), and University of Nevada, Reno for Faculty Travel Grant-FY23. EM-U is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas UNAM and has received a CONACyT Fellowship. We thank Dalia Zavala, Paola Palacios, Juan Pablo Pérez, Honoria Rueda-González, Inés Rosas-Chávez, Luis Zárate, and Bienes Comunales of Santiago Tlazala for the field assistance, Rafael Peña for his help in Hobos installation design, and Santiago Ramírez-Barahona for constructive comments to the manuscript. This research was supported by a grant from the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM) IA201419 granted to EAR. Collection permits were provided by the Secretaría del Medio Ambiente y Recursos Naturales SGPA/DGVS/5673/19.

## Author contributions

EAR conceived and designed the project. EAR, EM-U, TMG-M, VDA-A, MVT, and SLA-M collected all the samples and data. SLA-M and MDB performed *B. dendrobatidis* qPCR. MDB analyzed the data. All authors interpreted data. MDB and EAR wrote the first draft of the manuscript and all authors contributed to the improvement of the manuscript.

## Conflict of interest

We declare no conflict of interests.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Pairwise Pearson correlations of physicochemical and host-associated traits for *A. altamirani*. Variables selected with less than $r = 0.7$ are highlighted in red.

**Table S2.** Comparison of *Batrachochytrium dendrobatidis* prevalence and *B. dendrobatidis* infection load between sites and seasons for all individuals. Significant differences are shown in bold.

**Table S3.** Comparison of *Batrachochytrium dendrobatidis* prevalence and *B. dendrobatidis* infection load between sites and seasons for gilled individuals. Significant differences are shown in bold.

**Table S4.** Comparison of *Batrachochytrium dendrobatidis* prevalence and *B. dendrobatidis* infection load between sites and seasons for non-gilled individuals. Significant differences are shown in bold.

**Table S5.** Results of the 10 best fitting generalized linear models (GLM) for *B. dendrobatidis* presence. The most parsimonious model with the lowest AIC value (the best model) is shown in bold.

**Table S6.** Results of the best fitting generalized linear model (GLM) for *B. dendrobatidis Batrachochytrium dendrobatidis* presence in *A. altamirani*. Significant *P* values are shown in bold.

**Table S7.** Results of the ten best fitting generalized linear models (GLM) for *B. dendrobatidis* infection load. The most parsimonious model with the lowest AIC value (the best model) is shown in bold.

**Table S8.** Results of the best fitting generalized linear model (GLM) for *B. dendrobatidis* infection load in *A. altamirani*. Significant *P* values are shown in bold.

**Figure S1.** Water physicochemical variables (mean water temperature of sampling day, seasonal minimum and maximum temperature, mean pH, mean dissolved oxygen, and mean conductivity) across sites and seasons were *A. altamirani* individuals were sampled. Boxes represent 25 and 75 percentiles, the horizontal line indicates that the median and whiskers are maximum and minimum values, and points represent outliers. Letters signify significant differences obtained with post hoc Wilcoxon tests.

# *BATRACHOCHYTRIUM DENDROBATIDIS* OCCURRENCE IN DEAD AMPHIBIANS OF CENTRAL MEXICO: A REPORT OF *AMBYSTOMA ALTAMIRANI* AND *LITHOBATES MONTEZUMAE*

## PRESENCIA DE *BATRACHOCHYTRIUM DENDROBATIDIS* EN ANFIBIOS MUERTOS DEL CENTRO DE MÉXICO: UN INFORME DE *AMBYSTOMA ALTAMIRANI* Y *LITHOBATES MONTEZUMAE*

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**Received**: 2020-11-06. **Accepted**: 2021-02-03.

**Resumen.—** La quitridiomicosis, causada por los hongos *Batrachochytrium dendrobatidis* (*Bd*) y *B. salamandrivorans* (*Bsal*), es una enfermedad infecciosa relacionada con la muerte masiva de anfibios en todo el mundo. En este estudio, se analizaron cuatro individuos muertos y moribundos de *Ambystoma altamirani* y *Lithobates montezumae* para detectar la presencia de *Bd* y *Bsal*. Mediante el uso de PCR en tiempo real (qPCR) e histopatología, se detectó la presencia de *Bd* y la ausencia de *Bsal* en todos los individuos analizados. Estos resultados indican que la quitridiomicosis puede representar una amenaza para estas especies, y sugieren la urgencia de realizar futuros estudios que evalúen la infección por *Bd* en las poblaciones de *A. altamirani* y *L. montezumae*.

**Palabras clave.—** Quitridiomicosis, anfibios, declives, enfermedades infecciosas.

**Abstract.—** Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), is an infectious disease of amphibians linked to mass amphibian die-offs worldwide. In this study, we sampled four dead and dying individuals of *Ambystoma altamirani* and *Lithobates montezumae* to detect the presence of *Bd* and *Bsal*. By real-time PCR (qPCR) and histopathology methods, we found the presence of *Bd* and the absence of *Bsal* in all individuals sampled. Our study indicates that chytridiomycosis may act as a threat for these species and highlight that future surveys are urgently needed to evaluate the *Bd* infection on populations of *A. altamirani* and *L. montezumae*.

**Keywords.—** Chytridiomycosis, amphibians, declines, infectious disease.

Chytridiomycosis is cataloged as the worst infectious disease in vertebrates due to the great extent of affected species and the mass amphibian die-offs caused worldwide over the last century (Gascon et al., 2007). The disease is caused by two fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). Chytridiomycosis causes hyperkeratosis and hyperplasia, which can cause death and even catastrophic declines in susceptible species' populations (Voyles et al., 2009; Martel et al., 2013). In Mexico, *Bd* has been found in 83 amphibian species (Basanta et al., 2019; Bolom-Huet et al.,

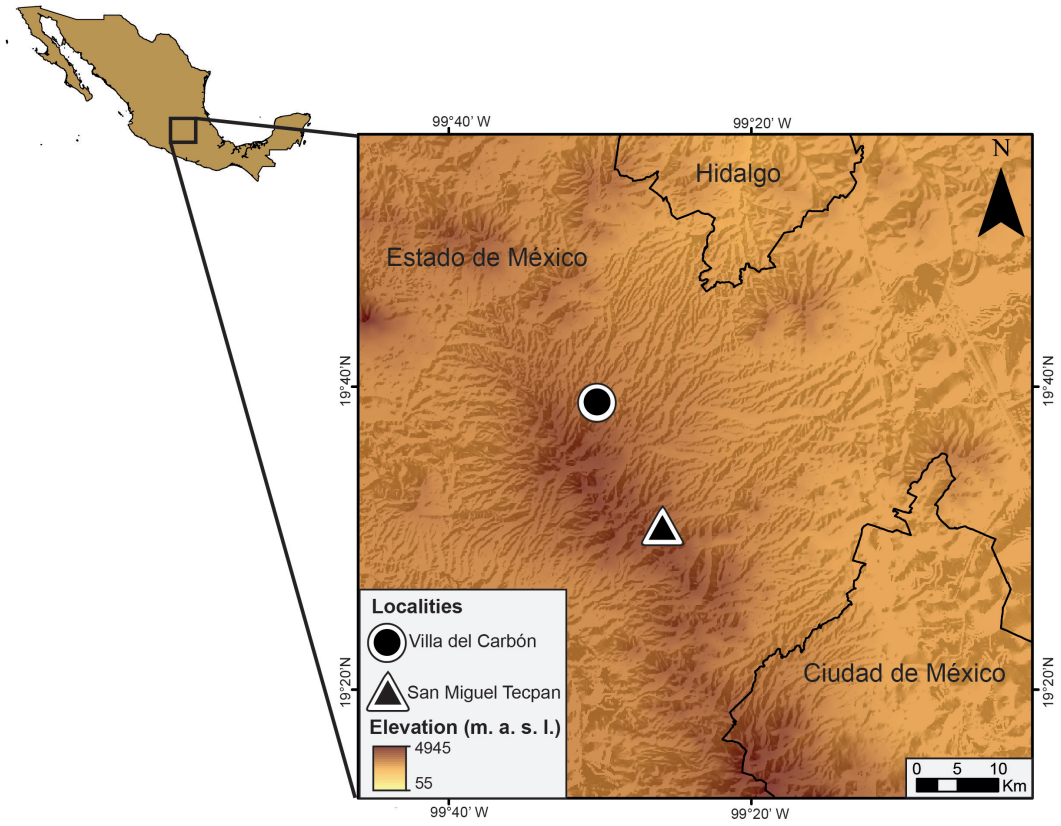


**Figura 1.** Mapa de las localidades Villa del Carbón y San Miguel Tecpan donde fueron encontrados los individuos moribundos o muertos de *A. altamirani* y *L. montezumae* infectados por *Bd*.
**Figure 1.** Map of the localities Villa del Carbón and San Miguel Tecpan where individuals of *A. altamirani* and *L. montezumae* were found moribund or dead, and infected by *Bd*.

2019; Hernández-Martínez et al., 2019), while the *Bsal* has not been detected yet in the country (Olivares-Miranda et al., 2020; Waddle et al., 2020).

Between January-July 2019 (winter and summer seasons) we found two dead and one dying individuals of *Ambystoma altamirani*, and one dead individual of *Lithobates montezumae* in the municipalities of Villa del Carbón, and Jilotzingo (at San Miguel Tecpan locality), Estado de México, all located in the northern part of Sierra de las Cruces in Central Mexico (Fig. 1). All specimens were found without obvious external causes of death or damage (e.g., predation or injury), and the dying individual showed chytridiomycosis signs such as lack of reflexes, stiffness, and extreme skin shedding (Fig. 2). The specimens of *A. altamirani* (N = 3) and *L. montezumae* (N = 1) were swabbed with a synthetic cotton swab following the protocol by Hyatt et al. (2007). All the individuals were fixed and stored in neutral 10% formalin. In the laboratory, DNA extraction from swab samples was performed using Prepman or Qiagen Blood and Tissue Kit DNA extraction (Table 1). Then, samples were assayed using real-time TaqMan PCR assays according to Boyle et al. (2004) and Martel et al. (2013) to detect *Bd* and *Bsal* presence, respectively. Each sample was run in duplicate with a negative control (5 uL sterile water) and four standards of DNA Gblocks (1, 100, 1000, and 10000 genome equivalents, GE) for separate assays of *Bd* and *Bsal*.

Multiple skin samples from fixed individuals were obtained for histological examination according to Berger et al. (1999). Briefly, skin samples were dehydrated in ethanol of increasing gradation, from 40% to 100%, clearing with xylene, and embedded in paraffin using a Tissue Embedding Center-Tissue-Tek®. Microtomy with disposable blades was carried out in a microtome Leica RM2125RT to obtain 4-6 µm thick sections which were stained with hematoxylin and eosin (Berger et al., 1999) or Schiff periodic acid histochemistry (PAS) and analyzed with an Olympus BX50 microscope equipped with a Lumenera digital camera and Infinity Analyze 6.3.0 software. The search

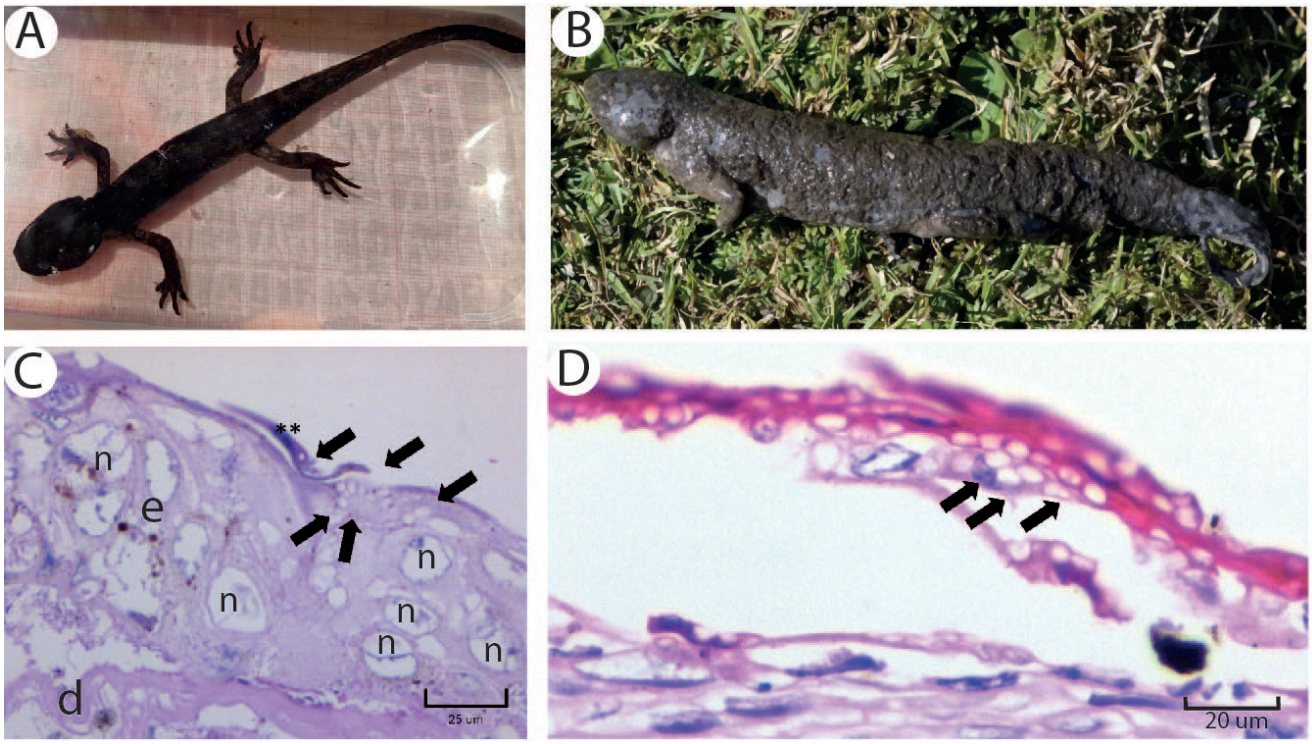


**Figura 2.** Individuos de *Ambystoma altamirani* infectados por *Bd*. A) individuo moribundo con desprendimiento de piel extremo, B) individuo muerto, C) piel con zoosporangios de *Bd*, D) fragmento de exfoliación epidérmica con zoosporangio incrustado. Dermis (d), epidermis (e), núcleo de una célula epitelial (n), zoosporangio (flechas), exfoliación epidérmica (**). Fotos de Eria A. Rebollar (A), Oscar L. Chávez (B), y microfotografías de Omar Betancourt y Armando Pérez-Torres (C-D).

**Figure 2.** Individuals of *Ambystoma altamirani* infected by *Bd*. A) dying individual with extreme skin shedding, B) dead individual, C) skin with *Bd* zoosporangia, D) fragment of epidermal exfoliation with an embedded zoosporangium. Dermis (d), epidermis (e), nucleus of an epithelial cell (n), zoosporangia (arrows), epidermal exfoliation (**). Photographs by Eria A. Rebollar (A), Oscar L. Chávez (B), and photomicrographs by Omar Betancourt and Armando Pérez-Torres (C-D).

for *Bd* zoosporangia was carried out at a total magnification of 400X. All specimens were deposited in the Colección Nacional de Anfibios y Reptiles, Instituto de Biología, UNAM (IBH).

The analyses of qPCR showed *Bd* presence and *Bsal* absence in all swab samples of *A. altamirani* and *L. montezumae* (Table 1). Dead individuals showed lower *Bd* infection loads than the dying individual (Table 1). Histopathology of skin showed evidence of fungal infection in all *A. altamirani* individuals. Spherical and ovoid zoosporangia, empty or containing zoospores, were identified in the superficial and partially detached keratinized cell layers of the epidermis (Fig. 2) with irregular thickening of the epidermis due to hyperplasia. The infected areas included zoosporangia ranging from 5 µm to 10 µm in diameter, mild to moderate hyperkeratosis, and areas of focal erosion adjacent to the infection. These observations agree with *Bd* infection as described by Berger et al. (1999). Skin histopathology of *L. montezumae* skin showed diffuse epidermal detachment related to postmortem changes, so the search for fungal infection was not feasible.

Our finding constitutes the first record of *Bd* in dead or dying amphibians of Central Mexico. The presence of dead and moribund specimens on different occasions and seasons of the year of *A. altamirani* suggests that these species could be susceptible to *Bd* infection. The low *Bd* infection load found in dead individuals may have been due to DNA degradation as a cause of the deteriorating state of the specimen, while the high infection load found in the two dying individual suggests that *Bd* may be one of its causes of death. *Ambystoma altamirani* and *L. montezumae* are threatened and endemic species of Mexico. The axolotl *A. altamirani* has a restricted distribution in Central Mexico, considered "Endangered" by the IUCN (IUCN, 2020a) and identified as "Threatened" by the Mexican law (NOM-059; SEMARNAT 2015). Meanwhile, the frog *L. montezumae* has a wide distribution in Central Mexico and it is considered a species of "Least Concern" by the IUCN (IUCN, 2020b) and subject to

**Tabla 1.** Individuos analizados para la detección de *Bd* y *Bsal* y depositados en la Colección Nacional de Anfibios y Reptiles, Instituto de Biología, UNAM (IBH).
**Table 1.** Individuals sampled for Bd and *Bsal* detection and deposited in the Colección Nacional de Anfibios y Reptiles, Instituto de Biología, UNAM (IBH).

| Species | Individual status | Collection date | Locality | Extraction method | Histological examination | Bd load GE | Bsal load GE | Voucher number |
|---|---|---|---|---|---|---|---|---|
| *Ambystoma altamirani* | Dead | January 2019 | Villa del Carbón | Not evaluated | Presence of zoosporangia | Not evaluated | Not evaluated | IBH32583 |
| *Ambystoma altamirani* | Dead | April 2019 | Villa del Carbón | Prepman | Presence of zoosporangia | 1.4 | 0 | IBH32584 |
| *Ambystoma altamirani* | Moribund | July 2019 | San Miguel Tecpan | Qiagen | Presence of zoosporangia | 337,927 | 0 | IBH32585 |
| *Lithobates montezumae* | Dead | January 2019 | Villa del Carbón | Prepman | Not evaluated | 329.4 | 0 | IBH32582 |

"Special Protection" by Mexican law (NOM-059; SEMARNAT, 2015). The main threats to both species are habitat loss, pollution of the streams where these species are distributed, and the presence of invasive fish species (Lemos-Espinal et al., 1999). Our study indicates that chytridiomycosis is an additional threat for these native species from Central Mexico. Previous studies of *Bd* detection on wild *Ambystoma* and *Lithobates* species in Mexico have found medium to high *Bd* prevalence, but without any dead individuals or those with signs of the disease chytridiomycosis (Frías-Alvarez et al., 2008; García-Feria et al., 2017; Peralta-García et al., 2018; Basanta et al., 2019).

Based on our results, future surveys are urgently needed to evaluate the prevalence and infection intensity in populations of *A. altamirani* and *L. montezumae* across their respective distributions, so that proper conservation strategies can be implemented for these species.

**Acknowledgments.–** MDB thanks the Posgrado en Ciencias Biológicas program, Universidad Nacional Autónoma de México, and CONACyT for a scholarship grant (CVU/Becario: 509293/288791). We thank M. García-Castillo for constructive comments regarding the manuscript, Luis Zárate for the field assistance, and Omar Hernández-Ordóñez for the help provided.

This research was supported by grants from the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM) IN203617 to GPO and IA201419 to EAR. Collection permits were provided by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT) SGPA/DGVS/003513/18, SGPA/DGVS/1496/19.

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