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“Infección por *Batrachochytrium dendrobatidis* y su relación con la comunidad bacteriana presente en la piel del ensamble de anfibios de la Reserva de la Biosfera Mariposa-Monarca”.

TESIS

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M. EN C. BISBRIAN ALHELÍ NAVA GONZÁLEZ
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DIRECTORA DE TESIS
DRA. IRERI SUAZO ORTUÑO
CO-DIRECTORA
DRA.GABRIELA PARRA-OLEA

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INDICE

RESUMEN

La pérdida de biodiversidad en la actualidad es vertiginosa. Aunque son muchos los factores que contribuyen, las EIDs (enfermedades infecciosas emergentes) se posicionan como uno de los más importantes. Entre ellas, la quitridiomicosis, producida por el hongo *Bd (Batrachochytrium dendrobatidis*) continúa como uno de los factores más agresivos en contra de la diversidad de especies de anfibios en el mundo. En México, la mayoría de los estudios se han centrado en especies del trópico y alta humedad, mientras que las áreas boscosas y elevadas, cuentan con poca información. Este estudio, se enfocó en la comunidad de anfibios ribereños de una región de alta montaña (por encima de 3000 msnm), la Reserva de la Biosfera de la Mariposa Monarca, y su estado en relación al hongo *Bd* considerando el papel que la comunidad bacteriana de la piel juega en la defensa de los organismos ante el patógeno a lo largo de un ciclo anual. Encontramos *Bd* en los seis sitios muestreados y registramos *Bd* en ocho de las 11 especies de anfibios evaluadas, incluidas dos especies en riesgo. Dos grupos funcionales mostraron la mayor prevalencia de *Bd*: salamandras acuáticas (AqS) (84%) y ranas semiacuáticas (SaqF) (76%). Se encontró una relación negativa entre la prevalencia de *Bd*, la temperatura y la precipitación, para ambos grupos. Esta relación fue marcadamente fuerte (0,95) durante el invierno, a temperaturas inferiores de 7 ° C y <50 mm de precipitación mensual. En contraste, encontramos las cargas de *Bd* más altas a temperaturas (> 10 ° C) y humedad, más altas (> 100 mm), especialmente para AqS y SaqF. Para el análisis de la comunidad bacteriana muestreamos *Bd* y las bacterias asociadas a la piel de dos especies de anfibios coexistentes (la salamandra *Ambystoma rivulare* y la rana *Rana neovolcanica*). El 63% de los individuos muestreados de la salamandra y el 80% de los individuos de ranas dieron positivo para *Bd*. Registramos 66 géneros de bacterias conocidos por tener efectos inhibitorios contra *Bd* que representan el 55% del porcentaje de la abundancia de géneros de bacteria identificados. Nuestros resultados indican que, si bien se comparten algunos de los taxones bacterianos más abundantes, la estructura de la comunidad bacteriana de la piel de las dos especies huésped es diferente, tanto en composición taxonómica como en abundancia relativa de taxones. *Pseudomonas* y *Streptrophomonas* se encontraron entre los cinco taxones bacterianos más abundantes en la piel de las dos especies hospederas estudiadas. Se ha registrado que ambos taxones bacterianos inhiben la intensidad de la infección por *Bd*. Detectamos que la riqueza bacteriana asociada a la piel, así como la abundancia de bacterias inhibidoras de *Bd*, estuvieron negativamente relacionadas con la intensidad de la infección por *Bd* (medida como el número de zoosporas de *Bd*) independientemente de las especies y las estaciones (secas versus lluvias). En general, la carga de *Bd* varió de 1.75 a 853 zoosporas. A pesar de la prevalencia relativamente alta de *Bd* en las dos especies hospederas, no se registraron individuos muertos o enfermos durante los muestreos en campo. . Nuestros resultados sugieren que la abundancia de bacterias inhibitorias de *Bd* en la piel de los anfibios puede ser sea una defensa biológica importante que limita la intensidead de la infección sin importar las especies hospederas y la estacionalidad en regiones de alta alitud. Los niveles relativamente bajos de carga de *Bd* registrados en la RBMM, aparentemente no comprometen la supervivencia de las especies hospederas. Por lo tanto, nuestros resultados sugieren que los individuos de las poblaciones estudiadas pueden sobrevivir y prosperar bajo una relación dinámica con las infecciones enzoóticas de *Bd*. Un aumento en las temperaturas ambientales en áreas de gran altitud, como se predice para el cinturón volcánico trans-mexicano, podría afectar negativamente a las comunidades de anfibios al hacerlas más vulnerables debido a un probable aumento en la carga de *Bd* y cambios en el porcentaje de abundancia de bacterias inhibidoras de *Bd*.

Palabras clave: Quitridiomicosis, Ambystoma, anfibiofauna, bosque de oyamel, microbiota

ABSTRACT

Nowdays, global biodiversity loss is a rampant phenomenon. Although, a number of factors contribute to this loss, EIDs (emergent infectious diseases) is one of the most important. Quitridiomicosis, is one of these illness, caused by the fungus (*Batrachochytrium dendrobatidis, Bd*). This disease is one of the most aggresive threats to the amphibian diversity around the globe. In Mexico, most studies have dealt with amphibian species from tropical lowland forests, whereas for species from high altitude forests, information is limited. The present study was focused on the riparian amphibian community from a high altitude region (above 3000 m). The Monarch Butterfly Biosphere Reserve (RBMM). The main objective was to assess the relation of this amphibian community to *Bd* infection and the role of the skin-associated bacterial community in the defense against this pathogen, along an anual cycle. We found *Bd* in the six sampled sites, and in eight of the 11 amphibian sampled species *Bd* was registered, including two species at global risk. Two functional groups showed the highest *Bd* prevalence: aquatic salamanders (AqS) (84%) and semiaquatic frogs (SaqF) (76%). A negative relationship between *Bd* prevalence and temperature and precipitation was registered for both groups. This relation was particularly marked (0.95) during the winter season, at temperatures below 7ºC and a monthly precipitation below 50 mm. Contrastingly, higher *Bd* loads were registered at temperatures above 10ºC and precipitation above 100 mm, especially in the AqS and SaqF groups. To analyze the skin-associated bacterial community we sampled *Bd* and bacteria of two co-ocurring amphibian species (the salamander *Ambystoma rivulare* and the frog *Rana neovolcanica*). Sixty-three percent of sampled individuals of the salamander and 80% of frog individuals were *Bd* positive. We registered 66 bacteria genera that present inhibitory effects against *Bd* infection. This number represent in abundance about 55% of the total of bacterial taxa registered. Results indicate that albeit sharing the most abundant bacterial taxa, the structure of the bacterial community of the two host species was different. This difference included the taxonomic composition and the relative abundance of taxa. *Pseudomonas* and *Streptrophomonas* were among the most abundant bacterial taxa in the skin of the two host species. These two bacterial taxa inhibit intensity of *Bd* infection. Skin-associated bacterial richness and abundance of inhibitory bacteria were negatively related to intensity of *Bd* infection (measured as the number of *Bd* zoospores) independent of species and seasons (rainy and dry). Overall, *Bd* load ranged from 1.75 to 853 zoospores. Albeit the relatively high *Bd* prevalence

registered in the two host species, no dead or sick individuals were registered during the field surveys. Our results suggest that abundance of *Bd* inhibitory bacteria might be an important biological defense against *Bd* infection, regardless of host species and seasonality effects in areas of high altitude. The relatively low levels of *Bd* load registered in the RBMM, apparently do not compromise the survival of host species. Therefore, our results suggest that individuals of the studied populations can survive and thrive under a dynamic relation to *Bd* enzootic infections. An increase in environmental temperatures in high altitude regions, as is predicted for areas in the trans-Mexican volcanic belt, might negatively affect amphibian communities by turning them more vulnerable, due to a posible increase in *Bd* load levels and changes in the abundance of *Bd* inhibitory bacterias.

III. INTRODUCCIÓN GENERAL

Los anfibios son uno de los grupos de vertebrados más antiguos e icónicos, ya que representan la transición entre el ambiente acuático y terrestre. La clase Anfibia consta de tres órdenes con especies que en la actualidad existen: Anura (ranas), Caecilia o Gymnophiona (cecilidos) y Caudata o Urodela (salamandras). De ellos, los anuros son los más diversos y exitosos ya que han sido capaces de colonizar la mayoría de los continentes con excepción de la Antártida. Si bien son organismos con una amplia distribución, la mayor riqueza de especies de anfibios se concentra en los trópicos, particularmente en la región Neotropical (Baillie et al., 2010).

Actualmente, se han descrito aproximadamente 8,118 especies en el mundo, de las cuales 7,165 son ranas, 739 son salamandras y 214 son cecílidos (AmphibiaWeb, 2020). Con nuevos descubrimientos, el número de especies continúa aumentando y a partir de 1985 el número total de especies reconocidas se ha incrementando en más de 60% (AmphibiaWeb, 2020). Sin embargo, los anfibios son la clase de vertebrados en mayor riesgo de extinción con aproximadamente el 41% de sus especies en riesgo (Monastersky, 2014). Este porcentaje, no considera 1,567 especies de las que no existe suficiente información para asignarlas a alguna categoría de conservación (IUCN, 2019) Considerando el número de especies de anfibios en riesgo más el número de especies con falta de información (DD deficiencia de datos) para listarlas en alguna categoría de riesgo, el porcentaje de especies potencialmente en riesgo se incrementa a 53% (IUCN, 2019).

Las causas de la pérdida de especies y declive de poblaciones de anfibios son diversas y complejas, frecuentemente asociadas a factores como la degradación, contaminación y destrucción de hábitats, el aumento en la exposición a rayos UV, la extracción y reubicación de ejemplares para uso comercial o consumo humano, el cambio climático, la introducción de especies exóticas, así como la aparición de enfermedades emergentes (Baillie et al., 2010; Blaustein y Wake 1995; Kiesecker et al., 2001; Lips, 2016; Stuart, 2004).

Un patógeno en particular, *Batrachochytrium dendrobatidis* (*Bd*), es actualmente responsable de la pérdida más grave de biodiversidad de anfibios (Berger et al., 1998; Cheng et al., 2011; Daszak et al., 1999, 2003; Skerratt et al., 2007). Descrito en 1999 por Longcore; se ha registrado tanto en individuos en campo como en ejemplares de colecciones científicas colectados varios años antes de la identificación del hongo, en lugares como Australia, África, Norte y

Centroamérica, El Caribe, Asia (Cheng et al., 2011; Burrowes et al., 2017; Frías-Álvarez et al., 2008; Lips et al., 1999, 2003, 2016; Berger et al., 2016; Aceves-Hernández y Dosta, 2014) y más recientemente en Madagascar (Bletz et al., 2015).

El *Bd* es un hongo microscópico perteneciente al orden Quitridiales, con la capacidad de secretar enzimas proteolíticas que degradan celulosa, quitina y queratina. Se ha encontrado en las células queratinizadas del estrato córneo de la piel de anfibios postmetamórficos, así como en las estructuras bucales de las larvas (Blaustein et al., 2005; Fellers et al., 2001; Berger et al., 1998, 2005a; Pessier et al., 1999). Presenta dos formas de vida dentro de su ciclo: la primera, también conocida como fase infecciosa, consiste de zoosporas uniflageladas con capacidad para desplazarse hasta 2cm en medio inmóvil hasta 24hrs las cuales se mueven por quemotaxis en busca de un hospedero (Moss et al., 2008). Al encontrar un sustrato (piel), las zoosporas se enquistan, el flagelo se reabsorbe y un tubo germinativo, crece y penetra las células de la piel del anfibio, tantas veces como sea necesario para alcanzar el estrato germinativo de la piel; una vez ahí, el tubo germinativo se hincha y permite el crecimiento del zoosporangio con algunos rizoides a su alrededor para formar la segunda fase de vida, una fase sésil conformada que se divide mitóticamente para crear nuevas zoosporas que maduran y salen al ambiente al mismo tiempo que la capa basal de piel alcanza la superficie (Greenspan et al., 2012; VanRooij et al., 2012; James et al., 2015; Berger et al., 2005b).

En estados avanzados, la enfermedad, denominada quitridiomicosis, genera síntomas como decoloración, ulceraciones, falta de apetito, posturas anormales y falta de reacción de escape (letargia) (Pessier et al., 1999; Berger et al., 1998; Nichols et al., 2001; Parker et al., 2002 Daszak et al, 2004). El organismo infectado puede sufrir: *1)* Desequilibrio osmótico causado por las perforaciones que generan los tubos de descarga de los zoosporangios (erosión) o por la hiperqueratosis de la piel; este desorden puede ocasionar un desbalance de electrolitos severo que redunda en falla cardiaca (Berger et al., 1998; Pessier et al., 1999; Voyles et al., 2009); *2)* Deficiencia en el intercambio de gases causado por la hiperplasia de la piel (Berger et al., 1998; Pessier et al., 1999); y *3)* Liberación de enzimas proteolíticas tóxicas para los anfibios (Berger et al., 1998). Adicionalmente, se ha reportado que el hongo es capaz de segregar micotoxinas que inhiben la proliferación de linfocitos y promueven apoptosis de los mismos, haciendo más eficiente el desarrollo de la infección (Fites et al., 2013).

En condiciones de laboratorio el hongo crece y se reproduce en un amplio rango de temperaturas (4º a 25 ºC) y de pH (4 a 8). Este rango de variación resulta en cambios en el número de esporangios y zoosporas producidos, importantes en la determinación del grado de infección de los individuos. Aunque se ha demostrado que el hongo es capaz de resistir la congelación (Gleason et al., 2008), la mayoría de las cepas muestran una marcada reducción en el crecimiento y alta mortalidad por debajo de los 10ºC y por arriba de los 28ºC (Longcore et al., 1999; Bradley et al., 2002; Woodhams et al., 2003; Piotrowski et al., 2004), por lo que el crecimiento óptimo se da entre los 17º y 25ºC con un pH de 6-7 (Piotrowski et al., 2004).

En campo, la mayoría de los episodios más severos de mortalidad en masa se ha registrado en regiones húmedas, frías y en elevadas altitudes (por arriba de los 1500 msnm) (Carey et al., 1999; Berger et al., 2004) que coinciden con zonas de altos valores de endemismo de anfibios (Ron, 2005; Becker y Zamudio, 2011). Además, se han identificado picos de infección que corresponden a los meses más fríos del año, así como una mayor sobrevivencia de los individuos infectados durante los periodos cálidos subsecuentes (Kriger y Hero, 2006; Retallick et al., 2004).

En eleuterodactilidos (*Eleuterodactylidae*) del Neotrópico se ha descrito un período cíclico de infección con tres períodos principales: estiaje, frío-húmedo y cálido-seco. La mayoría de los decesos han sido registrados en el período húmedo-frío, cuando la combinación de lluvias escasas y bajas temperaturas promueven la formación de unos cuantos estanques someros donde los individuos se concentran facilitando así la transmisión horizontal del patógeno (Longo et al., 2010). También se ha observado que existe una mayor persistencia y probabilidad de infección en anfibios que frecuentan cuerpos de agua permanentes con flujos moderados en comparación con aquellas especies que frecuentan cuerpos de agua con mayor movimiento o cuerpos someros donde la temperatura es mayor (Kriger y Hero, 2007; Bancroft et al., 2011; Ruggeri et al., 2015). Estas observaciones, tanto en campo como en laboratorio, son el soporte para la hipótesis de una relación entre la estacionalidad (variación en temperaturas y precipitaciones) y los patrones de infección por el hongo.

A pesar de las muchas poblaciones de anfibios erradicadas por el hongo (Berger et al., 1998; Bosch et al., 2001; Lips et al., 2006), existen sitios donde las especies persisten en números discretos, portando la enfermedad (Hanselmann et al., 2004; Briggs et al., 2005; Kriger y Hero, 2006a, 2006b; Rothermel et al., 2008). El estudio de estas poblaciones ha permitido la identificación de una extraordinaria variación de respuestas ante la exposición a *Bd* y con ello, la exploración de posibles estrategias para mitigar la infección de *Bd*. La mayoría de las especies presentan mayor vulnerabilidad y probabilidad de muerte durante los estadios juveniles pre y postmetamórficos (Fellers et al., 2001; Blaustein et al., 2005) mientras que otras especies presentan fuertes afecciones, principalmente en el estadio larvario (Parris y Beaudoin, 2004; Parris y Cornelius, 2004; Davidson et al., 2007). Se ha sugerido también que la variación estacional podría afectar la respuesta inmune de los anfibios, haciéndolos más susceptibles a la enfermedad durante ciertos períodos del año (Rodder et al., 2008; Savage et al., 2011; Ruggeri et al., 2015; Longo et al., 2015;).

Lo anterior sugiere que la infección por *Bd* tiene una relación con factores extrínsecos e intrínsecos del hospedero (Fisher et al., 2009). Entre los factores extrínsecos se cuentan numerosos factores ambientales como sustrato, temperatura, estacionalidad, grado de deforestación y elevación (Lips et al., 2003b; Berger et al., 2004; Raffel et al., 2010), mientras que los factores intrínsecos se relacionan con la dependencia de ambientes acuáticos, comportamiento, tamaño de la población y la inmunidad específica de los hospederos (Daszak et al., 2003; Lips et al., 2003b; Woodhams et al., 2003; 2007c; Kriger y Hero, 2007).

Referente a los factores extrínsecos, se han probado tratamientos anti fúngicos, incluyendo: exposición prolongada a temperaturas mayores a 28ºC, baños con formalin/malaquita y medicamentos fungicidas de uso veterinario como el Itraconazol al 0.01% (Nichols y Lamirande, 2001; Berger et al., 2009;). No obstante, el uso de estos tratamientos se restringe al ambiente de laboratorio, y aunque existen estrategias de control biológico que han probado ser eficientes en el campo, como es el caso del cladócero *Daphnia magna* que es capaz de consumir zoosporas de *B. dendrobatidis* (Buck et al., 2011), son pocos y muy recientes los estudios en el área y los riesgos potenciales a los ecosistemas siguen siendo altos. Más alarmante aún, los organismos expuestos al hongo en condiciones de laboratorio, tienden a re infectarse en el campo, lo que indica que no existe memoria inmunológica o desarrollo de resistencia (Stice y Briggs, 2010).

Los estudios de inmunidad se basan en observaciones de las propiedades bioactivas de los péptidos antimicrobianos (PAMs) secretados por las glándulas granulares de la piel de los anfibios, con actividad específica contra el hongo (Rollins-Smith et al., 2002a, 2002b; Rollins-Smith y Conlon, 2005). Dado que se secretan en la piel, éstos compuestos son considerados la primera línea de defensa del organismo (Rollins-Smith, 2009).

A la fecha se han purificado, caracterizado y probado más de 40 PAMs para evaluar su efectividad contra el hongo y establecer concentraciones inhibitorias mínimas (CIMs). En laboratorio se ha comprobado su eficacia contra el hongo, ya sea en soluciones puras o mezclas de varios péptidos. Sin embargo, se desconoce su efectividad *in vivo* ya que se han observado diferencias sustanciales entre los péptidos de diferentes especies de anfibios (Woodhams et al., 2007b; Rollins-Smith, 2009), y no se conoce si los organismos portadores son capaces de producir las CIMs necesarias en el medio silvestre.

Por otra parte, el microbioma bacteriano de la piel de los anfibios es considerada parte del sistema inmune de los anfibios, protegiendo a los hospederos mediante la producción de metabolitos antifúngicos (Rollins-Smith et al., 2002b; Harris et al., 2006; Lauer et al., 2007; Woodhams et al., 2007c; Brucker et al., 2008). Como otros sistemas inmunológicos, es posible que las diferencias en la composición del microbioma, influyan sobre las diferentes respuestas que presentan los individuos a la enfermedad.

Diferentes especies, tanto animales como vegetales, presentan asociaciones simbióticas donde los microorganismos juegan un papel importante desde el punto de vista ecológico. Dentro de algunas de estas asociaciones simbióticas existe un cierto grado de especificidad de los microorganismos con su hospedero (McFall-Ngai et al., 2013). Estudios en mamíferos (Ley et al., 2008), incluyendo primates (Yildirim et al., 2010) y plantas (Redford et al., 2010), son ejemplos de especificidad entre simbiontes microbianos y diferentes taxa. Sin embargo, poco se conoce sobre las comunidades microbianas naturales que se encuentran en asociación en la piel de los anfibios sanos y si éstas presentan antagonismo a la colonización de *B. dendrobatidis*.

En el caso de los anfibios, Culp et al. (2007) demostraron que existen especies de bacterias comunes entre especies de anfibios. Este grupo de investigadores estudió el microbioma de tres especies de anfibios, una salamandra adulta (*Notophthalamus viridescens*), y dos individuos en estadio larvario, *Rana catesbeiana* y *Plethodon cinereus.* El estudio se enfocó en las identidades del microbioma natural de los anfibios, y si ésta presentaba algún tipo de simbiosis con las especies de anfibios. Lograron identificar cinco cepas bacterianas en *N. viridescens*, tres en *R. catesbeiana* y cuatro en *P. cinereus* y en sus resultados revelaron que, en la composición del microbioma, se encuentra *Pseudomona florescens*, que tiene la capacidad de producir compuestos de propiedades antibióticas, además de fijar nitrógeno. Otro ejemplo de esto, es el uso del microbioma inherente en ranicultura como control biológico y así evitar el uso de antibióticos contaminantes (Mendoza et al., 2012). Se conoce que el microbioma de Rana (*Lithobates) pipiens, Rana muscosa, Hyalinobatrachium colymbiphyllum*, y las salamandras *Hemidactylium scutatum* y *Plethodon cinereus* es eficaz en la inhibición del crecimiento del hongo (Harris et al., 2006; Woodhams et al., 2007a; Walke et al., 2011). Por lo tanto, es evidente la importancia que tiene el microbioma que naturalmente ocurre en los organismos, para el control de patógenos en defensa de los mismos (McFall-Ngai et al., 2013).

Michoacán es la entidad federativa con el 5º lugar en diversidad de especies de anfibios del país y alberga a 54 especies, de las cuales, 30 son endémicas al país y siete al estado. Toda esta biodiversidad habita en apenas un 3% del territorio nacional (Alvarado-Díaz et al., 2013), lo que coloca al estado como altamente biodiverso. Desafortunadamente, la anfibiofauna de Michoacán no está exenta a la infección por *B. dendrobatidis*. Aunque a la fecha se ha registrado la presencia de este hongo solo en dos especies que habitan en el estado de Michoacán, en particular dentro de la Reserva de la Biósfera Mariposa Monarca: la salamandra *Ambystoma rivulare* y la rana *Rana neovolcanica* (Frías-Alvarez et al., 2008), la rapidez con que se propaga el hongo y la coexistencia de estas dos especies con otras 32 especies de anfibios en el Eje Neovolcánico Transversal indican la amenaza del riesgo de propagación de *Bd* en la parte norte del Estado de Michoacán, incluyendo la región de la Reserva de la Mariposa Monarca y sobre la importancia de evaluar la presencia del hongo y qué papel juega la comunidad bacteriana natural presente en la piel de los anfibios sobre la supresión en la infección fúngica.

En este estudio se evalúo la infección por *B. dendrobatidis* en el ensamble de anfibios de un hábitat de alta elevación como lo es la RBMM y la relación entre la presencia del hongo Bd y el microbioma de la piel de dos especies de anfibios (*A. rivulare* y *L. neovolcánica*) que habitan las riberas de seis cuerpos de agua dentro de la RBMM.

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IV. CAPÍTULO 1.

INFECCIÓN POR ***BATRACHOCHYTRIUM DENDROBATIDIS*** EN ANFIBIOS DE UN HABITAT DE ALTA ELEVACIÓN EN EL CINTURÓN TRANS-MEXICANO

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RESUMEN

La quitridiomicosis en anfibios es una enfermedad panzoótica vigente y tiene un amplio impacto mundial en los anfibios. La gravedad del declive de anfibios vinculado a la quitridiomicosis se asocia parcialmente con los rangos altitudinales de las especies. El estado de Michoacán, en México, alberga 54 especies de anfibios en una amplia gama de climas, incluidas las zonas de gran altitud (por encima de 3000 msnm), adecuadas para el crecimiento de *Bd*, como lo es la Reserva de la Biosfera de la Mariposa Monarca. Este estudio tiene como objetivo documentar los patrones de infección por *Bd* en la comunidad de anfibios ribereños de esta altitud, así como explorar las diferencias en las tasas de infección y la estacionalidad entre los grupos funcionales de los anfibios muestreados. Encontramos *Bd* en los seis sitios muestreados y registramos *Bd* en ocho de las 11 especies de anfibios evaluadas, incluidas dos especies en riesgo. Dos grupos funcionales mostraron la mayor prevalencia de *Bd*: salamandras acuáticas (AqS) (84%) y ranas semiacuáticas (SaqF) (76%). Se encontró una relación negativa entre la prevalencia de *Bd*, la temperatura y la precipitación, para ambos grupos. Esta relación fue marcadamente fuerte (0,95) durante el invierno, a temperaturas inferiores de 7 ° C y <50 mm de precipitación mensual. En contraste, encontramos las cargas de *Bd* más altas a temperaturas (> 10 ° C) y humedad más alta (> 100 mm), especialmente para AqS y SaqF. No se encontraron individuos muertos o enfermos durante los muestreos. Estos bajos niveles de patogenicidad probablemente se relacionan con las bajas temperaturas ambientales predominantes en este hábitat de gran altitud que impiden el crecimiento óptimo de *Bd,* particularmente durante el invierno. Nuestros resultados sugieren que un aumento en las temperaturas ambientales en áreas de gran altitud, como se predice para el cinturón volcánico trans-mexicano, podría afectar negativamente a las comunidades de anfibios al hacerlas más vulnerables debido a un probable aumento en la carga de *Bd*.

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Batrachochytrium dendrobatidis infection in amphibians from a high elevation habitat in the trans-Mexican volcanic belt

Bisbrian Alhelí Nava-González · Ireri Suazo-Ortuño· Gabriela Parra-Olea · Leonel López-Toledo · Javier Alvarado-Díaz

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Abstract Amphibian chytridiomycosis panzootic is ongoing and continuing to have extensive worldwide impact on amphibians. Severity of chytridiomycosis-associated amphibians declines is partly associated with species' altitudinal ranges. Michoacán state in México harbors 54 amphibian species on a wide range of climates, including high-altitude areas (above 3000 masl), suitable for *Bd* growth, such as the Monarch Butterfly Biosphere Reserve. This study aims to document patterns of *Bd* infection on the riparian amphibian community from this high altitude, as well as explore differences in infection rates and seasonality among amphibians' functional groups. We found *Bd* at all six sampled sites and registered *Bd* in eight of the 11 evaluated amphibian species, including two species at global risk. Two functional groups showed the highest *Bd* prevalence: aquatic salamanders (AqS) (84%) and semiaquatic frogs (SaqF) (76%). A negative relationship between *Bd* prevalence, temperature and rainfall were found for both groups. This relation was markedly strong (0.95) during the winter, at temperatures below 7°C and < 50 mm of monthly rainfall. Contrastingly, we found the highest *Bd* loads at higher temperature (> 10 °C) and moisture (> 100 mm), especially for AqS and SaqF. No dead or sick individuals were found throughout the whole sampling survey. These low pathogenicity levels were probably related to the predominating low environmental temperatures of this high-altitude habitat preventing optimal *Bd* growth, particularly during winter. Our results suggest that an increase in environmental temperatures in high-altitude areas, as is predicted for the trans-Mexican volcanic belt, might negatively affect amphibian communities by making them more vulnerable because of a likely increase in *Bd* load.

Keywords Chytridiomycosis in México · High elevation amphibians · *Bd* seasonality · Temperate areas · Fir forest amphibians

Introduction

Globally, about 41% of amphibian species are threatened with extinction (Stuart 2004). Population declines, as well as extirpation of populations, have frequently occurred in relatively pristine habitats, where anthropogenic disturbance is low (Berger et al.

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B. A. Nava-González · I. Suazo-Ortuño (✉) ·
L. López-Toledo · J. Alvarado-Díaz
Instituto de Investigaciones sobre los Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, México
e-mail: ireri.suazo@gmail.com

G. Parra-Olea
Instituto de Biología, Universidad Nacional Autónoma de México, Cd de México, México

1998; Lips et al. 2005; Young et al. 2001). Many of these events have been attributed to an emergent disease called chytridiomycosis which is caused by *Batrachochytrium dendrobatidis* (*Bd*) (Becker et al. 2017; Berger et al. 1998; Longcore et al. 1999; Piotrowski et al. 2004). *Bd* breaks down skin's keratin, disrupting the integument functions (Berger et al. 1998; Voyles et al. 2009) of the infected individuals. In some species, *Bd* infection inhibits host's immune response (Fites et al. 2013; Woodhams et al. 2007b) and in cases of acute infection, osmotic imbalance may result in host's death (Voyles et al. 2007, 2009). However, susceptibility to *Bd* varies among species. Under laboratory conditions, mortality rates of amphibians range from 0 to 100% depending on the amphibian species, host age, *Bd* genotype and dosage (Berger et al. 2005b; Daszak et al. 2003; Pessier 2002; Pessier et al. 1999). Differences in host susceptibility to *Bd* include immunogenic variation (Rollins-Smith et al. 2011; Woodhams et al. 2007b), microhabitat use (Lips et al. 2003b; Simpkins et al. 2017), level of association with water (Lips 1999) and host's thermoregulatory behavior (Daversa et al. 2018; Wood- hams et al. 2003). Because the transmission of *Bd* may occur by direct physical contact between individuals or by contact with motile zoospores, species that spend more time in water are expected to be more vulnerable to infection than those species that are mainly terrestrial (Greenspan et al. 2012). Thus, it is expected that *Bd* susceptibility among species will show a relation with life-history traits, and that species within functional groups with a completely aquatic life cycle or semiaquatic will show different vulnerability to *Bd*, being the aquatic species the more susceptible (Greenspan et al. 2012; Lips et al. 2003b; Longo et al. 2015; Rachowicz and Briggs 2007).

Although amphibian chytridiomycosis has a catastrophic impact, since it is the main cause of the decline of more than 500 species and the extinction of at least 90 species (Scheele et al. 2019), documented amphibian declines present marked differences in species susceptibility to *Bd*, some species being highly susceptible, disappearing once *Bd* is introduced, others being moderately susceptible, markedly declining, but not losing complete populations, and a few species showing tolerance to infection without developing clinical signs of disease (Berger et al. 1998; Daszak et al. 2003; Lips et al. 2006). Similar variation exists at the population level; individuals of some populations recover more readily from infection than others (Briggs et al. 2005; Davidson et al. 2003). Because *Bd* grows optimally in places with high humidity levels and with temperatures ranging from 17 to 25 °C (Piotrowski et al. 2004) and several studies report massive declines on areas with these environmental conditions, it has been suggested that *Bd* may markedly respond to seasonality (Horner et al. 2017; Piotrowski et al. 2004; Ruggeri et al. 2015; Stevenson et al. 2014).

Batrachochytrium dendrobatidis was first reported in México in 1972, and until 2013, it has been registered in 50 amphibian species (Mendoza-Almeralla et al. 2015; Savage et al. 2011). In México, *Bd* has been mostly studied in regions bellow 2500 m, with fewer studies in areas above that altitude (Mendoza-Almeralla et al. 2015). The Monarch Butterfly Biosphere Reserve (MBBR), located in the central portion of the trans-Mexican volcanic belt, was established to protect monarch butterflies throughout the wintering period, but it also protects flora and fauna inhabiting these fir forests (DOF 2010). This study aims to document *Bd* infection patterns on the amphibian species inhabiting high elevation habitats. Specifically, we report on patterns of infection prevalence and intensity, and the role of seasonality in these patterns. We hypothesize that *Bd*'s prevalence and intensity (load) will be higher during the summer, when environmental temperature ranges nearest to *Bd*'s optimal growth range (17–25 °C, Piotrowski et al. 2004) and functional groups associated with water will be more susceptible to infection.

Materials and methods

Field site

The study was conducted at the core zone of the Monarch Butterfly Biosphere Reserve (MBBR), located in the trans-Mexican volcanic belt on the border of Michoacán state and state of México, 100 km northwest of México City. The reserve's core zones (13,551 ha) are located above 3000 m, and the main vegetation cover consists of fir forests.
During the summer (11–17 June, 5–11 August), fall (10–16 October), and winter (5–11 December) of 2016, and winter (15–21 February) and spring (31 March–6 April) of 2017, we sampled six independent

streams, each surrounded by fir forest and alpine grasslands. Distance among streams was at least 1km from each other. Altitude of streams ranged from 3000 to 3500 m. Climate is humid cold with a mean annual temperature of 14 °C (range - 1.5 °C in December to 19.9°C in May) and a mean rainfall of 700–1250 mm. The six sampled streams were Arroyo Oyamel (19°37’ 57.38” N, 100°19’ 59.73” W), Arroyo La Calera (19°37’ 57.38” N, 100°19’ 35.84” W), Arroyo Jesús el Nazareno (19°37’ 37.55” N, 100°19’ 9.86” W), Arroyo del Llano el Establo (19°40’ 24.5” N, 100°15’ 59.6” W), Arroyo del Ejido Garatachía (19°40’ 20.0” N, 100°14’ 51” W) and Arroyo la Mesa (19°34’ 49.5” N, 100°12’ 44.1” W) (Fig. 1).

Sampling sites

A 100 × 100 m plot was randomly laid along each of the sampled streams. Width of transects was 50 m from the edge of each of the streams’ banks (left and right) toward uplands. Within this 10,000 m^2, four people visually searched for amphibians during 60 min (sampling effort = 4 person/h). Sampling was diurnal (09:00–10:00) and nocturnal (20:00–21:00). Transect design maximized species detection by allowing us to locate amphibians within the stream, at the stream banks and up to 50m upland away from the stream, during six consecutive days.

To collect skin samples for *Bd* detection, we followed the procedures of Hyatt et al. (2007), swabbing with sterile rayon-tipped swabs the ventral and inguinal regions of each collected individual. Swabs were placed and stored in vials with alcohol (70%) at - 20 °C and then dried to evaporate all residual ethanol prior to DNA extraction. Each swabbed individual was identified to species. After sampling was completed, on every site all sampling material, including collectors’ clothes, was disinfected with alcohol (70%) and chlorine bleach (6%) to prevent accidental *Bd* dispersal.

DNA extraction was carried out using PrepMan® Ultra (Applied Biosystems); tubes were centrifuged at 13,000 9 g for 3 min, incubated at 100°C for 10 min, and the supernatant was then aspirated from swabs and placed in a new, sterile 1.5-ml tube. Testing for *Bd* was performed using real-time PCR analysis (qPCR) on an Applied Biosystems StepOnePlus^{TM} Real-Time PCR System. The *Bd* strain used for standards was GPL1 isolated from *Pseudoeurycea leprosa* from the National Park La Malinche, México, and cultured at the Instituto de Biología UNAM. PCR reactions and amplification parameters followed Boyle et al. (2004). Each sample was run in triplicate. Results are presented as zoospore equivalents (ZE) calculated by multiplying the raw genomic output by 80, as extracts of DNA were diluted 80 times during extraction and the qPCR (Briggs et al. 2010; Vredenburg et al. 2010). A result was considered positive when the sample amplified (0.1 ZE) before cycle 39 for at least two times; mean of the two positive samples was used to calculate ZE (Kriger and Hero 2007). ZE can be used as an index of the intensity of infection (*Bd* load) for individuals sampled.

Data analysis

To evaluate the impact of *Bd* in the riparian amphibian community of MBBR, we used two indicators: (1) prevalence and (2) *Bd* load on the skin of individuals. We then explored their relationship with environmental variables (temperature and monthly rainfall) obtained from the Servicio Metereológico Nacional (http://smn.cna.gob.mxa). On the first statistical analysis, we tested *Bd* prevalence, defined as the number of positive cases divided by the total number of individuals tested per species against monthly mean temperature and monthly rainfall. Then, we analyzed *Bd* load against the same environmental variables.

We grouped the study species in four functional groups according to their particular habitat use: (1) aquatic salamanders (AqS), salamanders with a completely aquatic life cycle (i.e., neotenic individuals with external gills) (*Ambystoma rivulare*); (2) terrestrial frogs (TF), frogs with an arboreal and/or terrestrial habit (*Craugastor hobartsmithi, Craugastor occidentalis, Dryophytes plicatus* and *Plectrohyla bistincta*); (3) semiaquatic frogs (SaqF), frogs whose life cycle is associated with water, at least during the mating season (*Lithobates montezumae, L. spectabilis, L. neovolcanica*); and (4) terrestrial salamanders (Ts), salamanders whose life cycle is completely terrestrial (*Isthmura bellii, Pseudoeurycea leprosa* and *P. longicauda*) (Table 1).

Given the nature of the response variables, the analyses were conducted using generalized linear models (GLMs) with an ANCOVA design in which the environmental variables (temperature and monthly rainfall) were the numeric variables and the functional

Fig. 1 Location of sampling sites for evaluation of *Bd* infection in amphibians from the Monarch Butterfly Biosphere Reserve (RBMM) in México

group (AqS and SaqF) was the categorical variable. The functional groups TF and Ts were excluded from all analysis due to low sample size. For each environmental variable (temperature and monthly rainfall), we tested the relationship against prevalence and load between the two most abundant functional groups (AqS and SaqF). We developed a saturated model considering the main effect of the factors and the interaction. When a term was not significant, the model was reduced to use only significant terms. For prevalence, a binomial error and logit link function were used, while abundance was analyzed using a Poisson error and a logarithmic link function. When over-dispersion problems occurred, a proper rescaled model was then fitted (Crawley 2017). The GLMs were conducted with the Stats package of the R program version 3.5.1 (R Core Team 2018).

To explore whether attributes of the herpetofauna community contribute to explain the infection patterns throughout the year, we correlated species richness,

Table 1 Bd prevalence of the 11 riparian amphibian species collected at RBMM

Functional groups/Species	**Rainy Season**			**Dry Season**			Total
	June	August	October	December	February	April	
Aquatic salamanders (AqS)							
Ambystoma rivulare (DD)	0.75 (28)	0.73 (11)	0.76 (25)	0.83 (30)	0.90 (30)	0.97 (30)	0.84
Terrestrial Frogs (TF)							
Craugastor hobartsmithi (En)	–	–	–	–	0 (1)	–	0
Craugastor occidentalis (DD)	0 (1)	–	–	–	0 (1)	–	0
Dryophytes plicatus (LC)	0.06 (17)	0.33 (6)	0.43 (14)	0 (1)	–	–	0.24
Plectrohyla bisctincta (LC)	1.00 (2)	0.22 (9)	–	–	–	–	0.36
Semi Aquatic Frogs (SaqF)							
Lithobates montezumae (LC)	0.64 (14)	1.00 (1)	0 (1)	0.60 (5)	1.00 (6)	–	0.70
Lithobates spectabilis (LC)	0.43 (28)	0.67 (9)	1.00 (19)	0.93 (15)	0.93 (30)	0.70 (30)	0.76
Lithobates neovolcanica (Nt)	0.58 (19)	0.29 (17)	0.50 (2)	1.00 (2)	0 (1)	–	0.46
Terrestrial salamanders (Ts)							
Isthmura bellii (Vu)	–	–	1.00 (1)	–	–	–	1.0
Pseudoeurycea leprosa (LC)	–	–	0 (1)	–	–	–	0
Pseudoeurycea longicauda (En)	0.50 (2)	0 (3)	–	–	–	–	0.20

Species are clustered in functional groups; number in parenthesis refers to total individuals tested for *Bd*. For each species IUCN status is included in parenthesis

abundance of individuals and diversity (Shannon index) for each of the six sampling dates against *Bd* load (general mean, mean of species and the total sum of load from all individuals), however none of these correlations were significant and therefore we did not include any of these attributes in further analysis.

Results

We sampled a total of 412 individuals of 11 species, from the six surveyed locations within the nuclear protected area of BMMR. The overall abundance and species richness were highest at the beginning of the rainy season (June 16) with 109 individuals of eight species registered, whereas the lowest numbers were registered during the dry season (April 17), with 61 individuals of two species found. *Ambystoma rivulare* was the species with the highest abundance, but also with the highest variation in numbers, with 12 individuals collected in August and 53 in December. *Lithobates montezumae* and *L. neovolcanica* were found mainly during the rainy season and were absent only in one sampling survey during the dry season. *Pseudoeurycea leprosa, I. bellii* and *C. occidentalis* were the rarest species with only one individual collected of each species throughout all the sampling surveys. Most of the skin samples corresponded to *A. rivulare* (38%) and *L. spectabilis* (32%) (Table 1). We found infection in eight of the 11 species tested (73%) and 68% of the samples were *Bd* positive. Of the eleven sampled species, 36% (4) are considered at risk by the IUCN red list: two species as Endangered (*Craugastor hobartsmithi* and *Pseudoeurycea longicauda*), one as Vulnerable (*Isthmura bellii*) and one as Near Threatened (*Lithobates neovolcanica*). The rest of the species are listed in the Least Concern (5), Data Deficient (2) and Near Threatened (1) categories (Table 1).

C. hobartsmithi, C. occidentalis and *P. leprosa* were negative for *Bd*. Prevalence had a marked variation among species that presented infection, from a low of 0.20 in *P. longicauda* to a high of 1.0 in *I. belli* (Table 1). In the case of *I. belli*, however, only one individual was sampled. Overall (all species pooled), the community showed a *Bd* prevalence mean of 0.42 ± 0.1. Species with infected individuals registered in both rainy and dry season belonged to the AqS

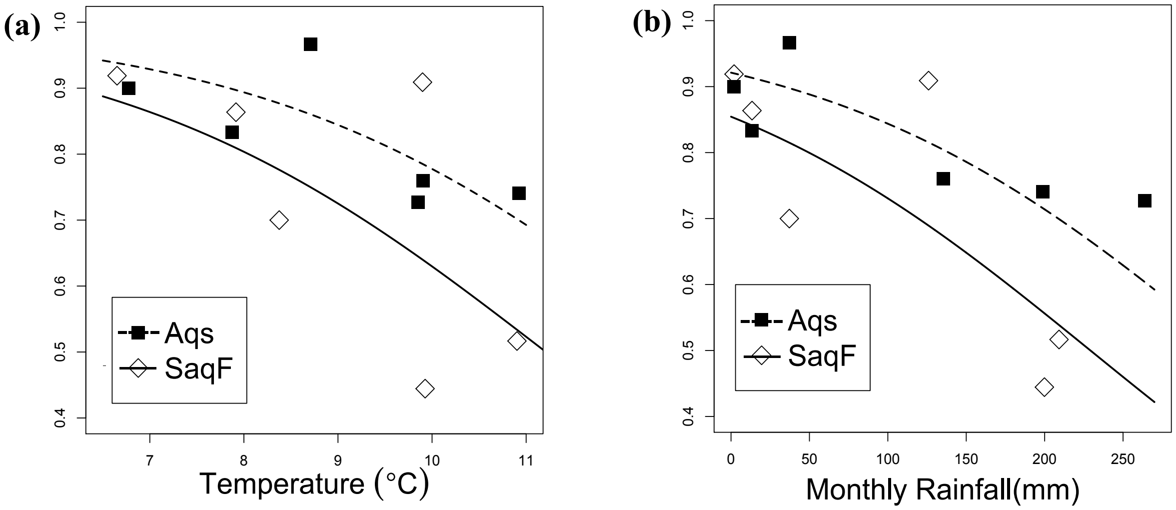

Fig. 2 Prevalence of *Batrachochytrium dendrobatidis* in the riparian amphibians of the Monarch Butterfly Biosphere Reserve (MBBR) in México. a Relation of *Bd* prevalence to temperature for the two most abundant functional groups: semiaquatic frogs (SaqF) and aquatic salamanders (AqS), in which the community was arranged ("Data analysis" section); b relation of *Bd* prevalence to monthly rainfall for the same groups. Regression lines for each group were fit from the GLM binomial

or the SaqF groups. Terrestrial frogs (TF) were registered mainly in the rainy season (N = 49, vs. 2 in the dry season), and terrestrial salamanders (Ts) were registered exclusively in the rainy season. Therefore, seasonal effect on prevalence was only considered for AqS and SaqF groups. *Ambystoma rivulare* (AqS group) showed a high and constant prevalence throughout the whole sampling surveys (mean = 0.84 ± 0.04, range 0.73–0.97). In the SaqF group, *L. montezumae* and *L. spectabilis* presented high, similar prevalence values (0.70 and 0.76, respectively), whereas, *L. neovolcanica* showed lower prevalence values (0.46). For the three species, the mean prevalence was higher in dry than in rainy season (*L. montezumae* 0.80 vs. 0.55, *L. spectabilis* 0.85 vs. 0.70, *L. neovolcanica* 1.0 vs. 0.46) (Table 1).

We found a negative, significant relationship between *Bd* prevalence and mean temperature, reaching the highest prevalence (up to 0.9) at 6.5 °C (Fig. 2). For the first GLM, comparing AqS and SaqF groups we observed a negative relationship between prevalence and mean temperature, although AqS salamanders showed higher prevalence values than SaqF frogs (Fig. 2a; Table 2a). Similarly, to the temperature analysis, we found a negative relationship between *Bd* prevalence and monthly rainfall (Fig. 2b). Additionally, GLM detects significant differences among the two most abundant functional groups, with higher significant prevalence in AqS (0.84) when compared to the SaqF functional group (0.64) (Fig. 2b; Table 2b).

Table 2 Results of the generalized linear models exploring the effects and interaction of temperature (T) and monthly rainfall (R) for the two most abundant functional groups (FG) of the amphibian community of Monarch Butterfly Biosphere Reserve (MBBR)

	χ^2	ρ
(a) Functional groups (AqS, SaqF)		
Temperature (*T*)	$27.7_{(1)}$	< 0.001
Functional groups (FG)	$7.1_{(1)}$	< 0.001
T : FG	ns	–
(b) Functional groups (AqS, SaqF)		
Rainfall (*R*)	$32.5_{(1)}$	< 0.001
Functional groups (FG)	$6.3_{(1)}$	< 0.001
R : FG	ns	–

χ^2 degrees of freedom in parenthesis and ρ-values are shown. ns indicates no significance of the term and it was removed from the final model

Although the presence of *Bd* was registered throughout the year for most of the sampled species, the highest *Bd* loads were observed at the onset of the rainy season. *Bd* reached its highest load (mean ± SE = 38,292 ± 8784 ZE) during the rainy season for SaqF frogs (particularly *L. neovolcanica* and *L. montezumae*) and TF frogs (for *P. bistincta*). In the

Table 3 Bd load (mean ± SE) of the 11 riparian amphibian species collected at RBMM

Species (Status)	**Rainy season**			**Dry season**			
	June	August	October	December	February	April	Total
Aquatic salamanders (AqS)							
Ambystoma rivulare	9172 ± 1733	79 ± 23	610 ± 122	100.5 ± 18.3	94.3 ± 17.2	31.5 ± 5.8	1816.7 ± 146.4
Terrestrial Frogs (TF)							
Craugastor hobartsmithi	–	–	–	–	0	–	0
Craugastor occidentalis	0	–	–	–	0	–	0
Dryophytes plicatus	0.7 ± 0.2	788 ± 321.8	52.7 ± 14.1	0	–	–	144.2 ± 23.4
Plectrohyla bisctincta	8558 ± 6051	30818±10272	–	–	–	–	26771 ±8072
Semi Aquatic Frogs (SaqF)							

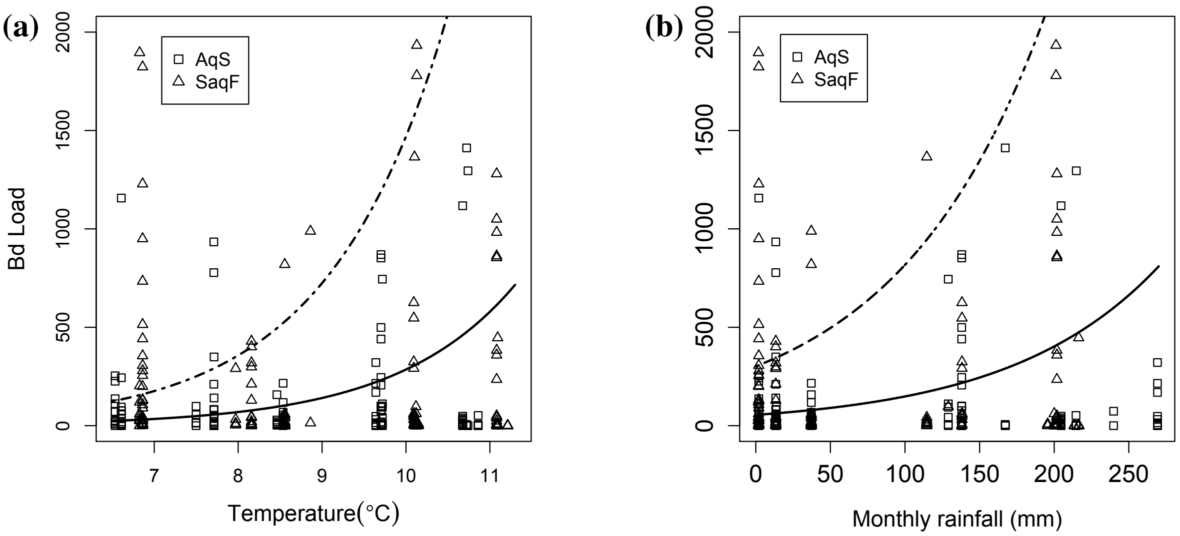

Fig. 3 *Batrachochytrium dendrobatidis* load in the riparian amphibians as a function of climatic variables and functional groups in the Monarch Butterfly Biosphere Reserve (MBBR) in México. Lines correspond to each group shown in the legend box. In **a** relation of *Bd* load to temperature for two functional groups (AqS and SaqF); **b** relationship of *Bd* load to monthly rainfall for same functional groups. Regression lines for each group were fit from the GLM Poisson

cool-dry periods, load decreased 90% in *L. montezumae* and 73% in *L. neovolcanica*. Comparing *Bd* load between warm-rainy periods with cool-dry periods, in the SaqF group load diminished 80% during the cool- dry periods (Table 3). In the AqS group, *A. rivulare* showed a decrease in *Bd* load of 97% during the cool- dry periods.

We found that the two functional groups differed significantly in *Bd* load, with SaqF frogs displaying the highest load (Fig. 3a; Table 4). In contrast to prevalence, we found that *Bd* load associated positively with precipitation and temperature. Specifically, we found a positive relationship with temperature, with highest intensities at a temperature range of 10–11 °C, and the lowest at temperatures below 7 °C, with values dropping to \ 300 ZEs (Fig. 3a; Table 4). We found that infection intensity was also related to monthly rainfall (Fig. 3b). The highest intensities were found at higher rainfall values, especially over 200 mm (Fig. 3b). The GLM indicated the same trend with monthly rainfall among the two most abundant functional groups as seen for mean temperature (Table 4).

Discussion

Table 4 Results of the Poisson generalized linear models exploring the effects and interaction of temperature (*T*) and monthly rainfall (*R*) with the two most abundant functional groups (FG) on *Batrachochytrium dendrobatidis* load within the amphibian community of Monarch Butterfly Biosphere Reserve (MBBR)

	a) Functional groups (AqS, SaqF)	
	χ^2	ρ
Temperature (*T*)	$27.7_{(1)}$	< 0.001
Functional group (FG)	$11.71_{(1)}$	< 0.001
T : FG	ns	–
Rainfall (*R*)	$20.0_{(1)}$	< 0.001
Functional groups (FG)	$11.6_{(1)}$	< 0.001
R : FG	ns	–

χ^2 degrees of freedom in parenthesis and ρ-values are shown. *ns* indicates no significance of the terms and it was removed from the final model

Batrachochytrium dendrobatidis has caused declines in amphibian populations in all continents of the world, except in Asia, where this disease was probably originated (O'Hanlon et al. 2018). Severity of amphibian's populations decline has been associated with environmental conditions and host life-history traits. High environmental humidity, a condition likely to promote *Bd* growth, since it desiccates and dies under dry conditions, has been associated with high severity declines. Therefore, severity of decline has been greatest in species frequently occurring in wet regions and those living in aquatic habitats (Burrowes et al. 2004). Although it has been reported that in high-altitude environments, species decline is moderate (Scheele et al. 2019), there is no information about the causes of this moderate decline. Since 1972, when *Bd* was reported for the first time in México, it was considered a dangerous threat for the Mexican amphibian fauna, especially for those species at global risk (Mendoza-Almeralla et al. 2015). *Bd* has caused notorious diminishments on populations in the southern states of the country, especially for salamanders on tropical lowlands (Lips et al. 2005; Parra-Olea et al. 2014). However, there is limited information for temperate montane regions in the country. For example, few studies (Frías-Alvarez et al. 2008; Kinney et al. 2011) have been carried out at places higher than 3000 m. These studies have reported four *Bd* infected species (*Lithobates neovolcanica, Ambystoma rivulare, Pseudoeurycea cephalica* and *P. leprosa*) that represent 8% of the 50 infected species reported in México until 2013 (Mendoza-Almeralla et al. 2015). Our study showed that of the 11 species that have been registered above 3000 masl in the MBBR, 73% (8 species) were infected with *Bd*, adding two new frog species for areas at these altitudes (*Dryophytes plicatus* and *Plectrohyla bistincta*).

Although, we reported infection in 8 of the 11 sampled species, the three *Bd* free species (*Craugastor hobartsmithi, C. occidentalis* and *P. leprosa*), considering the small sample size (1 individual per species), should not be conclusively considered as *Bd* free. For example, *P. leprosa* has been reported with *Bd* infection in other regions of México (Mendoza-Almeralla et al. 2015). Therefore, it is important to monitor possible vulnerability of these species to *Bd* infection. This is particularly relevant for rare and endangered species such as *C. hobartsmithi*, but it is also important for those species that tested positive for *Bd* (*Pseudoeurycea longicauda* and *Isthmura bellii*) and that are also rare and at global risk (IUCN 2019).

We did not register dead or sick amphibians in any of the sampling surveys. The apparent lack of clinical signs of chytridiomycosis suggests that this community is persisting with enzootic infections of *Bd*, which could be a result of: (1) innate and/or acquired defense mechanisms (Savage et al. 2011; Woodhams et al. 2007a), which are yet to be studied in our sampled species. These acquired defense mechanisms might be a response to a previous unregistered infection event; (2) presence of skin bacterial commensals with anti- fungal properties (Harris et al. 2006); (3) behavioral and ecological factors that reduce the likelihood of infection and disease, such as microhabitat selection and/or reproductive mode (Briggs et al. 2010; Lips et al. 2003b); (4) variation in *Bd* virulence (Berger et al. 2005a; Fisher et al. 2009), considering that genetic identity of *Bd* strains in our study region is currently unknown; and (5) limited *Bd* pathogenicity as a result of non-optimal environmental temperatures for *Bd* growth (Piotrowski et al. 2004).

We registered a relationship between seasons (monthly temperature and rainfall) and *Bd* infection among two functional groups in all surveyed sites. The proportion of infected individuals (prevalence) increased in the two most abundant functional groups, when monthly temperature or rainfall decreased. The pattern found in our study, of higher prevalence associated with lower temperatures (< 7 °C) and precipitation (< 50 mm), is different to the general pattern described in other studies, in which *Bd* infection is positively associated with conditions of high levels of humidity and low temperature (in these studies low temperatures are considered within a range of 10–18 °C; e.g., Adams et al. 2017; Longo et al. 2010; Longo and Zamudio 2017; Ruggeri et al. 2018).

Comparing prevalence among groups, AqS presented significantly more infected individuals than SaqF. This is consistent with increased exposure to pathogen via water sources (Guayasamin et al. 2014; Lips et al. 2003a, b). AqS group was conformed by the ambystomatid *A. rivulare*, a facultative neotenic species, inhabiting the high-altitude streams in the core area of the RBMM. All sampled individuals of this species (N = 150) were collected within streams and showed a high (0.84) and constant prevalence throughout the whole sampling surveys, with no differences between seasons.

We found low prevalence and high load *Bd* levels during the warm-rainy periods of the year.

These findings contrast with other studies that report higher levels of *Bd* prevalence and load during the cool-dry periods (Longo et al. 2010; Longo and Zamudio 2017; Voyles et al. 2017). The lower *Bd* load registered during cool-dry periods in our study might be related to environmental temperatures. Although Piotrowski et al. (2004) indicated that temperatures lower than 10 °C limit optimal *Bd* growth (but still allows *Bd* to persist at low densities; Rachowicz and

Briggs 2007) and that optimal growth occurs at temperatures between 17 to 25 °C, in our study we registered a decrease in prevalence with increasing temperatures (6.5 to 11 °C). According to Voyles et al. (2017) there might be a variation among *Bd* strains in thermal tolerance, and temperate strains can present high growth at temperatures as low as at 2–3 °C. The fungus appears to maintain pathogenicity over a broad range of temperatures and responds to decreasing temperatures with life-history trade-offs that increase fecundity as maturation rate slows and increase infectivity as growth decreases (Woodhams et al. 2008)

The study area in the RBMM presents altitudes higher than 3000 masl, and therefore, average temperatures throughout the year are below 14 °C, and during the cool-dry periods, mean temperature is 7.9 °C, a temperature level markedly below that required by *Bd* for optimal growth. Therefore, our results suggest that during cool-dry periods more individuals are infected, whereas during warm-rainy periods, the prevalence is lower, but environmental conditions (temperature) are more favorable for *Bd* growth (higher load). We hypothesize that during cool-dry periods, individuals of the SaqF group congregate in humid microhabitats, and therefore, although at low *Bd* loads, infection by physical contact among individuals is high (Lips et al. 2003b; Longo et al. 2010). In the case of the AqS group, temperature in streams inhabited by *A. rivulare* is very likely too cold during the cool-dry periods of the year for *Bd* optimal growth, and this is reflected in a markedly low load.

Our results suggested that the combined effects of seasonal variations, high elevation of the study area and differences in habitat use by the studied amphibian species resulted in a *Bd* persistence throughout the year, with high prevalence and high load values alternating between seasons. Although in general, *Bd* prevalence is high, the subclinical manifestations of the studied amphibian community to *Bd* infection might be a result of the general low levels of load registered. The registered low levels of *Bd* load are probably a result of the low environmental temperatures present in the studied area. These temperatures in general are lower than the temperature levels required by Bd for optimal growth. Therefore, the moderate declines registered in high elevation amphibian species around the globe might be a result of the environmental temperature constraining *Bd* growth. This situation of reduced *Bd* pathogenicity might change if environmental temperatures are modified. Studies of climate change in mountainous regions have predicted an increase of 2–3 °C for the 2050 year in the trans-Mexican volcanic belt (Villers-Ruiz and Castañeda-Aguado 2013), where our studied amphibian community lives. This climatic threat might be expected to impact high elevation amphibian populations around the globe.

Conclusions

Worldwide, chytridiomycosis is still a serious problem for many amphibian species and is present in the amphibian populations inhabiting high elevation habitats (above 3000 masl) in México. Although no dead or sick individuals were registered throughout the sampling surveys, our results showed that the riparian amphibian community at high elevation sites in the core area of the RBBM is infected with *Bd*. (Eight of 11 sampled species were positive to *Bd*.) The community showed prevalence mean of 0.42 with two functional groups (aquatic salamanders and semi- aquatic frogs) showing infected individuals in both rainy and dry season. Prevalence was negatively associated with environmental temperature and precipitation with highest prevalence at low temperatures (< 7.0 °C) and precipitation (< 50 mm). In contrast to prevalence, *Bd* load associated positively with precipitation and temperature. Although *Bd* presence was registered throughout the year for most of the sampled species, the highest *Bd* loads were observed during the warm-rainy season. In the SaqF group *Bd* load diminished 80% during the cool-dry periods, whereas in the AqS group, *A. rivulare* showed a decrease in *Bd* load of 97% during the cool-dry periods. Specifically, *Bd* load had a positive relationship with temperature, with highest intensities at a temperature range of 10–11 °C (mean load = 1465.8 ± 285.7), and the lowest at temperatures below 7.0 °C, with values dropping to < 300 ZEs. Considering that environ- mental temperatures in our study area were in general lower than the temperature levels required by *Bd* for optimal growth, we concluded that MBBR’s amphibian communities are in a vulnerable situation, particularly if *Bd* pathogenicity changes as a result of an increase in temperatures as a consequence of global change.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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V. CAPÍTULO II.

INHIBICIÓN DE LA INFECCIÓN DE *Bd* POR LA COMUNIDAD BACTERIANA DE LA PIEL EN POBLACIONES SILVESTRES DE LOS ANFIBIOS *Rana neovolcanica* Y *Ambystoma rivulare*

Este artículo ha sido enviado como:

Nava-González, B.A., I. Suazo-Ortuño, P. Bibian-López, Y. Maldonado-López, L. Lopez-Toledo1, L. Raggi, G. Parra-Olea and J. Alvarado-Díaz. 2020. Inhibition of *Bd* infection by skin bacterial communities in wild amphibian populations of Rana neovolcanica and Ambystoma rivulare. Enviado para revisión.

RESUMEN

Se conoce que varias bacterias asociadas con la piel de los anfibios inhiben la intensidad de infección del patógeno fúngico *Batrachochytrium dendrobatidis* (*Bd*). También se ha postulado que la estructura de la comunidad bacteriana asociada a la piel, está relacionada con la intensidad de la infección por *Bd*. Sin embargo, nuestra comprensión de la dinámica microbiana del huésped y su importancia para limitar la intensidad de *Bd* es limitada. Muestreamos *Bd* y las bacterias asociadas a la piel de dos especies de anfibios coexistentes (la salamandra *Ambystoma rivulare* y la rana *Rana neovolcanica*) de un sitio tropical de gran altitud en el centro de México. El sesenta y tres por ciento de los individuos muestreados de la salamandra y el 80% de los individuos de ranas dieron positivo para *Bd*. En general, registramos 622 géneros de bacterias asociadas con la piel, de los cuales 66 son conocidos por tener efectos inhibitorios contra *Bd*. Estos taxones inhibidores representaron una abundancia aproximada del 55% en relación con la abundancia bacteriana total. Nuestros resultados indican que, si bien se comparten algunos de los taxones bacterianos más abundantes, la estructura de la comunidad bacteriana de la piel de las dos especies huésped es diferente, tanto en composición taxonómica como en abundancia relativa de taxones. *Pseudomonas* y *Streptrophomonas* se encontraron entre los cinco taxones bacterianos más abundantes en la piel de las dos especies hospederas estudiadas. Se ha registrado que ambos taxones bacterianos inhiben la intensidad de la infección por *Bd*. Detectamos que la riqueza bacteriana asociada a la piel, así como la abundancia de bacterias inhibidoras de *Bd*, estuvieron negativamente relacionadas con la intensidad de la infección por *Bd* (medida como el número de zoosporas de *Bd*) independientemente de las especies y las estaciones (secas versus lluvias). En general, la carga de *Bd* varió de 1.75 a 853 zoosporas. A pesar de la prevalencia relativamente alta de *Bd* en las dos especies hospederas, no se registraron individuos muertos o enfermos durante los muestreos en campo. Los niveles relativamente bajos de carga de *Bd* registrados, aparentemente no comprometen la supervivencia de las especies hospederas. Por lo tanto, nuestros resultados sugieren que los individuos de las poblaciones estudiadas pueden sobrevivir y prosperar bajo una relación dinámica con las infecciones enzoóticas de *Bd*.

Inhibition of *Bd* infection by skin bacterial communities in wild amphibian populations of *Rana neovolcanica* and *Ambystoma rivulare*

Bisbrian Nava-González[1], Ireri Suazo-Ortuño[1*], Perla Bibian López[2], Yurixhi Maldonado-López[3], Leonel Lopez-Toledo[1], Luciana Raggi[4] , Gabriela Parra-Olea[5] and Javier Alvarado-Díaz[1]

[1]Instituto de Investigaciones sobre los Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo, Avenida Juanito Itzícuaro SN, Nueva Esperanza, 58330 Morelia, Michoacán, México.

[2]Universidad Tecnológica de Morelia. Avenida Vicepresidente Pino Suárez 750, Cd. Industrial, 58200 Morelia, Michoacán, México.

[3]CONACyT-Instituto de Investigaciones sobre los Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo, Avenida Juanito Itzícuaro SN, Nueva Esperanza, 58330 Morelia, Michoacán, México

[4]CONACYT-Instituto de Investigaciones Agropecuarias y Forestales, Universidad Michoacana de San Nicolás de Hidalgo, Avenida Juanito Itzícuaro SN, Nueva Esperanza, 58330 Morelia, Michoacán, México.

[5]Instituto de Biología, Universidad Nacional Autónoma de México, Cd de México, México.

Corresponding author: Ireri Suazo-Ortuño, e-mail: ireri.suazo@umich.mx; ireri.suazo@gmail.com

Abstract

A number of skin-associated bacteria and its community structure are known to inhibit the infection intensity of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) in amphibians. However, we still lack understanding of host microbial dynamics and its role against *Bd*. We sampled skin-associated bacteria and *Bd* from two co-occurring amphibian species from a tropical high-altitude site in central Mexico. Sixty-three percent of sampled salamandra individuals and 80% of frog individuals tested positive for *Bd*. We registered 622 skin-associated bacterial genera, from which 66 are known to inhibit *Bd*. These inhibitory taxa represented about 55% relation to total bacterial abundance. Our results indicate that, albeit sharing some of the most abundant bacterial taxa, bacterial community structure from the skin of the two hosts' species is different, in both taxonomic composition and relative abundance of taxa. Pseudomonas and *Streptrophomonas* were among the five most abundant bacterial taxa, and have been reported to inhibit *Bd*. Skin-associated bacterial richness as well as abundance of *Bd*-inhibitory bacteria were negatively related to *Bd* infection (*Bd* load) independent of species and seasons (dry vs. rainy). Overall, *Bd* load ranged from 1.75 to 853 zoospores. Despite the relatively high *Bd* prevalence in the two hosts' species, no dead or sick individuals were registered during field surveys. The relatively low levels of *Bd* load registered, apparently do not compromise survival of host species. Therefore, our results suggest that individuals of the populations studied are able to survive and thrive under a dynamic relation with enzootic infections of *Bd*.

Key-words

Batrachochytrium dendrobatidis, bacterial communities, hosts' health, microbial communities, skin microbioma, amphibians, host resistance

Introduction

All animals and plants harbor microbial communities that frequently have crucial roles in maintaining hosts' health (McKenzie et al. 2012). The skin associated bacterial communities of amphibians likely provide the first line of defense against pathogens. Lower disease risk in vertebrates has been associated with various features of the microbiome, including bacterial species richness (Longo, et al. 2015), specific microbial community assemblages (Becker, 2015), and the presence of microbes that produce metabolites that inhibit pathogens growth (Hol, et al. 2015). A specific pathogen of global importance is the fungus *Batrachochytrium dendrobatidis* (*Bd*), which has been a major factor in the global decline of amphibian populations and extinction of species (Fisher et al. 2012). Of 1,240 amphibian species sampled up to 2013, 516 (42%) were infected with *Bd* (Olson et al., 2013). Different amphibian species and populations within species frequently present variations in their vulnerability to *Bd* infection (Kilpatrick et al., 2010). Although there are multiple factors that might have a role in amphibian resistance to *Bd*, including host genetics, immunology (Carey et al., 1999), skin peptides (Rollins-Smith 2009) and environmental factors (Belden and Harris, 2007), bacterial communities living on amphibians' skin may also have a crucial role in the host resistance (Harris et al., 2006). Several bacteria found on amphibian skin are known to produce chemical compounds that are able to inhibit growth of *Bd* and to reduce amphibian mortality under laboratory conditions. *Janthinobacteriium lividum*, for example, was successfully used to mitigate *Bd* infection in *Rana muscosa* (Harris et al., 2009), and *Pedobacter cryocontis* reduced Bd load infection in the same frog species (Woodhams et al., 2012). Up to 2015, there were about 255 anti-*Bd* bacterial operational units (OTUs) identified from the skin of 37 species of amphibians that had been deposited in a reference database (Woodhams et al., 2015). Activity of protective skin bacteria may be influenced by environmental conditions such as temperature. Daskin et al. (2014) reported that anti-*Bd* bacterial skin symbionts reduced anti-*Bd* activity at cool temperatures in three species of the frog Litoria.

There is a paucity of information whether in the wild, different species of co-occurring amphibians, and therefore, likely coming from similar microbial background present resembling skin microbial communities. There is also limited information about whether specific microbial assemblages are involved in resistance to *Bd* in wild amphibian populations, as well as on the role of environmental conditions on the efficiency of anti-*Bd* bacterial skin symbionts. Characterizing these patterns is important to understand evolutionary and ecological processes structuring functionally microbial skin assemblages, which may help in the design of conservation strategies (Muletz-Wolz et al. 2017).

In the central region of the trans-Mexican volcanic belt several amphibian species have been reported to be infected with Bd, including co-occurring populations of the facultatively neotenic salamander Ambystoma rivulare and the semi-aquatic frog *Rana neovolcanica* (Nava-González et al., 2020). The goal of the present study was to evaluate if two populations of co-occurring species (the frog *R. neovolcanica* and the salamander *A. rivulare*) presented different skin bacterial communities and if taxonomic composition and structure of these communities influenced host's resistance to *Bd*. Additionally we evaluated the effect of seasonality on *Bd* resistance.

Considering that different amphibian species present different levels of susceptibility to *Bd* infection and that skin-associated bacteria have the potential to influence this susceptibility, we hypothesized that skin bacterial composition and structure of *R. neovolcanica* and *A. rivulare* were different and that species richness and abundance of inhibitor bacteria was negatively related to *Bd* load, an indicator of the severity of disease symptoms of the host. We also hypothesized that environmental conditions associated to seasons modulate the relationship between bacterial community and *Bd* infection.

Materials and methods

Field site

Our study was conducted at the core zone of the Monarch Butterfly Biosphere Reserve (MBBR), located in the trans-Mexican volcanic belt on the border of Michoacán state and state of Mexico. The reserve's core zones (13,551 ha) are located above 3,000 m and the main vegetation cover consists of fir forests. Climate is humid-cold with a mean annual temperature of 14ºC (range = - 1.5ºC in December to 19.9ºC in May) and a mean rainfall of 700 to 1250 mm3.

We sampled six independent streams (Table 1), each surrounded by fir forest and alpine grasslands in six different periods between 2016 and 2017 (three occasions in rainy and three in dry season). Streams were separated by a distance of at least 1 km. Altitude at the streams ranged from 3,000 - 3,200 m.

Sampling sites

Along each of the sampled streams a 100 x 100 m plot was positioned at random. Each transect was 50 m wide from stream banks in a straight line towards uplands. Four people visually searched each transect for one hour. Sampling was carried out at daylight (09:00-10:00) and at night (20:00-21:00). To determine presence of Bd and collect skin bacteria we took skin samples of *Ambystoma rivulare* and *Rana neovolcanica* according to the methods of Hyatt et al. (2007). With a sterile rayon-tipped swab, the ventral and inguinal regions of each collected individual was swabbed. Each swab was placed and stored in vials with alcohol (70%) at –20ºC. Before DNA extraction swabs were dried to evaporate residual ethanol. To prevent Bd dispersal, after sampling was completed on every plot sampling material was disinfected with alcohol (70%) and chlorine bleach (6%).

DNA extraction

We used swab samples to describe skin bacterial communities and determine the presence of Bd in wild co-occurring populations of the salamander *A. rivulare* and the frog *R. neovolcanica*. We standardized the number of swabs per individual and body region and used new gloves to avoid sample contamination (Hyatt et al., 2007). Individuals were not rinsed previous to swabbing; therefore, we assume that bacterial communities were consisted of resident and transient taxa. For DNA extraction of both, *Bd* and bacterial community we used Prep Man® Ultra (Applied Biosystems). Tubes were centrifuged for 3 min at 13 000 × g, incubated for 10 min at 100°C. The supernatant was then aspirated from swabs and placed in a sterile 1.5 ml tube. For *Bd* testing we used Real-Time PCR analysis (qPCR) on an Applied Biosystems StepOnePlusTM Real-Time PCR System. The *Bd* strain used for standards was GPL1 isolated from *Pseudoeurycea leprosa* from the National Park La Malinche, México and cultured at the Instituto de Biología UNAM. For PCR reactions and amplification parameters we used the methods of Boyle et al. (2004) and each sample was run in triplicate.

To measure *Bd* intensity of infection (*Bd* load) we calculated zoospore equivalents (ZE) by multiplying the raw genomic output by 80 as DNA extracts were diluted 80 times during extraction and the qPCR (Briggs et al., 2010; Vredenburg et al., 2010). A result was considered positive when the sample amplified (0.1 ZE) before cycle 39 for at least two times; mean of the two positive samples was used to calculate ZE (Kriger and Hero, 2007).

Skin bacterial community metagenomics

Bacterial 16S rRNA metagenomics profiling was performed amplifying V3 variable region of the 16S rRNA gene with the primer set V3-338f and V3-533r and sequenced in an Illumina Miniseq at Centro de Investigaciones en Alimentación y Desarrollo A.C. (CIAD) facilities in Mazatlán, México. Reads were assembled with Flash v.1.2.7 software (Magoc & Salzberg, 2011), and VSEARCH (Rognes et al., 2016) was used for further processing, obtaining an abundance matrix of bacterial OTUs0.03 (Operational Taxonomic Units clusterized at 97% identity) that was normalized using the metagenome Seq method (McMurdie & Holmes, 2014). Taxonomic annotation was performed using VSEARCH against RDP database v11.5 (Cole et al. 2014).

Data analysis

The *Bd* load and bacterial community were analyzed using two independent and complementary methods. For the first, we used univariate analysis and for the second multivariate analysis were conducted. The univariate analysis aimed to evaluate the role of the skin bacterial community in the restraining of *Bd* infection. We used the following parameters: Bacterial Shannon Index (BSI), expected richness (Chao 1) and observed species richness (OSR) that were calculated from the OTUs matrix with phyloseq package of R software (McMurdie & Holmes 2013). The percentage of inhibitory bacteria taxa (TIB) and the percentage of abundance of these taxa (AIB) registered in the two sampled species of amphibians were calculated. Each of the bacteria taxa registered was searched in a reference database of antifungal amphibian skin-associated bacteria (Woodhams et al., 2015). The number of sampled bacteria taxa listed in the database was used to calculate the percentage of inhibitory taxa of the total bacterial sample. The percentage of abundance of these inhibitory taxa was also calculated in relation to the total bacterial abundance of the samples. To explore the relationship between total bacterial community and *Bd* load in the two amphibian's species and the two different seasons we used Generalized Linear Models (GLMs) with an ANCOVA design. We developed one model for each of the parameters of the total bacterial community (observed taxa richness, Chao 1 and Shannon Index) as the continuous explanatory variables and the amphibian species (*A. rivulare* and *R. neovolcanica*) and the seasons (dry and rainy) were the categorical variables and *Bd* load was the response variable. We then developed separately a model for the percentage of inhibitory bacterial taxa (IBT) and the percentage of abundance of inhibitory bacteria (AIB) and *Bd* load in the two amphibian's species and the two different seasons using also GLMs with an ANCOVA design in which the response variable was *Bd* load and the parameters of inhibitory bacteria were the continuous explanatory variables and the amphibian species and the seasons were the categorical variables. We developed saturated models considering the main effect of the factors and the two and three interactions among the factors. When a term was not significant, the model was reduced to use only significant terms. For GLMs we used a Poisson error and a logarithmic link function. For each relationship we obtained a model of the form y = mx + b, where m is the slope and b the ordinate. When significant, differences between slopes were tested among species and season. The regression lines presented in the results were obtained from the GLMs. We conducted GLMs analysis with the R statistical software R 3.6.

The multivariate methods were used to compare the structure composition of bacterial community in different categories of Bd load (ZE= 0, ZE>0.01, ZE>100) among the two different amphibian species and seasons. For metagenomic data, vegan R library v2.4-6 (Dixon, 2003) was used to generate a non-parametric multidimensional scaling plot (NMDS) with OTUs0.03 matrix and a statistical analysis was performed with the ANOSIM function to evaluate groups using the Bray-Curtis distance and 999 permutations.

Results

Regarding *Bd* infection status, of 27 sampled individuals of *A. rivulare* and 25 of *R. neovolcanica*, 17 (63 %) and 20 (80 %), respectively, were *Bd* positive. Of these *Bd* positive individuals, 21 occurred in the rainy season (10 *A. rivulare*, 11 *R. neovolcanica*), and 16 in the dry season (7 *A. rivulare*, *9 R. neovolcanica*). Overall, we registered 5,837 skin-associated bacterial taxa, 3,361 from *A. rivulare* and 4,729 from *R. neovolcanica*. Of these bacterial OTUs, 29 phyla, 52 classes, 105 orders, 207 families and 622 genera were annotated (Fig. 1). Of the annotated bacterial OTUs, we registered 66 inhibitory *Bd* bacterial genera, representing on average about 55% of the total abundance of bacterial genera (54% for *A. rivulare* in the rainy season, 61% for this species in the dry season, 53% for *R. neovolcanica* in the rainy season, 57% for this species in the dry season). Of these identified inhibitory genera, Pseudomonas, Stenotrophomonas, *Microbacterium, Variovorax* and Massilia were the most abundant taxa in both, *A. rivulare* and *R. neovolcanica* (Table 2, Fig. 2). We observed that high bacterial species richness and Chao1 resulted in low *Bd* loads (e.g. ZE= 1.75), while at low species richness we found *Bd* loads as high as ZE=853. This negative relationship was significant and the GLMs indicated that this pattern was independent of amphibian species, season and the interactions between these factors (Table 3, Fig. 3a,b). Contrastingly, Shannon index did not explain any of the variations of *Bd* loads. In relation to analysis of *Bd* inhibitory bacteria, we also found a negative relationship in the case of abundance (percentage); at high levels of inhibitory bacterial abundance, low *Bd* loads were registered. This pattern was independent of amphibian species and season (Table 3, Fig. 3c). Percentage of inhibitory bacterial taxa did not show a significant relationship with *Bd* loads.

Microbial profiles separate by host species, and for *R. neovolcanica* there was a separation for rain and dry season (Fig. 4). Although ANOSIM values do not stress a significant difference for the 12 analyzed groups (3 *Bd* load categories, 2 host species, 2 seasons), there was a tendency for the bacterial community of *A. rivulare* to group in rainy season (Fig. 4). We observed a more dispersal community in the dry season for this species. For *R. neovolcanica*, a clear group of high *Bd* load in the dry season was observed (Fig. 4).

Discussion

Although the understanding of the dynamics between *Bd* infection and skin microbial ecology in amphibians is rapidly growing (e.g. Jani & Briggs, 2014, Longo et al., 2015), the number of field studies on the modulation of this relationship by *Bd* inhibitory bacteria, bacterial community structure and seasonal effects is still limited. In this study we address some aspects of these knowledge gaps by assessing the relation between structure of skin bacterial communities, number and abundance of *Bd* inhibitory bacteria, and seasonal influences on *Bd* infection load of two co-occurring amphibian species. Our results suggested that richness of the skin bacterial community diminishes the intensity of *Bd* infection in both the salamander and the frog species studied. We also found that abundance of *Bd* inhibitory bacteria affects the intensity of *Bd* infection.

The number of identified skin-associated bacterial OTUs for each of the studied species, 3,361 for *A. rivulare* and 4,729 for *R. neovolcanica* falls within the range reported in other amphibian species (e.g. 2,667 for *Craugastor fitzingeri* to 10,192 for *Alytes obsetricans*) (Rebollar, 2016; Bates et al., 2018; Bellden, 2015). We registered a significant negative relationship between infection load (number of *Bd* zoospores) and skin bacterial community richness independent of host species and seasons. Several studies have reported negative relationships between skin bacterial community richness and *Bd* load in amphibians (e.g. Longo et al., 2015; Piviota-Scott et al., 2017).

Considering the most abundant bacterial genera for each of the two host's species, only four that ranked among the 10 most abundant genera were shared. Additionally, NMDS plots showed little overlap in bacterial community structure, with different dispersion between the two host species. In *R. neovolcanica*, the seasonal (rain vs. dry) skin bacterial community structure was also evident in NMDS plots (Fig. 4). Several studies (e.g. Belden et al. 2015; Rebollar et al. 2016; Bates et al. 2018) have also reported differences in composition and structure of the skin-associated bacterial communities within assemblages of amphibian species. Differences in composition and structure of skin microbial communities among amphibians species may be associated to host traits, such as chemical composition of the skin mucus (Wells, 2007), host behavior, skin shedding rate and diet (Meyer et al., 2012). Although we cannot be certain, differences in phylogenetic position (host species are included in two distantly related orders, Caudata and Anura) might be, at least partially, causing differences in skin bacterial communities between *A. rivulare* and *R. neovolcanica*. Rebollar et al. (2016) suggest that amphibian species that are phylogenetically closely related might have similar skin chemical conditions, and therefore, similarities in bacterial communities. Additionally, although the studied populations of the two species are co-occurring, adult stages are associated to different habitats; *A. rivulare* primarily inhabits aquatic habitats and *R. neovolcanica* riparian habitats. Therefore, some traits associated to differences in bacterial community among host species, such as diet and behavior may contribute to the microbial differences registered in the two species. Finally, if the environmental bacterial source in both types of habitat is different, it would be reasonable to expect differences in the skin bacterial communities between the two host's species.

Albeit differences in skin bacterial communities, the most abundant bacterial genus for the two hosts' species was Pseudomonas. This bacterial genus is dominant in the skin-associated bacterial communities of several *Bd* non-susceptible amphibian's species (Rebollar et al., 2016). The second and third most dominant genus in the skin bacterial community of *R. neovolcanica* and *A. rivulare*, respectively, was *Streptrophomonas*. Both, *Pseudomonas* and *Stretrophomonas* have been reported to inhibit the intensity of *Bd* infection (Muletz-Wolz et al., 2017). At the level of phyla, microbial skin communities in both host species were dominated by the same three phyla (*Proteobacteria, Actinobacteria* and *Bacterioidetes*). The two dominant bacterial genus, Pseudomonas and *Streptrophomonas* belong to Proteobacteria, the phylum that dominates skin bacterial communities in amphibians (Kueneman et al., 2013) and that comprise about 80% of bacteria with known anti-*Bd* effects (Woodhams et al., 2015). Of the bacterial genus registered, 66 (55% of total bacterial abundance) are considered to be inhibitory of the intensity of *Bd* infection (Woodhams et al., 2015). Three and five of these genera were among the 10 most abundant bacteria in *A. rivulare* and *R. neovolcanica*, respectively. Additionally, *Pseudomonas*, an inhibitory bacterium was the most abundant genus in both species. Intensity of *Bd* infection measured as *Bd* load ranged for both species between 1.75 to 853 ZE (average ZE = 152 +-35). Longo et al. (2018) consider *Bd* infection to be low in *Eleutherodactylus coqui* when load is less than 100 *Bd* zoospores, and Jani and Brig (2014) consider a *Bd* load of 10,000 Bd cells to be the threshold at which *Rana sierrae* succumbs to chytridiomycosis. Therefore, we can assume, that in general *Bd* infection intensity in both of our studied species ranged from low to moderate.

We registered a tendency of higher percentage of abundance of *Bd* inhibitory bacteria in the dry season (*A. rivulare*: rainy season 54% vs. 61% dry season; *R. neovolcanica*: rainy season 53% vs. 57% dry season). In the dry season we also registered for both host species a smaller number of *Bd* positive individuals (*A. rivulare* 10 in rainy season vs. 7 in the dry season; *R. neovolcanica* 11 in rainy season vs. 9 in dry season). A lower *Bd* load was also registered during the dry season in our study site in the assemblage of amphibians, including the two host's species of our study (Nava-González et al., 2020). These findings suggest that environmental conditions such as low temperature (< 7ºC) and low humidity (< 50 mm of rain), as well as the higher abundance of inhibitory bacteria might be causal factors in the decrease of *Bd* load in the dry season. Additionally, we also registered a higher bacterial richness in the dry season for *A. rivulare*, but no for *R. neovolcanica*.

Longo et al. (2015) registered a higher skin bacterial richness and higher *Bd* load in *Lithobates yavapaeienis* during the winter. Therefore, our results suggest that there might be a synergic effect of temperature, bacterial richness and abundance of inhibitory bacteria that results in a lower intensity of *Bd* infection during the dry season. This pattern is more evident in the case of *A. rivulare*.

Although 63 % of sampled individuals of *A. rivulare* and 80 % of *R. neovolcanica* were *Bd* positive, no dead or apparently sick individuals were registered during the field surveys. The relatively high percentages of infected individuals of both host species and the low to moderate intensity of level of *Bd* load that apparently do not kill or sicken infected individuals suggests that both host species are able to survive and thrive under a dynamic relation with enzootic infections of *Bd*. Our results suggest that abundance of *Bd* inhibitory bacterial genera in the skin of both host species might be an important biological defense that limits infection intensity of *Bd* regardless of host species and seasons.

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Author contributions statement

ISO and BNG conceived the ideas and designed methodology; BNG collected the data; BNG, PBL, YML, LLT and LR analyzed the data; ISO led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Tables and Figures

Table 1. Geographic location of the sampled streams in the core zone of the Monarch Butterfly Biosphere Reserve (MBBR).

Streams	N	W
Arroyo Oyamel	19º 37' 57.38''	100º 19' 59.73''
Arroyo La Calera	19º 37' 57.38''	100º 19'35. 84''
Arroyo Jesús el Nazareno	19º 37' 37.55	100º 19' 9.86''
Arroyo del Llano el Establo	19º 40' 24.5''	100º15'59. 6''
Arroyo del Ejido Garatachía	19º 40' 20.0''	100º 14' 51''
Arroyo la Mesa	19º 34' 49.5''	100º 12' 44.1''

Table 2. Abundances (%) of the genus registered in skin samples of *A. rivulare* and *R. neovolcanica* in the Monarch Butterfly Biosphere Reserve during the rainy and dry season. Only the first twenty more abundant species, which represent 60-85 % are shown. Below the total number of genus registered are included. The *Bd* inhibitory effect is shown (In) for each genus based on Woodhams et al. (2015).

	Ambystoma				***Rana***		
Genus	**Rain**		**Dry**		**Rain**		**Dry**
***Pseudomonas* (In)**	33.75	***Pseudomonas* (In)**	30.4	***Pseudomonas* (In)**	30.35	***Pseudomonas* (In)**	37.88
Photobacterium	11.41	***Stenotrophomonas* (In)**	9.35	***Microbacterium* (In)**	7.79	***Stenotrophomona* (In)**	14.81
Moraxella	8.78	***Microbacterium* (In)**	6.19	***Stenotrophomonas* (In)**	7.63	***Variovorax* (In)**	4.80
Sulfurospirillum	6.80	*Limnohabitans*	4.57	*Devosia*	3.64	***Microbacterium* (In)**	2.79
Actinopolymorpha	6.67	*Oxalicibacterium*	3.92	*Ochrobactrum*	2.68	*Devosia*	2.01
Oceanicaulis	3.19	*Devosia*	3.08	***Variovorax* (In)**	1.86	***Massilia* (In)**	2.00
Tepidisphaera	2.96	*Ochrobactrum*	3.02	*Luteolibacter*	1.11	*Ochrobactrum*	1.22
Knoellia	2.70	***Variovorax* (In)**	2.23	***Massilia* (In)**	1.06	*Duganella*	1.00
Anaerotruncus	2.37	***Massilia* (In)**	1.46	*Propionibacterium*	0.63	*Limnohabitans*	0.93
Luteibacter	1.76	*Duganella*	0.67	*Duganella*	0.54	*Propionibacterium*	0.67
Nosocomiicoccus	1.14	*Roseomonas*	0.45	*Roseomonas*	0.47	*Roseomonas*	0.44
Methyloceanibacter	0.84	*Propionibacterium*	0.32	*Acinetobacter*	0.44	*Janthinobacterium*	0.21
Nitrolancea	0.50	*Rhizobium*	0.28	*Rhizobium*	0.35	*Arthrobacter*	0.19
Portibacter	0.36	*Salinibacterium*	0.26	*Arthrobacter*	0.32	*Undibacterium*	0.19
Oscillibacter	0.36	*Kaistia*	0.25	*Advenella*	0.30	*Simplicispira*	0.18
Enterococcus	0.36	*Curtobacterium*	0.24	*Pedobacter*	0.28	*Curtobacterium*	0.18
Novosphingobium	0.32	*Leifsonia*	0.24	*Curtobacterium*	0.26	*Oxalicibacterium*	0.16
Peptostreptococcus	0.29	*Arthrobacter*	0.22	*Leifsonia*	0.26	*Serpens*	0.15
Neorhizobium	0.29	*Sphingomonas*	0.20	*Sphingomonas*	0.26	*Luteolibacter*	0.14
Micrococcus	0.29	*Serpens*	0.19	*Salinibacterium*	0.25	*Rhizobium*	0.13
Total	**85.1**		**67.5**		**60.5**		**70.10**
Number of genus	**597**		**389**		**447**		**362**

Table 3. Relationship between total bacterial community and *Bd* load used Generalized Linear Models (GLMs) with an ANCOVA design.

Total bacterial Community	X^2	*P*
Observed species richness (Osr)	$4.8_{(1)}$	<0.05
Bacteria Shannon Index (BSI)	ns	-
Chao 1	$6.6_{(1)}$	< 0.01
Inhibitory Bacterial		
Abundance of Inhibitory Bacteria (AIB)	$9.45_{(1)}$	< 0.001
Species of Inhibitory Bacteria (SIB)	ns	-

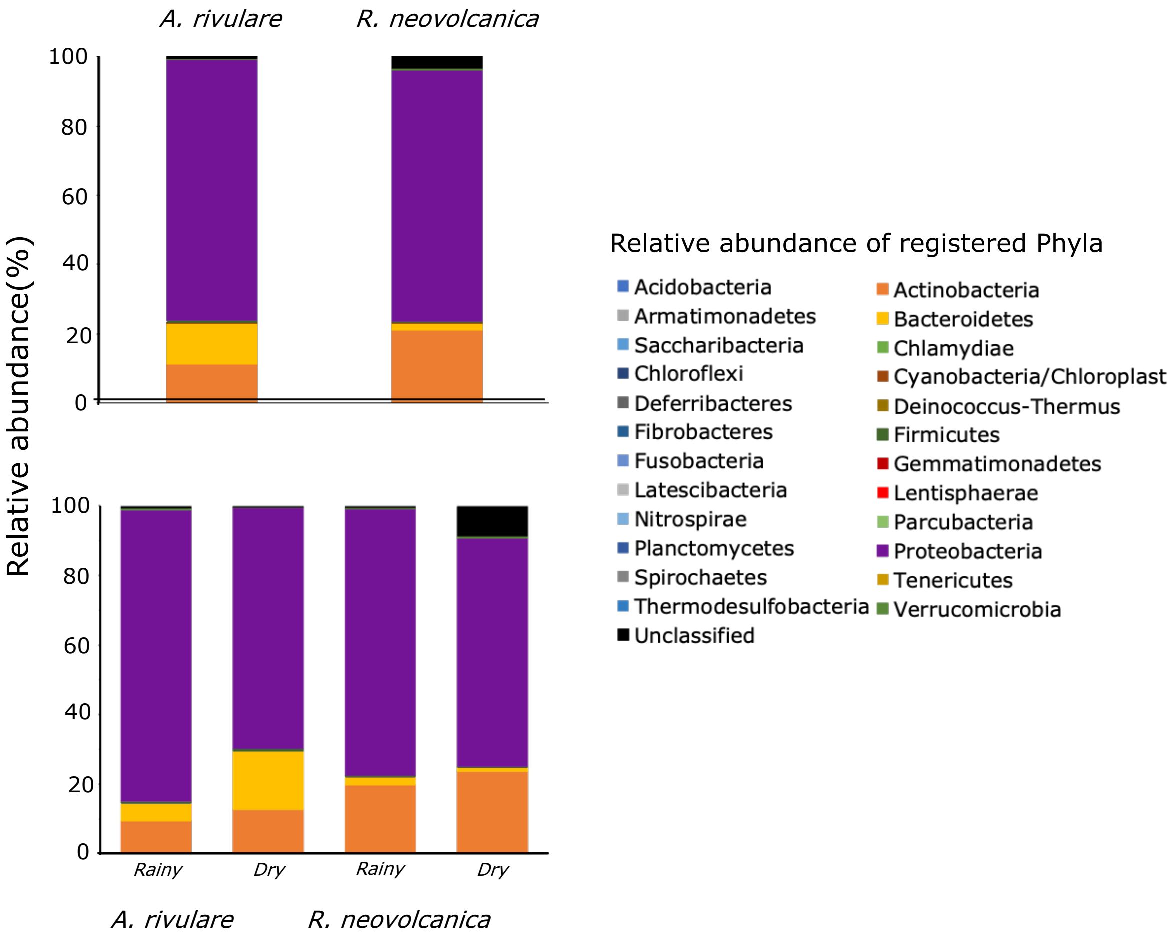

Figure 1. Relative abundance of Phyla registered in skin samples of *A. rivulare* and *R. neovolcanica* in the Monarch Butterfly Biosphere Reserve during the rainy and dry season.

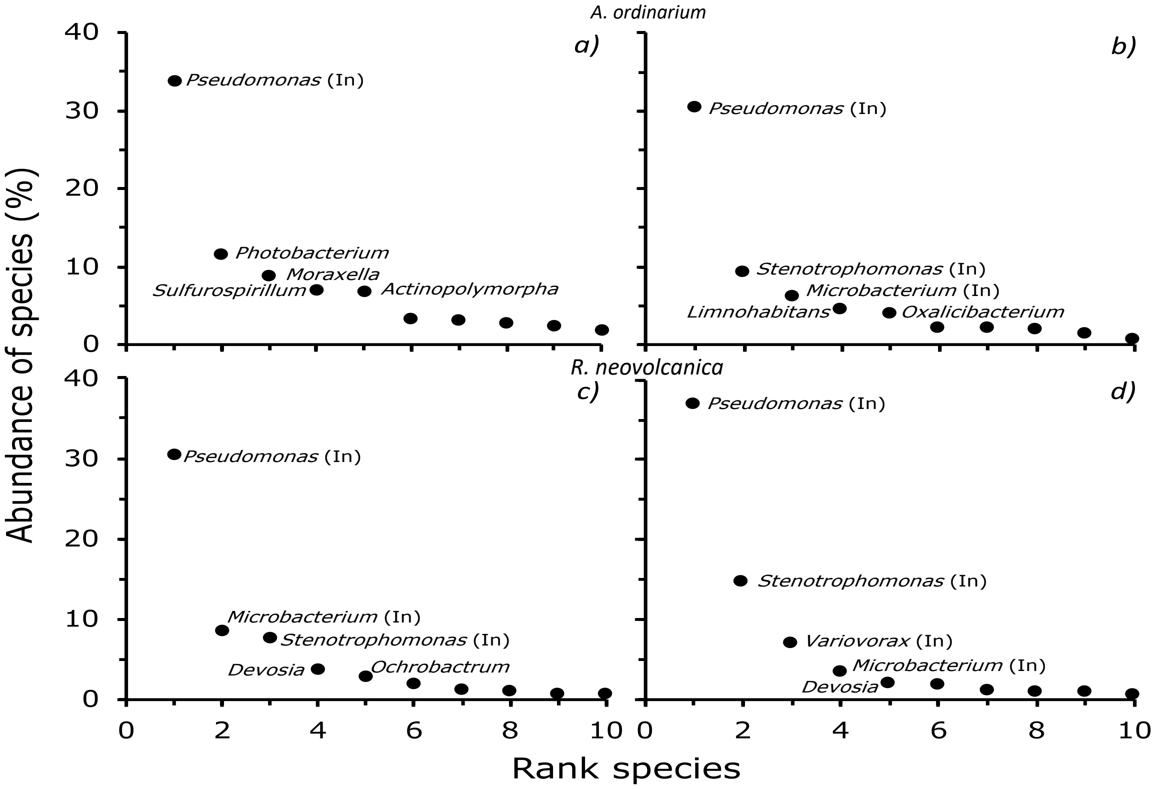

Figure 2. Rank-abundance curves for the community of genus of bacteria in two amphibian species in the Monarch Butterfly Biosphere Reserve, Mexico: *A. rivulare* in the upper and *R. neovolcanica* in the bottom charts during the rainy season (a and c) and the dry season (b and d).

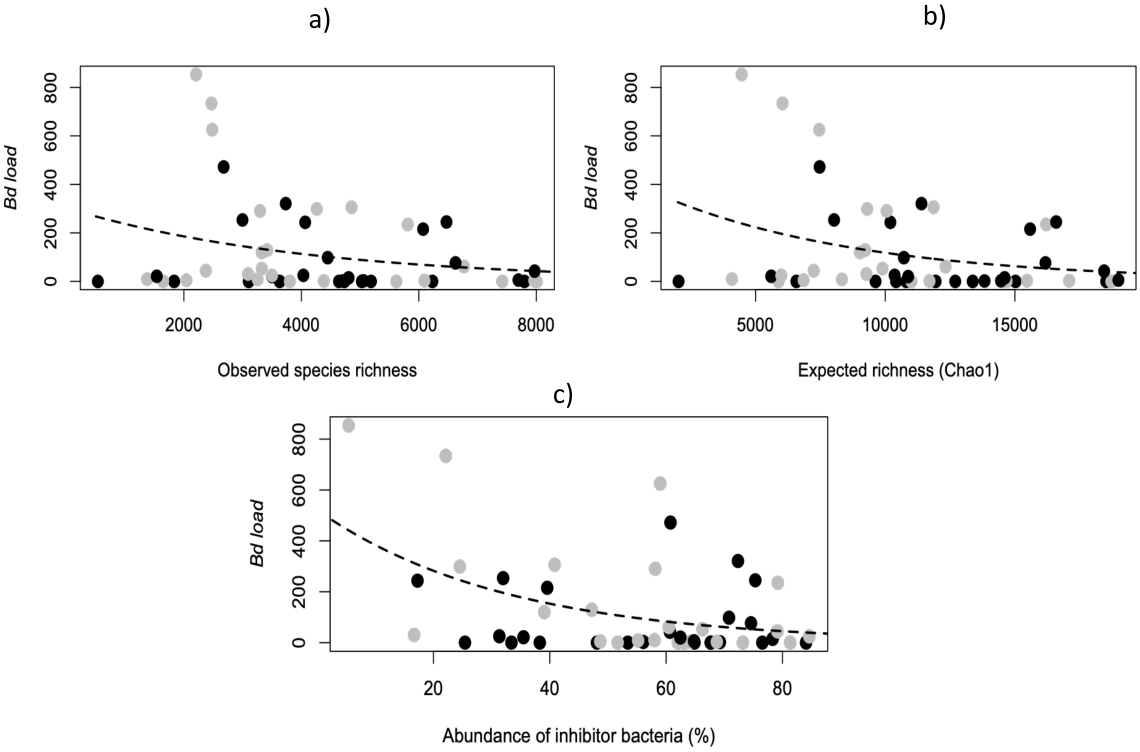

Figure 3. Relationship among *Bd* load and bacterial species richness, Chao1 and abundance (percentage) of *Bd* inhibitory bacteria accord the GLMs.

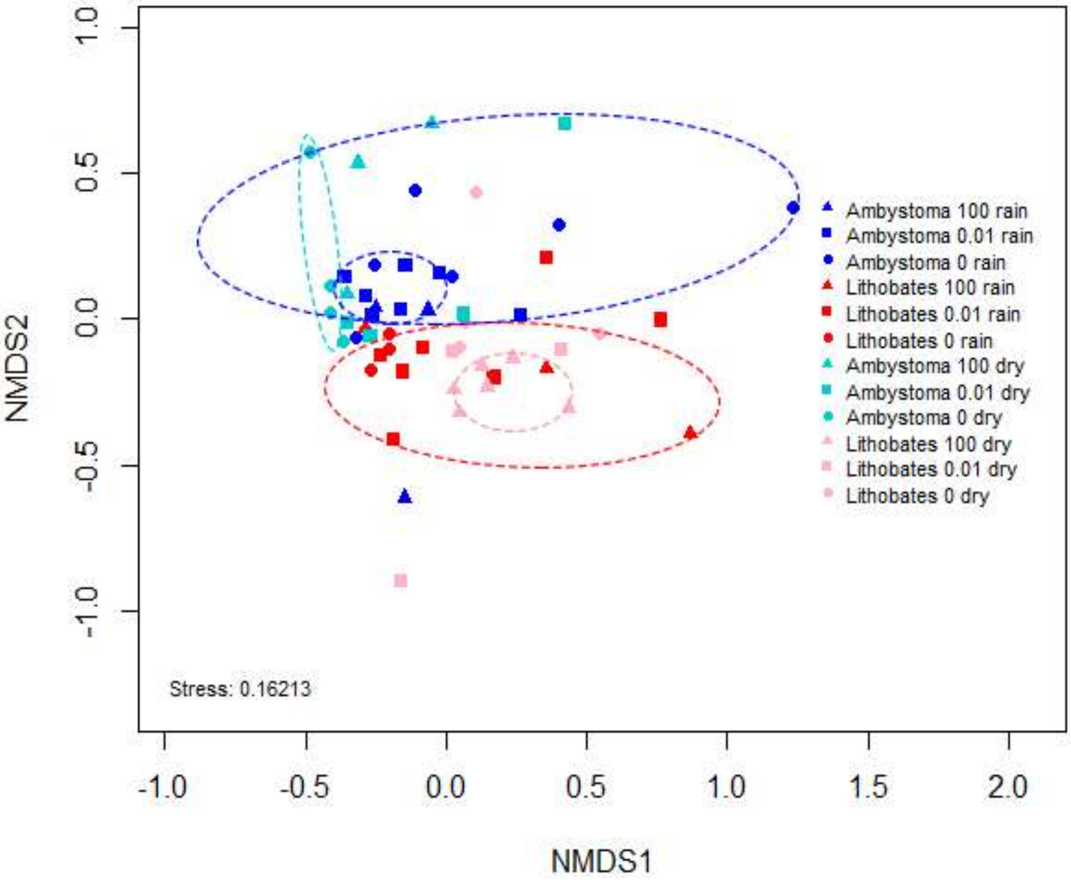

Figure 4. ANOSIM analysis of microbial profiles separate by host species and season for 12 analyzed groups (3 *Bd* load categories, 2 host species, 2 seasons). We observe in red colors *Rana neovolcanica* and in blue colors *Ambystoma* rivulare. In triangles high *Bd* load, in squares low *Bd* load, and in circles no *Bd*. Microbial community seem to structure depending on both species and season, and *Bd* seems to be correlated with the *R. neovolcanica* bacterial community.

DISCUSIÓN GENERAL

Las enfermedades infecciosas emergentes (EIDs por sus siglas en inglés) son uno de los factores de pérdida de biodiversidad más importantes de la actualidad (Daszak,1999). La Quitridiomicosis, causada por el hongo *Batrachochytrium dendrobatidis* (Bd) es una EIDs que afecta a los anfibios (Daszak et al. 2003; Scheele et al., 2019) considerándosele como un patógeno que de manera reciente (desde 1998) se ha convertido en un factor importante del declive de las poblaciones de anfibios a nivel mundial, adicional a la degradación y destrucción de los hábitats (Daszak et al., 1999). Tan solo en Latinoamérica, se ha demostrado que este hongo ha afectado las poblaciones de 43 especies de anfibios, principalmente para el caso de Costa Rica y Panamá (Lips et al., 2003). En el caso de México, se ha reportado la presencia de este hongo en los estados de Guerrero (Santos-Barrera, 2009), Chiapas, Oaxaca, (Parra-Olea et al., 1999; Rovito et al., 2009; Quintero-Díaz, 2015), Puebla (Santos-Barrera, 2004), Sonora (Hale, 2006; Santos-Barrera, 2004), México, Michoacán, Morelos, D.F. (Frías-Alvarez et al., 2008) y Veracruz (Murrieta-Galindo et al., 2014). A pesar de que los estudios sobre *Bd* en los anfibios se han incrementado sustancialmente, es necesario seguir aumentando el conocimiento sobre la distribución y afecciones de esta enfermedad, sobre todo por que muchas especies y poblaciones de anfibios han sido erradicadas por éste hongo (Scheele et al., 2019) y por los efectos sinérgicos que el *Bd* podría tener sobre los anfibios como consecuencia del cambio climático y el continúo deterioro y destrucción de los hábitas por las actividades humanas.

Aún y cuando muchas poblaciones han sido erradicadas por el *Bd* se ha documentado que existen sitios donde las especies persisten portando la enfermedad sin síntomas aparentes (Savage et al., 2011; Woodhams et al. 2007). El estudio de estas poblaciones ha permitido conocer el grado de susceptibilidad de las especies a *Bd*, así como detectar diferencias inter e intra específicas en la susceptibilidad, además se ha podido documentar que la variación entre los miembros de una misma especie pueden estar asociados a los estadios de desarrollo (Briggs et al., 2005) o a la estacionalidad, sugiriendo que tanto factores intrínsecos e extrínsecos pueden afectar la respuesta inmune de los anfibios, dejándolos suceptibles a la enfermedad durante ciertos períodos del año (Savage et al., 2011),

La microbiota bacteriana de la piel de los anfibios es considerada parte del sistema inmune y de los factores intrínsecos de los anfibios, protegiendo a los hospederos mediante la producción de metabolitos antifúngicos (Brucker et al., 2008; Harris et al., 2006; Lauer et al., 2007; Woodhams et al., 2007). Como otros sistemas inmunes, se ha hipotetizado que las diferencias en la composición de esta microbiota, es responsable de las diferentes respuestas que presentan los individuos a la enfermedad, aunque se conoce poco sobre las comunidades microbianas naturales que se encuentran en asociación en la piel de los anfibios sanos y sí estas presentan antagonismo a la colonización de *B. dendrobatidis*.

Tanto el peligro latente que presenta el *Bd* para las diferentes especies de anfibios a nivel mundial -en particular para la especies que se distribuyen en los rangos climáticos óptimos para el hongo, como para aquellas especies en las que se predice que sus hábitats sufrirán cambios en la temperatura como resultado del calentamiento global- como el potencial asociado que representa el conocer la interacción entre el *Bd* y la microbiota asociada a la piel de los anfibios abre una línea de investigación relevante para evaluar la relación entre la infección por *Bd* y la comunidad bacteria de la piel de los anfibios en condiciones naturales considerando diferencias entre especies coexistentes y la estacionalidad, en particular en sitios donde *Bd* se comporta como una enfermedad enzoótica.

En este sentido el estado de Michoacán, que gracias a sus condiciones geográficas y climáticas alberga una gran diversidad de especies de anfibios que se distribuyen desde las planicies costeras a nivel del mar hasta elevadas altitudes como la región de la Reserva de la Biósfera Mariposa Monarca (RBMM), que es además altamente vulnerable a los efectos del cambio climático (Alvarado-Díaz et al. 2013) fue un modelo ideal para probar la respuesta del ensamble de anfibios, en particular de grupos funcionales acuáticos y terrestres, a la infección por *Bd* y su relación con la comunidad bacteriana de la piel de los anfibios.

Los resultados del presente estudio muestran que de las 11 especies que fueron registradas arriba de los 3000 msnm en la RBMM, 73% (8 especies) estuvieron infectadas con *Bd*. Debido a que éstas tres especies (*Craugastor hobartsmithi, C. occidentalis* y *P. leprosa)* fueron registradas con solo individuo sería riesgoso reportarlas como especies libres de *Bd* ya que algunas de ellas ya han sido reportadas con *Bd* en algunas otras regiones del país (Mendoza-Almeralla et al., 2015). Por lo que es importante monitorear, la posible vulnerabilidad de estas especies a la infección por *Bd*. Esto es particularmente relevante para especies raras y en peligro como C. *hobartsmithi*, pero también es relevante para otras especies que fueron positivas para *Bd* (*Pseudoeurycea longicauda* e *Isthmura bellii*) y que también se encuentran en riesgo de acuerdo con la IUCN (2019). Durante el periodo de muestreo, no se registró ninguna especie con señales clíicas aparentes de la enfermedad, lo que sugiere que el ensamble de anfibios persiste como una comunidad enzoótica en relación con la infección de *Bd.* Entre otros factores, ésta respuesta puede ser resultado de sistema inmuune fortalecido producto de eventos epizóticos previos no registrados o por la presencia de comunidades bacterianas en la piel de los anfibios con características antifúngicas (Harris et al., 2006; Woodhams et al., 2007; Savage et al. 2011). Se registró una relación entre las estaciones (temperatura y lluvias) y la infección por *Bd* entre los dos grupos funcionales en todos los sitios estudiados. La proporción de individuos infectados (prevalencia) incrementó cuando la temperatura y la lluvia decrecieron. El patrón encontrado en éste estudio fue que existe una alta prevalencia (proporción de individuos infectados) asociada con temperaturas bajas (<7ºC) y precipitación (<50 mm). Estos resultados son diferentes con el patrón general reportado para otros estudios, en el cual la infección por *Bd* está positivamente asociado con condiciones de altos niveles de humedad y bajas temperatura con rangos que van de 10-

18 ºC (Longo et al., 2010; Adams et al. 2017; Longo and Zamudio 2017; Ruggeri et al. 2018). Al comparar la prevalencia entre los grupos funcionales, el grupo AqS (salamandras acuáticas) presentaron significativamente más individuos infectados que el grupo (SaqF) ranas semiacuáticas. Estos resultados son consistentes con lo reportado en otros estudios, en los que se observa un incremento en la exposición al patógeno en las especies acuáticas (Lips et al., 2003, b; Guayasamin et al. 2014). El grupo funcional AqS estuvo integrado por el ambistomátido *Ambytoma rivulare*, que es una salamandra neoténica facultativa, que habita los arroyos de montaña de la zona núcleo de la RBMM. Todos los individuos muestreados, durante el presente estudio (N=150) fueron colectados dentro de los arroyos en su fase larvaria y mostraron una alta y constante prevalencia de *Bd* durante todo el periodo de muestreo. En relación con la carga de *Bd*, en general se econtraron bajos niveles de carga durante los periodos calientes y lluviosos del año. Estos resultados contrastan con los altos niveles de prevalencia y carga reportados durante los periodos fríos y secos en otras regiones estudiadas (Longo et al., 2010; Voyles et al. 2017). Las diferencias con nuestro estudio, quizá se deban a las temperaturas ambientales que se registran en la región de la RBMM y a la tolerancia termal de la cepas de *Bd*. Piotrowski et al. (2004) indican que temperaturas debajo de los 10ºC limitan el crecimiento óptimo de *Bd* (pero le permite vivir a bajas desidades; Rachowicz y Briggs, 2007) y que el crecimiento óptimo ocurre a temperaturas entre los 17 y 25 ºC. A diferencia de éstos reportes, nosotros encontramos que la prevalencia decreció cuando incrementaron los temperaturas de 6.5 a 11 ºC. De acuerdo con Voyles et al. 2017, las cepas templadas de *Bd* pueden tolerar temperaturas tan bajas como los 2-3 ºC. El área de

estudio en la RBMM presenta altitudes más altas que 3000 msnm, por lo que, la temperatura media mensual a través del año es más baja que los 14 ºC, una temperatura marcadamente más baja que la reportada para el crecimiento óptimo del hongo (Piotrowski et al., 2004). Por lo que nuestros resultados sugieren que durante la época fría y seca del año más individuos son infectados pero con poca carga de *Bd* y durante los periodos calientes y lluviosos, la prevalencia es baja pero las condiciones ambientales (temperatura arriba de 10 ºC) son más favorables para el crecimiento de *Bd* y por lo tanto los individuos mantienen mayor carga. Es probable que durante los periodos secos y fríos, donde se reduce la disponibilidad de agua, los individuos del grupo funcional SaqF (ranas semiacuáticas) se congrequen en los lugares más húmedos, ocasionando que aunque tengan una baja carga de *Bd* por las restricciones de temperatura, más individuos se encuentren infectados por el incremento en el contacto físico entre ellos (Lips et al., 2003; Longo et al., 2010). En el caso de las salamandras acuáticas el agua de los arroyos puede alcanzar temperaturas extremadamente frías lo que puede ocasionar la baja carga de *Bd* observada en éste periodo, además de que la reducción en el caudal de los arroyos puede ocasionar mayor hacinamiento en los lugares más profundos. Los resultados de la primera parte de éste estudio sugieren que en la RBMM los efectos combinados de la variación estacional, la alta elevación, las diferencias en los tipos de hábitats utilizados por las especies estudiadas resultan en la persistencia de *Bd* a través del año, con alta prevalencia y bajas cargas alternando entre estaciones y sin manifestaciones clínicas de la enfermedad durante el año.

Debido a que los estudios en campo sobre la modulación entre la relación de las bacterias inhibitorias de la piel de los anfibios y la carga de *Bd* son aún muy escasos, en éste estudio se utilizamos dos especies de anfibios (*Ambystoma rivulare* y *Rana neovolcanica*) que habitan en la RBMM. Para ello, evaluamos la comunidad bacterian de su piel, el número y abundancia de especies inhibitorias de *Bd* que han sido reportadas previamente en la literatura y la influencia de la estación sobre la carga de *Bd.* Identificamos 3.361 OTUs de bacterias asociadas a la piel de *A. rivulare* y 4,729 para *R. Neovolcanica*. Éstos números son similares a los reportados para otras especies de anfibios (e.g. 2,667 for *Craugastor fitzingeri* to 10,192 for *Alytes obsetricans*) (Rebollar, 2016; Bates et al., 2018; Belden, 2015). Similar a lo reportado para otros anfibios (e.g. Belden et al. 2015; Rebollar et al. 2016; Bates et al. 2018) en nuestro estudio se encontró que de los 10 géneros más abundantes de bacterias solo cuatro géneros estuvieron compartidos entre las dos especies, por lo que en general la composición y estructura de la comunidad bacteriana asociada a la piel es diferente en ambas especies. Estas diferencias pueden estar asociadas a las características propias de cada especie como la composición se sus secreciones, comportamiento, características de la piel y dieta (Wells, 2007; Meyer et al., 2012), así como a diferencias filogenéticas (las especies estudiadas pertenecen a dos ordenes diferentes, Caudata y Anura). De acuerdo con Rebollar et al. (2016) las especies que son filogenéticamente relacionadas pueden tener condiciones químicas de la piel similares y por lo tanto comunidades bacterianas parecidas. Interesantemente, aún y cuando las dos especies presentaron comunidades bacterianas diferentes, en ambas especies, los géneros más abundantes de bacterias fueron las

Pseudomonas seguidos por *Streptrophomonas*, ambas perteneciente al filum Proteobacteria y aunque éste filum es el más dominante en las comunidades bacterianas de la piel de los ánfibios (Kueneman et al., 2013), los dos géneros han sido reportados como inhibidoras de la intensidad de *Bd* infection (Muletz-Wolz et al., 2017). El 55% de los géneros de bacterias registrados encontrados en la piel de nuestras especies estudiadas son considerados como inhibidores de la intensidad de infección por *Bd* (Woodhams et al., 2015). Considerando los 10 géneros más abundantes de la comunidad bacteriana, tres géneros éstos géneros de bacterias inhibidoras se encontraron en *A. rivulare* y 5 géneros de bacterias inhibidoras en *R. neovolcanica*. Se encontró una tendencia a presentar el mayor porcentaje de abundancia de bacterias inhibidoras en la estación de secas (*A. rivulare*: en la estación de lluvias presentó 54% contra 61% que presentó en la estación de secas y *R. neovolcanica*: en la estación de lluvias 53% contra 57% en la estación de secas). De acuerdo con Nava-González et al. 2020 (capítulo I) se encontró una menor carga de *Bd* tanto en el grupo funcionale AqS (salamandras acuáticas) como en el grupo funcional (SaqF) ranas semiacuáticas en la estación de secas, lo que sugiere que las condiciones ambientales tales como las bajas temperaturas (<7 ºC) y humedad (<50 mm) en ésta estación están relacionados con el decremento en la carga de *Bd* y que el alto porcentaje de bacterias inhibitorias en la estación de secas puede ser un factor importante que actúa en su disminución. Adicionalmente, también encontramos que la riqueza de la comunidad bacteriana de la piel aumentó de la estación de lluvias a la estación de secas para el caso de *A. rivulare* (lluvias 4373 vs 4919 secas) aunque en el caso de *R. neovolcanica* hubo una disminución (lluvias 4903 vs 3253 secas). De acuerdo con Longo et al. (2015) en la piel de *Lithobates yavapaeienis* la riqueza bacterial incrementó del verano al invierno, en la cual la infección por *Bd* es mas intensa. Por lo tanto, es posible que para nuestro caso de estudio un efecto sinérgico esté ocurriendo entre la temperatura, la riqueza de géneros de bacterias y la abundancia de bacterias inhibidoras de *Bd*, que puede estar constriñendo el crecimiento del hongo durante la estación de secas, aunque éste patrón se observa más claramente para *A. rivulare*, por lo que se abre una línea de investigación relevante.

Interesantemente, un alto porcentaje de individuos de *A. rivulare* y *R. neovolcanica* fueron positivos para *Bd* durante el presente estudio indicando que el *Bd* coexiste en una condición enzoótica sin señales aparentes de enfermedad o muerte. Nuestros resultados sugieren que la abundancia de bacterias inhibitorias de *Bd* en la piel de los anfibios puede ser sea una defensa biológica importante que limita la intensidead de la infección sin importar las especies hospederas y la estacionalidad en regiones de alta alitud. Por lo tanto, el moderado declive de los anfibios registrado en las elevaciones altas (> 3000 m) alrededor del planeta quizá sean el resultado de éstas interacciones. Éste balance en las interacciones hospedero-patógeno de patogeneicidad y condición enzoótica puede cambiar en regiones montañosas donde se predice un incremento de 2 a 3 ºC para el 2050 años debido al cambio climático como es el caso de la RBMM donde habita la comunidad de anfibios de nuestro estudio.

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ANEXO I

Bacterias a la defensa de los anfibios

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Bacterias a la defensa de los anfibios

Escrito por Bisbrian Alhelí Nava-González e Ireri Suazo-Ortuño

ARTÍCULO

Bacterias a la defensa de los anfibios

Bisbrian Alhelí Nava-González e Ireri Suazo-Ortuño

Tlaconete pinto, un pletodóntido severamente amenazado, en el que se ha reportado el patógeno ***Batrachochytrium dendrobatidis*** *conocido como Bd, en la Reserva de la Biósfera Mariposa Monarca (Fotografía: Nava-González B.A).*

Los anfibios son un grupo de animales vertebrados que existen en la tierra desde hace más de 350 millones de años. Los sapos o ranas, las salamandras y los cecílidos conforman el grupo de los anfibios y en su conjunto comprenden más de siete mil especies que viven en casi todo el planeta, con excepción de los casquetes polares y algunas islas oceánicas.

Los anfibios en riesgo de extinción

Si bien son organismos con una amplia distribución, la mayor riqueza de especies de anfibios se concentra hacia los trópicos, los que se encuentran fuertemente amenazados, como lo señala la Unión Internacional para la Conservación de la Naturaleza (IUCN por sus siglas en inglés), que coloca al 42% de las especies descritas dentro de alguna de las tres categorías de más alto riesgo: peligro crítico, en peligro o vulnerable. De acuerdo a criterios alternativos para asignar niveles de riesgo a la extinción, como el Índice Planeta Vivo (Living Planet Index) se estima que cerca del 80% de las poblaciones de anfibios a nivel mundial han disminuido su abundancia o se han extinguido.

Las causas del declive son diversas y complejas, asociadas a factores como la pérdida del ecosistema por degradación, contaminación y destrucción de hábitats, el aumento en la exposición a rayos UV, la extracción y reubicación de ejemplares para uso comercial o consumo humano, el cambio climático, la introducción de especies exóticas, así como también la aparición de nuevos patógenos.

Un patógeno culpable del riesgo de extinción

Un patógeno en particular es *Batrachochytrium dendrobatidis* conocido como *Bd,* un hongo microscópico responsable de la pérdida más grave de la biodiversidad entre los anfibios del planeta. Este hongo pertenece al orden Chytridiales (Familia: Incertae sedis), que como parte de su patogenicidad es capaz de secretar enzimas que degradan celulosa, quitina y queratina, causando la enfermedad denominada quitridiomicosis. Mayormente ataca en las células de la piel de los anfibios y las estructuras bucales de los renacuajos, y en estados avanzados de la enfermedad genera síntomas como decoloración, ulceraciones, falta de apetito, posturas anormales y falta de reacción de escape como signos evidentes en los organismos enfermos. En la figura 1 puedes ver el ciclo de vida de este hongo, capaz de desarrollarse en diferentes anfibios y provocarles la muerte.

En campo, la mayoría de los episodios más severos de mortalidad en masa se han observado en regiones relativamente frías a elevadas altitudes (por arriba de los 1000 metros sobre el nivel del mar). Además, se han identificado picos de infección que corresponden a los meses más fríos del año, así como una mayor supervivencia de los individuos infectados durante los periodos cálidos subsecuentes. La mayoría de los decesos se registran cuando la combinación de lluvias escasas y bajas temperaturas promueven la formación de unos pocos estanques someros donde los individuos se agrupan y resguardan. El hacinamiento promueve la transmisión del patógeno.

También se ha observado que existe una mayor persistencia y probabilidad de infección en anfibios que frecuentan cuerpos de agua permanentes con flujos moderados en comparación con aquellas especies que frecuentan cuerpos de agua con mayor movimiento o cuerpos de agua donde la temperatura es mayor.

A pesar de las numerosas poblaciones de anfibios erradicadas por el hongo, existen sitios donde las especies persisten portando la enfermedad. El estudio de estas poblaciones ha permitido la identificación de una extraordinaria variación de respuestas ante la exposición del hongo, y con ello la exploración de posibles soluciones para controlar la enfermedad.

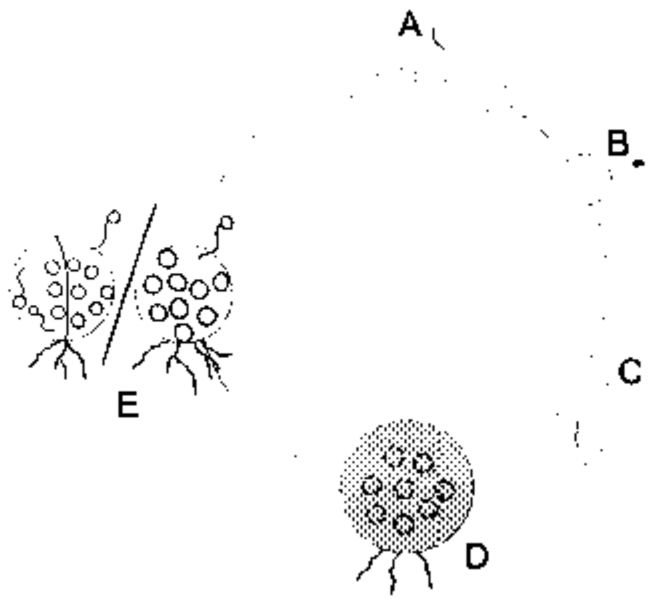

Figura 1. Ciclo de vida de Batrachochytrium dendrobatidis en cultivo, bajo estas condiciones sigue los estadios de vida A-E: A) Zoospora móvil flagelada; B) zoospora enquistada; C) quiste de zoospora con rizoides; D) esporangio inmaduro; E) zoosporangio maduro monocéntrico con un tubo de descarga (derecha) y dos tubos de descarga (izquierda) en un talo colonial con varios esporangios (Modificado de Berget et al., 2005).

Control de la enfermedad

En condiciones de laboratorio se han probado tratamientos antifúngicos, entre los que se cuentan los siguientes: exposición prolongada a temperaturas mayores a los 28oC, baños con formalín/malaquita y medicamentos fungicidas de uso veterinario, particularmente el uso de Itraconazol al 0.01% que ha probado ser uno de los más eficaces. No obstante, el uso de estos tratamientos se restringe al trabajo en laboratorio y aunque existen estrategias de control biológico que han probado ser eficientes en el campo, como es el caso de algunos crustáceos como las pulgas de agua dulce (*Daphnia magna*) que son capaces de consumir las zoosporas del hongo *Bd*, son pocos y muy recientes los estudios en este tema, y los riesgos potenciales a los ecosistemas siguen siendo altos. Más alarmante aún, los organismos expuestos al hongo en condiciones de laboratorio tienden a infectarse nuevamente en el campo, lo que indica que no existe memoria inmunológica o desarrollo de resistencia.

Toma de muestra de la comunidad bacteriana y del hongo Bd en la piel de la rana **Lithobates spectabilis** (Fotografía: Nava-González B.A).

Bacterias guerreras

Pero no todo está perdido para los anfibios en su lucha contra el *Bd*. Estudios recientes muestran que un tipo de glándulas llamadas granulares que poseen los anfibios secretan sustancias que en algunos casos presentan propiedades bioactivas específicas contra el hongo; y considerando que se secretan en la piel, éstas son considerados la primera línea de defensa del organismo contra la infección del hongo. Además de estas secreciones, en la piel de los anfibios habita una comunidad muy extensa de bacterias conocido como microbioma y representa la única línea de defensa que no es directamente producida por los anfibios.

Se sabe que diferentes especies, tanto animales como vegetales, viven asociadas con microorganismos en lo que se conoce como relación simbiótica. En muchas relaciones simbióticas, los participantes se necesitan mutuamente, ya sea para mantenerse con vida o para satisfacer determinadas necesidades. Específicamente, el papel benéfico de las bacterias para sus hospederos ha sido el foco de atención en estudios tanto de humanos como de animales silvestres, ya que pueden jugar un papel importante al proveerlos de protección contra patógenos.

Recientemente, se ha encontrado que varias especies de bacterias en la piel de los anfibios juegan un papel relevante en la defensa contra patógenos al producir sustancias de tipo antibiótico. De esta manera, las bacterias pueden actuar como guardianes de los anfibios, protegiéndolos contra la infección del *Bd* mediante la producción de metabolitos antifúngicos.

Sin embargo, cada especie de anfibio tiene diferente sensibilidad a ser infectada por el hongo. Esto ha sido previsto empíricamente, debido a que a nivel mundial no todas las especies contraen la enfermedad y las que lo hacen no se infectan con la misma intensidad. Por lo tanto, los investigadores se han cuestionado sí las características fisiológicas y ecológicas de cada especie juegan un papel importante para inhibir al patógeno. Así fue como se iniciaron varias investigaciones que condujeron a relacionar la presencia simbiótica de la comunidad bacteriana con la incidencia del hongo *Bd.*

La evidencia indicó que la diversidad y estructura del microbioma de la piel de los anfibios está influenciada por varios factores tales como la identidad de las especies, el estadio de desarrollo (larvas o adultos), la temperatura del aire y del agua, la estacionalidad, la ubicación geográfica y por la propia comunidad microbiana presente en el medioambiente.

Por lo tanto, se piensa que la comunidad de bacterias de la piel de los anfibios es diferente entre las estaciones del año y en consecuencia la protección contra el hongo *Bd* varía a lo largo del año. Un ejemplo de esto se encontró en la ranita *Eleutherodactylus coqui*, en la que al parecer los cambios estacionales en la comunidad bacteriana limitan la infección en la estación húmeda y caliente del año. Otro ejemplo, es el caso de la ranita australiana *Philoria loveridgei*, en la que se observó que al disminuir la diversidad de bacterias en la piel incrementó la infección por el hongo.

Probióticos una terapia alternativa

La mayoría de nosotros hemos escuchado hablar del beneficio de los probióticos para la salud humana. Desde este punto de vista, los probióticos son un grupo de bacterias que nos ayudan a preservar un entorno saludable, en particular a nivel de nuestro tracto digestivo. Por lo que el uso de probióticos es cada vez más frecuente en suplementos alimenticios.

En el caso de los anfibios, se ha observado que, en algunas especies, al eliminar las bacterias de la piel, los individuos presentan mayor mortalidad ante la exposición al *Bd* que los individuos en los que las bacterias no son manipuladas.

Plectrohyla bisctincta, *especie de anfibio amenazado por Bd en México (Fotografía: Nava-González B.A.)*

Es interesante que en experimentos usando probióticos en anfibios inoculados con bacterias que producen metabolitos anti *Bd* se reduce el número de individuos infectados, así como su mortalidad. Así por ejemplo, en experimentos de campo que involucraron la inoculación de probióticos de bacterias anti *Bd* como *Janthinobacterium lividum* en la rana *Rana muscosa* en la Sierra Nevada de E.U., mostraron que las ranas tratadas con baños de probióticos tuvieron picos de infección más bajos que las ranas control; mientras que las ranas control no se recuperaron de la infección, un año después, el 39% de los individuos tratados con probióticos se recuperaron, lo que sugiere que los tratamientos con probióticos permiten a los individuos sobrevivir al evitar que el *Bd* alcance un umbral letal.

Aunque aún es necesario llevar a cabo más investigaciones para entender el papel de las bacterias y el uso de probióticos adecuados para el control del *Bd,* los resultados de estudios previos indican que las bacterias tienen un enorme potencial para ayudar a las poblaciones vulnerables de anfibios en su lucha contra la infección del *Bd.*

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