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Le microbiome de la peau des amphibiens dans la pyramide des maladies

Adriana Paula Bernardo Cravo

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Adriana Paula Bernardo Cravo. Le microbiome de la peau des amphibiens dans la pyramide des maladies. Ecologie, Environnement. Université Paul Sabatier - Toulouse III, 2021. Français. NNT : 2021TOU30064 . tel-03552375

HAL Id: tel-03552375

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THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par :
Adriana Paula Bernardo Cravo

le 20.07.2021

Titre :

Le Microbiome de la Peau des Amphibiens dans la Pyramide des Maladies
(The Amphibian Skin Microbiome in the Disease Pyramid)

École doctorale et discipline ou spécialité :
ED SDU2E : Écologie fonctionnelle

Unité de recherche :
Laboratoire écologie fonctionnelle et environnement

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Le Microbiome de la Peau des Amphibiens dans la Pyramide des Maladies

The Amphibian Skin Microbiome in the Disease Pyramid

En vue de l'obtention du Doctorat de l'Université Fédérale Toulouse Midi-Pyrénées

Délivré par: l'Université Toulouse III Paul Sabatier (UT3 Paul Sabatier)

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Acknowledgment

If I had to thank all the people who have helped me, in some way, since I left Brazil, I would need to write a book. Not only since I left, but also while I was still there. My family, my friends, my colleagues, my bosses, or anyone who has helped me to get where I am today deserves and will be remembered.

This thesis closes one of the most important cycles of my life: first time living abroad, first time seeing snow, first time going to the mountain, first time sleeping in a tent, first time sledding, first time making friends from different cultures and first time working abroad with excellent references that made every day of this work worthwhile.

Mostly, I am grateful for the privilege of having this opportunity. At the beginning of this year, 12.8% of the Brazilian population was living below the extreme poverty line, and having the chance to do a PhD abroad, in the middle of the pandemic, with government resources of a country like Brazil is an incomparable privilege. Therefore, I will use this space to thank, specifically, the people who made this opportunity possible and who helped me get here.

First, for obvious reasons, I thank my entire family for investing in my education since the beginning of my life, as well as supporting me in every decision I made. Again, I am extremely lucky to have a life in which I always had everything I needed (and more), I cannot be grateful enough for that. I also can't thank Dirk enough, who agreed to have an interview with me, back in 2017, after receiving an email from a total stranger who had found his name on Google. If it wasn't for him, I certainly wouldn't be writing this thesis. From then on, the list of 'thanks' only increases: Adeline, also my supervisor who was present at all moments of the work and who always brought excellent discussions (besides helping me with French and French bureaucracy - extremely valuable help!); Antonis, who welcomed me in his group in Leipzig and who helped me to better understand the world of microorganisms, during all our long meetings (besides making very good Greek food). And finally (which is not 'finally'), Judit and Oliver, for the company in the mountains, in Cazavet and during our discussions about work and life!

All the people mentioned here deserve a much more extensive thank you! As well as those I haven't mentioned, but who were equally important. I hope that I have been able - or that I will be able - to thank everyone that brought me here.

Abstract

This PhD work aimed to explore factors impacting the relationship between the environment, the host, the host microbiome and the pathogens, starting with the transition of the disease triangle to the disease pyramid and exploring the relationships of all these elements, based on field work data.

We analyzed skin microbiome composition of tadpoles of three amphibian species (*Alytes obstetricans*, *Rana temporaria* and *Bufo bufo*) in a mountain range (the Pyrenees) and how the species *A. obstetricans* was additionally linked to the presence and disease status of the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd), responsible for the amphibian disease chytridiomycosis. The skin microbiome is an essential component of amphibians' health, considering its role in the amphibian immunity. I considered a combination of different factors, including: climatic variables (length of the growing season, number of consecutive dry days and simple precipitation index); lake characteristics (geographic coordinates, lake surface and altitude); anthropogenic proxies (tourism pressure, livestock presence and fish introduction); as well as several Bd variables (Bd-positive and negative populations, enzootic and epizootic populations, infected and uninfected individuals).

I found that the presence and relative abundance of abundant bacterial taxa in the tadpole skin responded to both growing season length and simple precipitation index. Considering that Bd infection is more usually acquired at the tadpole stage, such impact could interfere in host susceptibility to Bd or even other pathogens. Tourism pressure and livestock did not impact bacterial taxa abundance. However, some bacterial taxa differed significantly in abundance between lakes with and without fish; these taxa were all more abundant in lakes with fish, indicating a dominance of a few taxa and potentially a less balanced microbiome. Lastly, amphibian species was also significant for shaping the bacterial community.

To evaluate the interaction between the pathogen Bd and the skin microbiome of *A. obstetricans*, a species known to be susceptible to chytridiomycosis, I analyzed both bacterial and micro eukaryotic skin communities. I compared: uninfected vs. infected populations, enzootic (persistent) vs. epizootic (declining) populations, as well as (in infected populations) infected vs. uninfected individuals, for the whole microbiome and

detected specific ASVs that were significantly different in abundance and frequency in the various communities. The bacterial community showed different alpha diversity patterns over time in enzootic, epizootic and uninfected lakes. Such variation suggests a selection process acting on the diversity of the skin bacterial community of tadpoles from epizootic populations, which may allow some individuals to survive and, potentially, form an endemic population.

With my work, I could cover the different and complex interrelationships between host, host microbiome, environment and pathogen, and how alterations in one of these factors (or in a combination of them) can alter disease outcome and therefore, host health. The results found here are of great use for defining future conservation strategies against chytridiomycosis.

Résumé

Ce travail de thèse vise à explorer les facteurs ayant un impact sur la relation entre l'environnement, l'hôte, le microbiome de l'hôte et les pathogènes, en commençant par la transition du triangle des maladies à la pyramide des maladies et en explorant les relations de tous ces éléments, sur la base de données issues de travaux de terrain.

Nous avons analysé la composition du microbiome de la peau des têtards de trois espèces d'amphibiens (*Alytes obstetricans*, *Rana temporaria* et *Bufo bufo*) dans une chaîne de montagnes (les Pyrénées) et comment le microbiome d' *A. obstetricans* était liée à la présence du champignon pathogène *Batrachochytrium dendrobatidis* (Bd), responsable de la chytridiomycose, une maladie des amphibiens. Le microbiome de la peau est une composante essentielle de la santé des amphibiens, compte tenu de son rôle dans l'immunité des amphibiens. J'ai considéré une combinaison de différents facteurs, y compris : les variables climatiques (durée de la saison de croissance, nombre de jours secs consécutifs et indice simple de précipitation), les caractéristiques du lac (coordonnées géographiques, surface du lac et altitude), les proxies anthropogéniques (pression touristique, présence de bétail et introduction de poissons), ainsi que plusieurs variables liées à Bd (population Bd-positive ou négative, population enzootique ou épizootique, individus infectés ou non infectés).

J'ai constaté que la présence et l'abondance relative de taxons bactériens abondants dans la peau des têtards répondaient à la fois à la durée de la saison de croissance et à l'indice de précipitation simple. Considérant que l'infection par Bd est plus généralement acquise au stade de têtard, un tel impact pourrait interférer dans la sensibilité de l'hôte à Bd ou même à d'autres pathogènes. La pression touristique et le bétail n'ont pas eu d'impact sur l'abondance des taxons bactériens. Cependant, certains taxons bactériens différaient significativement en abondance entre les lacs avec et sans poissons ; ces taxons étaient tous plus abondants dans les lacs avec poissons, indiquant une dominance de quelques taxons et potentiellement un microbiome moins équilibré. Enfin, les espèces d'amphibiens ont également joué un rôle important dans le façonnement de la communauté bactérienne.

Pour évaluer l'interaction entre l'agent pathogène Bd et le microbiome cutané d'*A. obstetricans*, une espèce connue pour être sensible à la chytridiomycose, j'ai analysé les

communautés cutanées bactériennes et micro eucaryotes. J'ai comparé : les populations non infectées vs. infectées, les populations enzootiques (persistantes) vs. épizootiques (en déclin), ainsi que (dans les populations infectées) les individus infectés vs. non infectés, pour l'ensemble du microbiome et j'ai détecté des ASV spécifiques dont l'abondance et la fréquence étaient significativement différentes dans les diverses communautés. La communauté bactérienne a montré différents patterns de diversité alpha au fil du temps dans les lacs enzootiques, épizootiques et non infectés. Une telle variation suggère un processus de sélection agissant sur la diversité de la communauté bactérienne cutanée des têtards issus de populations épizootiques, ce qui peut permettre à certains individus de survivre et, potentiellement, de former une population endémique.

Avec mon travail, j'ai couvert les différentes interrelations complexes entre l'hôte, le microbiome de l'hôte, l'environnement et le pathogène, et comment les altérations d'un de ces facteurs (ou d'une combinaison d'entre eux) peuvent modifier l'issue de la maladie et donc, la santé de l'hôte. Les résultats trouvés ici sont d'une grande utilité pour définir les futures stratégies de conservation contre la chytridiomycose.

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Glossary

Acclimation response: is a reversible change of a physiological trait in response to an environmental change.

Emerging infectious disease: a disease that either has appeared and affected a population for the first time, or has existed previously but is rapidly spreading, either in terms of the number of individuals getting infected, or to new geographical areas. Emerging infectious diseases are caused by pathogens that are increasing in their incidence, geographic or host range, and virulence.

Holobiont: a symbiotic system composed of the host and its microbial partners (bacteria, archaea, viruses and eukaryotes).

Host microbiome: microbial communities living on (e.g. skin microbiome) or in (e.g. gut microbiome) the host. Skin and gut microbiomes are currently better described, but other host microbial communities exist, e.g. in the mouth, nose, pharynx, and respiratory and urogenital tracts.

Microbiome: communities of microorganisms including bacteria, yeast, fungi, protists and archaea in combination with their genomes.

Microbiota: collection of microorganisms that exist in a given environment, habitat, or host (inside or on host).

Microorganisms: microscopic organisms that exist as single cells or cell clusters. They include bacteria, protozoa, archaea, microscopic fungi, and microscopic algae. We also include viruses, which are microscopic but not cellular, although there is ongoing scientific discussion regarding their characterization as “micro-organisms”.

OTU: “Operational taxonomic unit”, used to refer to a cluster of DNA sequences of microorganisms, which are grouped by sequence similarity of a defined taxonomic marker gene present in their DNA.

ASV: “*Amplicon sequence variant*”. ASV methods infer the biological sequences in the sample prior to the introduction of amplification and sequencing errors, and distinguish sequence variants differing by as little as one nucleotide.

Pathobiome: initially, the pathobiome concept was defined as the pathogenic agent integrated within its microbial community (i.e. the pathogen interacting with the environmental microbiome [13]). This concept has now evolved to host-associated microorganisms (prokaryotes, eukaryotes and viruses) negatively impacting the health of the host, with the interaction between the host and its pathobiome inevitably moderated by the environment within the host and immediately surrounding it [14].

Phenotypic plasticity: the extent to which an organism can change its physiology, behavior, morphology and/or development in response to environmental cues.

Re-emerging infectious disease: a known infectious disease increasing in incidence after a period of reduced incidence, or even disappearance, of the disease.

Enzootic and epizootic *Bd* dynamics: *enzootic* populations are capable of living in the same environment as *Bd* without suffering a severe decline, while *epizootic* populations experience significant decrease in numbers after the arrival of *Bd* in their habitat.

Introduction générale

Menaces actuelles sur la faune sauvage et maladies infectieuses émergentes

La perte de biodiversité s'est considérablement accrue au cours des deux dernières années et cette tendance devrait se poursuivre, principalement en raison du changement d'usage des terres, du changement climatique, des dépôts d'azote, des échanges biotiques (lorsqu'une espèce se déplace ou est déplacée d'un biota à un autre, comme le déplacement des poissons pour l'empoisonnement) (Sala, 2000) et des maladies infectieuses émergentes (Scheele et al., 2019). Les conséquences pour les espèces sauvages dépendent toutefois de l'interaction de ces variables et des facteurs de stress locaux supplémentaires dans chaque biome (Sala, 2000).

Parmi les menaces qui pèsent sur la faune sauvage, le changement climatique a été identifié comme l'une des plus importantes pour la faune sauvage et les sociétés humaines. Non seulement il affecte directement les espèces animales et végétales, mais il renforce également d'autres facteurs de stress environnementaux, tels que le changement d'usage des terres (Opdam et Wascher, 2004) et les espèces envahissantes (Hellmann et al., 2008). Le changement climatique et l'altération des habitats sont responsables de la menace de l'extinction d'un quart des espèces actuellement connues (IPBES, 2019), avec des conséquences ultimes pour la vie humaine en raison de la fourniture (ou de l'absence de fourniture) de services écosystémiques (Gowdy, 2020), et de l'altération de la fréquence des zoonoses émergentes, par exemple (Daszak et al., 1999). Les maladies transmises par les moustiques, comme la dengue et la fièvre jaune, qui ont besoin de plans d'eau pour accomplir leur cycle, ont tendance à augmenter lorsque les précipitations extrêmes sont plus fréquentes (Slenning, 2010 ; Raffel et al., 2013). Les maladies des animaux domestiques, telles que la fièvre catarrhale ovine et la fièvre de la vallée du Rift africaine, deux maladies virales qui affectent le bétail (Slenning, 2010 ; Jones et al., 2019), devraient voir leur fréquence augmenter en raison du réchauffement des habitats des vecteurs et des changements météorologiques dans l'océan Indien, respectivement, entraînant d'importantes pertes économiques. Les maladies peuvent provenir de différentes sources (Daszak et al., 2000) : (a) l'émergence

du pathogène et le transfert à partir d'animaux domestiques, comme le virus de la maladie de Carré qui a provoqué l'extinction des populations de chiens sauvages d'Afrique ; (b) être liées à une intervention humaine (translocation d'hôtes et/ou de parasites), comme le virus Ebola, en raison du contact possible entre les humains infectés et les primates ; ou (c) ne pas être associées avec des humains ou des animaux domestiques, comme la chorioretinite virale, connue pour provoquer cécité et mortalité chez les kangourous (Daszak et al., 2000).

Conservation des amphibiens et menaces

Les populations d'amphibiens sont menacées depuis des décennies, 43 % des espèces connues montrent un déclin continu, un chiffre presque deux fois supérieur à celui des espèces de mammifères (23 %) (Stuart et al., 2004). Cela fait des amphibiens le groupe de vertébrés actuellement le plus menacé, notamment en raison de leur physiologie ectothermique et de la propagation d'une maladie fongique pathogène (Fisher et al., 2012 ; Scheele et al., 2019). Des déclins sévères suivis d'extinctions locales ont été détectés dans les années 1990 en Australie et aux Amériques (Berger et al., 1998), puis sur tous les continents. Les causes de ces déclins mondiaux sont diverses, notamment : (1) la présence de produits toxiques dans l'environnement, qui peuvent provoquer une absorption incomplète des ressources de l'œuf et une déformation des larves (Horne et Dunson, 1994), (2) la prédation, qui est intensifiée par l'introduction de poissons exotiques aux stratégies prédatrices inconnues pour les amphibiens indigènes (Vredenburg, 2004), (3) la dégradation de l'habitat, comme l'assèchement des zones humides et la suppression consécutive des sites de reproduction avec division de la population (Becker et al, 2007), et les changements du climat local (Wake et Vredenburg, 2008), (4) le rayonnement ultraviolet, qui peut entraîner un ralentissement de l'éclosion des œufs (Blaustein et al., 2003) et des anomalies de développement et de physiologie chez les embryons (Hatch et Blaustein, 2003), (5) le changement climatique, qui peut renforcer l'impact d'autres menaces, telles que la propagation d'espèces invasives (Blaustein et al, 2003 ; Beebee et Griffiths, 2005), qui concurrencent également les amphibiens dans leur habitat naturel, et (6) les maladies infectieuses émergentes, comme la chytridiomycose et la ranavirose.

Comme expliqué ci-dessus, le changement climatique devrait fortement influencer l'émergence et la propagation des agents pathogènes, et augmenter la fréquence des épidémies, en particulier pour les agents pathogènes ayant des cycles de vie complexes avec des animaux ectothermes comme hôtes (Paaijmans et al., 2013). Parmi eux, le champignon chytride *Batrachochytrium dendrobatidis* (ci-après Bd) est l'un des agents pathogènes affectés par les altérations climatiques, provoquant la maladie connue sous le nom de chytridiomycose (un autre agent pathogène, *B. salamandrivorans* est responsable de la même maladie chez les salamandres), qui représente la plus grande perte de biodiversité jamais enregistrée attribuable à une maladie (Scheele et al., 2019).

Batrachochytrium dendrobatidis

Le champignon Bd a pris la tête de la perte de la biodiversité due à un agent pathogène, provoquant le déclin de plus de 500 espèces d'amphibiens, l'extinction de 90 espèces dans le monde (Scheele et al., 2019) et atteignant 54% des espèces d'amphibiens connues en 2019 (Fisher et Garner, 2020). Elle a été caractérisée comme « la pire maladie infectieuse jamais enregistrée chez les vertébrés en termes de nombre d'espèces impactées, et de sa propension à les conduire à l'extinction » par l'UICN (UICN, 2005). L'agent pathogène est originaire d'Asie, il y a entre 50 et 120 ans, la péninsule coréenne étant le centre de la diversité génétique de Bd et une seule lignée, la Bd-GPL, étant responsable de la panzootie actuelle (O'Hanlon et al., 2018). L'expansion de la lignée Bd-GPL coïncide avec l'expansion mondiale du commerce des amphibiens à des fins d'animaux de compagnie exotiques, médicales et alimentaires au 20ème siècle (Fisher et al., 2009), ce qui indique une forte influence humaine sur la situation actuelle des amphibiens. La propagation du pathogène a été rapide depuis lors (Figure 1).

Le cycle de vie de Bd comprend deux étapes : la première est constituée de zoospores mobiles qui infectent activement les tissus kératinisés des amphibiens (qu'il s'agisse des pièces buccales des têtards ou des tissus cutanés des post-métamorphes), et la seconde étape comprend un thalle sessile (corps), où de nouvelles zoospores subissent une maturation pour être relâchées dans l'environnement ou pour réinfecter l'hôte (Berger et al., 2005) (Figure 2). Compte tenu de la mobilité des zoospores, un contact direct avec des individus infectés n'est pas nécessaire pour propager la maladie, même si la propagation peut être plus rapide en cas de contact physique. La présence de Bd dans

l'habitat est déjà suffisante pour provoquer une transmission indirecte à la population (Courtois et al., 2017). Chez les têtards, la chytridiomycose peut entraîner la perte des pièces buccales kératinisées, tandis que chez les individus post-métamorphiques, elle peut provoquer une hyperplasie des cellules kératinisées, qui peuvent être 12 fois plus épaisses qu'un tissu sain (Berger et al., 1998) et, par conséquent, entraîner de graves conséquences sur leur osmorégulation en raison de l'épaississement de la couche cutanée (Kilpatrick et al., 2010), conduisant à un arrêt cardiaque (Voyles et al., 2009).

L'impact du champignon pathogène sur les espèces d'amphibiens est toutefois variable et dépend de nombreux facteurs différents (biotiques, abiotiques, associés à l'hôte et/ou à la population et intrinsèques à Bd) (Figure 3). Les facteurs abiotiques, tels que les variables liées au climat et l'exposition aux ultraviolets (Walker et al., 2010) peuvent agir ensemble dans les zones où se trouvent des réservoirs de Bd et une forte densité d'espèces hôtes (Briggs et al., 2010), ajoutant des facteurs significatifs qui peuvent conduire la population au bord de l'extinction. La présence de prédateurs des zoospores de Bd (tels que les rotifères et les Parameciidae) dans l'environnement est également un facteur important pour définir la probabilité d'infection (Schmeller et al., 2014). Les variables intrinsèques à Bd, telles que la lignée, peuvent également affecter la virulence de Bd et provoquer des épidémies plus graves, en particulier lorsqu'elles colonisent une espèce et/ou une population sensible (en raison de la composition de leur microbiome cutané, de leur stade de vie et de la réponse immunitaire de l'hôte) (Fisher et Garner, 2020). Tout cela, combiné aux variables de température et de précipitations (Ruthsatz et al., 2020), peut être à l'origine d'une gamme diversifiée d'expression de la maladie. Récemment, une plus grande importance a été accordée aux facteurs environnementaux et climatiques et à leur interaction avec la défense immunitaire de l'hôte dans le contexte de la dynamique des maladies.

Un des facteurs climatiques les plus importants ayant un impact sur la propagation de Bd est la température, en raison de la croissance de Bd dans une plage de température optimale (Bradley et al., 2019). Bd peut se développer et se reproduire entre 4°C et 25°C, mais sa croissance peut être maximale sous des températures douces (17-25°C) (Piotrowski et al., 2004). Cependant, dans le cas de changements de température, les agents pathogènes en général sont capables de s'acclimater plus rapidement que leurs hôtes ectothermes, ce qui entraîne un retard dans la défense immunitaire de l'hôte (Raffel et al., 2013). Pour les larves d'amphibiens, un seul changement de 5°C vers le haut (15°C

à 20°C) ou vers le bas (20°C à 15°C) est déjà un facteur significatif, respectivement d'augmentation ou de diminution de l'infection par Bd (Bradley et al., 2019), ce qui indique la sensibilité de cette interaction hôte-pathogène. Avec un climat plus variable et une augmentation des températures moyennes, les amphibiens pourraient être plus sensibles aux infections à Bd en raison de différentes combinaisons de facteurs (par exemple, de plus grandes zones avec des températures optimales pour Bd, un retard dans la défense de l'hôte pour s'acclimater à de nouvelles conditions, l'influence de la température et des précipitations sur la physiologie des amphibiens, et la destruction de l'habitat), agissant comme une combinaison de facteurs de stress pour les amphibiens.

Bd et dynamique de la maladie

Après l'établissement de Bd, différentes espèces et même différentes populations de la même espèce peuvent présenter des réactions distinctes à l'infection, allant de la tolérance ou de la résistance (populations enzootiques), à des déclin massifs (populations épizootiques) (Figure 4). En général, des mécanismes de tolérance permettent aux individus de survivre et de maintenir leur aptitude en présence d'un agent pathogène, même avec des charges pathogènes élevées, tandis que les hôtes et/ou populations résistants (=enzootiques) présentent une diminution de la charge en pathogène, via sa neutralisation ou sa destruction (King et Divangahi, 2019). Les hôtes et/ou populations sensibles subissent un déclin sévère (=épizootie), pouvant conduire à des extinctions rapides.

De nombreux facteurs peuvent être responsables de la réponse des populations à Bd, comme le montre la figure 3 et comme l'évoquent les chapitres 2 et 4. Outre la croissance et la virulence de l'agent pathogène, pour provoquer un déclin significatif, voire l'extinction d'une population lors d'une épizootie, Bd ou tout autre agent pathogène a besoin d'un mode alternatif de transmission qui n'est pas compromis lorsque les hôtes sont morts (Smith et al., 2006). Cela peut se produire lorsque les hôtes infectés ont un contact illimité avec d'autres individus et lorsqu'il existe un réservoir du pathogène dans l'habitat (Wilber et al., 2017). En fait, il a été constaté que la transmission en fonction de la densité et du contact des hôtes était un modèle plus précis pour décrire la dynamique et l'infection par Bd, les individus hôtes étant infectés sur une large gamme de températures (Wilber et al., 2017). D'autres facteurs favorisant de manière significative

la propagation de Bd peuvent inclure le réservoir environnemental de zoospores et la résistance de l'hôte à Bd (Wilber et al., 2017).

Un des facteurs spécifiques de l'hôte ayant un impact sur la résistance de l'hôte est le microbiome de la peau, en raison de son rôle dans l'immunité des amphibiens (Rollins-Smith, 2020). Le microbiome cutané des amphibiens a été largement étudié en ce qui concerne sa composition à divers stades de développement (Prest et al., 2018), le temps (Bletz et al., 2017b), les saisons (Longo et al., 2015) et les espèces (Medina et al., 2019) et dans différentes conditions, notamment l'altitude (Muletz Wolz et al., 2018), la température et les précipitations (Ruthsatz et al., 2020), la perturbation de l'habitat (Jiménez et al., 2020), la présence de contaminants (Costa et al., 2016), la captivité (Sabino-Pinto et al., 2016), les maladies (Jani et al., 2021) et même les modèles bioclimatiques mondiaux (Kueneman et al., 2019).

Récemment, le corpus d'études reliant la chytridiomycose et le microbiome cutané des amphibiens a considérablement augmenté. Cependant, on ne sait toujours pas dans quelle direction l'impact est le plus fort, si c'est la chytridiomycose qui modifie la dynamique du microbiome (Jani et Briggs, 2014 ; Bates et al., 2019 ; Becker et al., 2019), si c'est la dynamique du microbiome qui modifie la sensibilité de l'hôte à Bd (Woodhams et al., 2007 ; Harris et al., 2009 ; Walke et al., 2015) ou si les deux événements peuvent se produire simultanément à la même échelle.

Étant donné que certaines populations et même certains individus d'espèces en déclin peuvent faire preuve de résistance ou de tolérance à Bd, j'ai exploré comment la composition initiale du microbiome cutané peut faciliter ou entraver la chytridiomycose. Harris et ses collègues (2009) ont découvert que les hôtes enrichis avec l'espèce bactérienne *Janthinobacterium lividum* étaient plus efficacement protégés contre Bd en raison de la production de violaceine, un métabolite anti-chytride. La composition initiale du microbiome était donc essentielle pour déterminer l'issue individuelle de la maladie (Harris et al., 2009), un schéma que l'on retrouve également chez les espèces panaméennes (Becker et al., 2015). La composition de la peau des amphibiens peut également déterminer la coexistence avec Bd dans les populations qui sont entourées de populations éteintes ayant subi un événement épizootique, un résultat expliqué par la proportion d'individus avec des bactéries antifongiques (Lam et al., 2010). Ces résultats indiquent que, tout comme les humains, les amphibiens peuvent bénéficier de l'immunité de groupe (Woodhams et al., 2007 ; Lam et al., 2010), lorsqu'un minimum de 80% des

individus (Lam et al., 2010) ont, au moins une espèce bactérienne avec des propriétés antifongiques (Woodhams et al., 2007 ; Lam et al., 2010). Ces résultats mettent à nouveau en évidence les interactions entre les caractéristiques de l'hôte (telles que la densité) et les fonctions du microbiome associées à la peau. Cependant, la dysbiose, c'est-à-dire l'altération du microbiome de l'hôte, après l'établissement de Bd peut également être observée. Dans une étude menée au Brésil, le débordement de Bd des populations aquatiques infectées vers les populations terrestres a été responsable d'une infection qui a radicalement modifié la composition du microbiome cutané des individus nouvellement infectés (Becker et al., 2019). Le même résultat a été trouvé dans un ensemble combiné d'expériences sur le terrain et en laboratoire, où un événement épizootique de Bd a provoqué une mortalité de 100 % des post-métamorphes et a modifié de manière significative le microbiome cutané dans les deux conditions (sur le terrain et en laboratoire), classant Bd comme un moteur de dysbiose bactérienne (Jani et Briggs, 2014). Le même phénomène a été rapporté avec la chytridiomycose affectant les salamandres (Bates et al., 2019).

Si la plupart des recherches sur ce sujet se sont concentrées sur le microbiome cutané des métamorphes et des adultes, la littérature sur les pré-métamorphes n'est pas aussi étendue (Kueneman et al., 2014 ; Ellison et al., 2019a ; Jimenez et al., 2019 ; Jervis et al., 2021). La façon dont les têtards répondent aux différentes conditions environnementales et à l'impact pathogène est, cependant, extrêmement pertinente pour comprendre si des changements similaires peuvent causer le même impact sur les populations d'amphibiens de différents stades de vie ou si les têtards répondent d'une manière différente, compte tenu de leur microbiome pré-métamorphique (Bataille et al., 2018).

Bd dans les régions montagneuses

Les montagnes sont l'une des zones les plus fortement soumises aux pressions climatiques et anthropiques (Schmeller et al., 2018). Dans ces régions, les températures ont déjà augmenté d'un degré entre 1970 et 2001 (Epstein, 2001), L'augmentation des températures moyennes et de l'urbanisation des hautes terres y a aussi augmenté la fréquence des maladies transmises par les insectes (Epstein, 2001). Les événements extrêmes (tels que les fortes pluies), plus fréquents en raison du changement climatique,

peuvent être des mécanismes importants de propagation des agents pathogènes (Smith et al., 2011), qui sont alors en mesure de réaffecter leurs ressources de croissance et de reproduction et, par conséquent, d'accroître leur virulence (Frenken et al., 2017).

En raison du changement climatique, les températures dans les régions montagneuses se déplacent vers l'optimum de croissance de Bd (Alan Pounds et al., 2006), ce qui permet à Bd de coloniser des zones où les populations d'amphibiens sont naïves. Dans ces circonstances, les agents pathogènes sont à même de s'adapter du froid au chaud plus rapidement que leurs hôtes, ce qui entraîne une plus grande sensibilité à la maladie lorsque les amphibiens sont confrontés à des températures plus chaudes dans les habitats infectés (Bradley et al., 2019). Par conséquent, on a déjà constaté que Bd infecte des amphibiens dans une large gamme d'altitudes, avec des épidémies plus graves en altitude (Walker et al., 2010 ; Medina et al., 2017). Dans les Pyrénées, par exemple, Bd a été détectée dans les années 2000 (O'Hanlon et al., 2018) et les populations d'amphibiens ont connu des déclin, principalement *A. obstetricans*, dus à Bd. Les projections futures indiquent un changement important dans la date du dégel printanier dans les Pyrénées, et il a été démontré que cela augmente la prévalence et la gravité des épidémies (Clare et al., 2016). Dans cette région, une introduction unique et une propagation ultérieure (ou des introductions multiples d'un clone de Bd identique) sont responsables de la répartition de Bd observée aujourd'hui (Walker et al., 2010 ; Clare et al., 2016 ; Courtois et al., 2017 ; Bates et al., 2018). Les populations d'amphibiens montagnards ont également été affectées dans de nombreuses autres chaînes de montagnes à travers le monde (Muths et al., 2008 ; Hirschfeld et al., 2016 ; LaBumbard et al., 2020).

Écologie des amphibiens

Les amphibiens sont connus pour jouer différents rôles dans le fonctionnement des écosystèmes, avec des conséquences importantes lorsque leurs populations sont menacées. Les têtards, par exemple, sont des brouteurs importants dans les cours d'eau, et une perte presque totale de leurs populations (98 %) peut entraîner une augmentation significative (deux fois supérieure à la normale) de la biomasse d'algues et de détritus, ainsi qu'une réduction de 50 % de l'absorption d'azote (Whiles et al., 2013). Pour l'écosystème, cela signifie une réduction significative de la respiration du cours d'eau et

moins d'activité biologique dans les sédiments. En outre, les amphibiens contribuent (1) à la lutte contre les maladies transmises par les moustiques grâce à la prédation, (2) à la structure de l'écosystème par le creusement du sol et la bioturbation aquatique, (3) ainsi qu'à la décomposition, fournissant du phosphore supplémentaire par l'excrétion de déchets (Hocking et Babbitt, 2014), entre autres, comme l'impact sur les consommateurs primaires dans le réseau alimentaire et le flux d'énergie dans les cours d'eau (Ranvestel et al., 2004). En raison de leur rôle essentiel dans les écosystèmes d'eau douce, les amphibiens sont également connus comme des « ingénieurs de l'écosystème » (Wood et Richardson, 2010).

Les amphibiens sont des animaux ectothermes qui ont besoin d'un niveau élevé d'humidité dans leur habitat pour satisfaire leurs besoins physiologiques, tels que la performance fonctionnelle (Greenberg et Palen, 2021) et l'échange de gaz et de fluides à travers leur peau perméable, un organe essentiel pour leur homéostasie. Les bonnes conditions de température et d'humidité sont également nécessaires pour leur protection immunitaire, qui commence sur la peau des amphibiens (davantage de détails au chapitre 2).

Après le stade de têtard, les individus subissent une métamorphose et ils passent d'un environnement aquatique à un environnement terrestre. Ce processus se déroule généralement en peu de temps et s'accompagne de changements importants dans tout le corps, comme le développement des organes internes, ainsi que la kératinisation et l'augmentation des cellules épidermiques de la peau (Vitt and Cardwell, 2013). Tout comme le reste du corps des amphibiens, leur microbiome cutané subit également un changement au cours de cette période, le microbiome cutané post-métamorphose différant de celui des têtards (Kueneman et al., 2014).

Chapter 1: General introduction

Current wildlife threats and emerging infectious diseases

Biodiversity loss is increasing substantially in the last couple of years and this tendency is foreseen to continue, mainly due to land-use change, climate change, nitrogen deposition, biotic exchange (when a species moves or is moved from one biota to another, such as fish displacement and stocking) (Sala, 2000), and emerging infectious disease (Scheele et al., 2019). The consequences for wildlife species are, however, dependent on the interaction of these variables and additional local stressors in each biome (Sala, 2000).

Among threats to wildlife, climate change has been identified as one of the greatest to both wildlife and human societies. Not only does it directly affect animal and plant species, but it also reinforces other environmental stressors, such as land use change (Opdam and Wascher, 2004) and invasive species (Hellmann et al., 2008). Climate change and habitat alterations are responsible for threatening one quarter of the currently known species with extinction (IPBES, 2019), with ultimate consequences for human life due to the provision (or lack of provision) of ecosystem services (Gowdy, 2020), and alteration of emerging zoonoses frequency, for example (Daszak et al., 1999). Mosquito-borne diseases, such as dengue and yellow fever, that need water bodies to complete their cycle tend to increase as extreme rainfalls are more frequent (Slenning, 2010; Raffel et al., 2013). Domestic animal diseases, such as bluetongue and African Rift Valley fever, two viral diseases that affect livestock (Slenning, 2010; Jones et al., 2019), are also foreseen to increase in frequency due to warmer habitats for vectors and changes in the Indian Ocean weather, respectively, causing large economic loss. Disease can come from different sources (Daszak et al., 2000): (a) emergence and transfer from domestic animals, such as the canine distemper virus that caused the extinction of African wild dogs populations; (b) related to human intervention (host and/or parasite translocation), e.g. ebola virus, due to the possible contact between infected humans with primates; or (c) without any human or domestic animal association, like the viral chorioretinitis, known for causing blindness and mortalities in kangaroos (Daszak et al., 2000).

Amphibian conservation and threats

Amphibian populations have been threatened for decades, with 43% of the known species experiencing ongoing decline, a number that is almost twice that in mammal species (23%) (Stuart et al., 2004). This makes amphibians the currently most threatened vertebrate group, especially due to their ectothermic physiology and the spread of a fungal disease (Fisher et al., 2012; Scheele et al., 2019). Severe declines followed by local extinctions were detected in the 1990s in Australia and the Americas (Berger et al., 1998) and later on in all the continents. The causes for the worldwide declines are diverse, including: (1) presence of toxics in the environment, what may cause incomplete absorption of egg yolk and larvae deformation (Horne and Dunson, 1994), (2) predation, which is intensified by introduction of exotic fish with unknown predatory strategies to native amphibians (Vredenburg, 2004), (3) habitat degradation, such as draining of wetlands and the consequential removal of breeding sites and population split (Becker et al., 2007), and changes in the local climate (Wake and Vredenburg, 2008), (4) ultraviolet radiation, that can cause slow rate of egg hatching (Blaustein et al., 2003) and developmental and physiological abnormalities in embryos (Hatch and Blaustein, 2003), (5) climate change, that may enhance the impact of other threats, such as spread of invasive species (Blaustein et al., 2003; Beebee and Griffiths, 2005), which will also compete with amphibians in their natural habitat, and (6) emerging infectious diseases, such as chytridiomycosis and ranaviriosis.

As explained above, climate change is expected to strongly influence pathogen emergence and spread, and increase outbreak frequency, especially for pathogens with complex life cycles with ectothermic animals as hosts (Paaijmans et al., 2013). Among them, the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd) has been one of the pathogens affected by climatic alterations, causing the disease known as chytridiomycosis (another pathogen, *B. salamandrivorans* is responsible for the same disease in salamanders), which represents the greatest ever recorded loss of biodiversity attributable to a disease (Scheele et al., 2019).

Batrachochytrium dendrobatidis

Bd has taken the lead in biodiversity loss due to a pathogen, causing the decline of more than 500 amphibian species, the extinction of 90 species worldwide (Scheele et al., 2019) and reaching 54% of the amphibian species known by 2019 (Fisher and Garner, 2020). It has been characterized as “the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction” by IUCN (Gascon, C., 2007). The pathogen originated in Asia, between 50 and 120 years ago, with the Korean peninsula being the centre of *Bd* diversity and one single lineage, the *Bd*-GPL, being responsible for the current panzootic (O’Hanlon et al., 2018). The expansion of the *Bd*-GPL lineage coincides with the global expansion of amphibian trades for exotic pet, medical, and food purposes in the 20th century (Fisher et al., 2009), indicating a strong human influence on current amphibian declines. The spread of *Bd* has been fast since then (Figure 1).

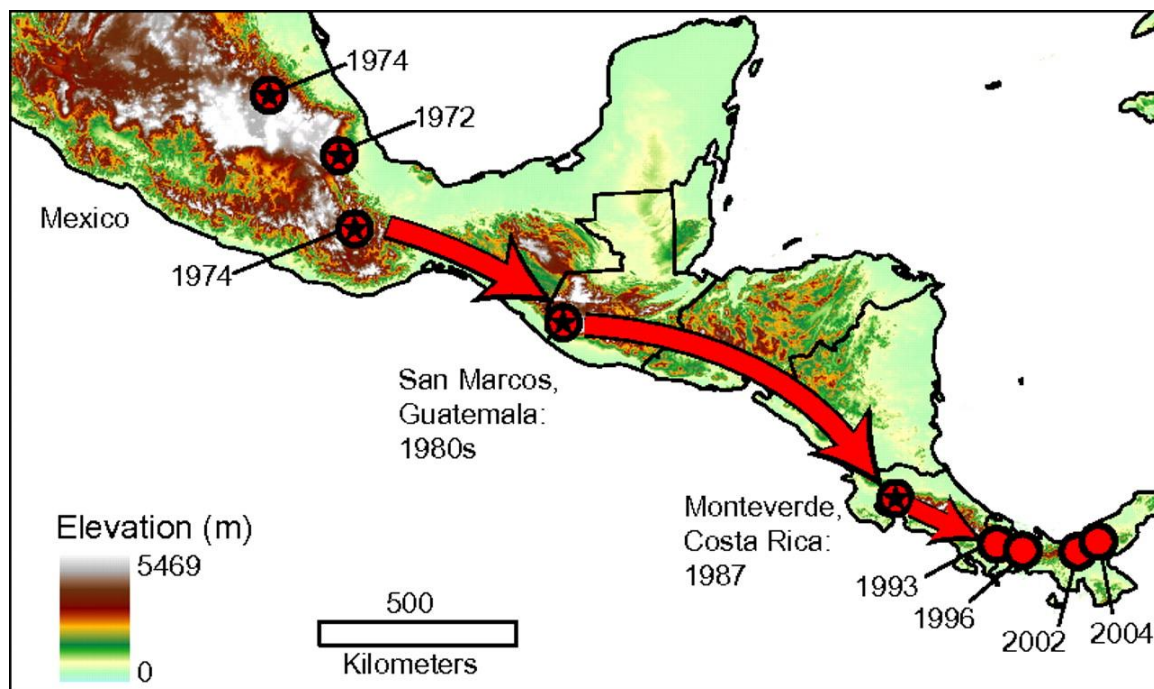


Figure 1: Example of spatial and temporal spread of *Bd* from Mexico to lower Central America. Both red circles with and without stars indicate *Bd*-positive sites (Figure from Cheng et al., 2011).

The life cycle of *Bd* includes two life stages: the first one are motile zoospores that actively infect keratinized tissues of amphibians (be it mouthparts in tadpoles or skin tissue in post metamorphs), and the second stage includes a sessile thallus (body), where

new zoospores undergo maturation to be released to the environment or to reinfect the host (Berger et al., 2005) (Figure 2). Considering the mobility of the zoospores, a direct contact with infected individuals is not necessary to spread the disease, even though it can be faster when physical contact occurs. The presence of Bd in the living site is already enough to cause indirect transmission to the population (Courtois et al., 2017). In tadpoles, chytridiomycosis can cause loss of the keratinized mouthparts, while in post-metamorphic individuals, it can cause hyperplasia of keratinaceous cells, that can be 12 times thicker than a healthy tissue (Berger et al., 1998) and, therefore, cause severe consequences for their osmoregulation due to the thickening of the skin layer (Kilpatrick et al., 2010), ultimately leading to cardiac arrest (Voyles et al., 2009).

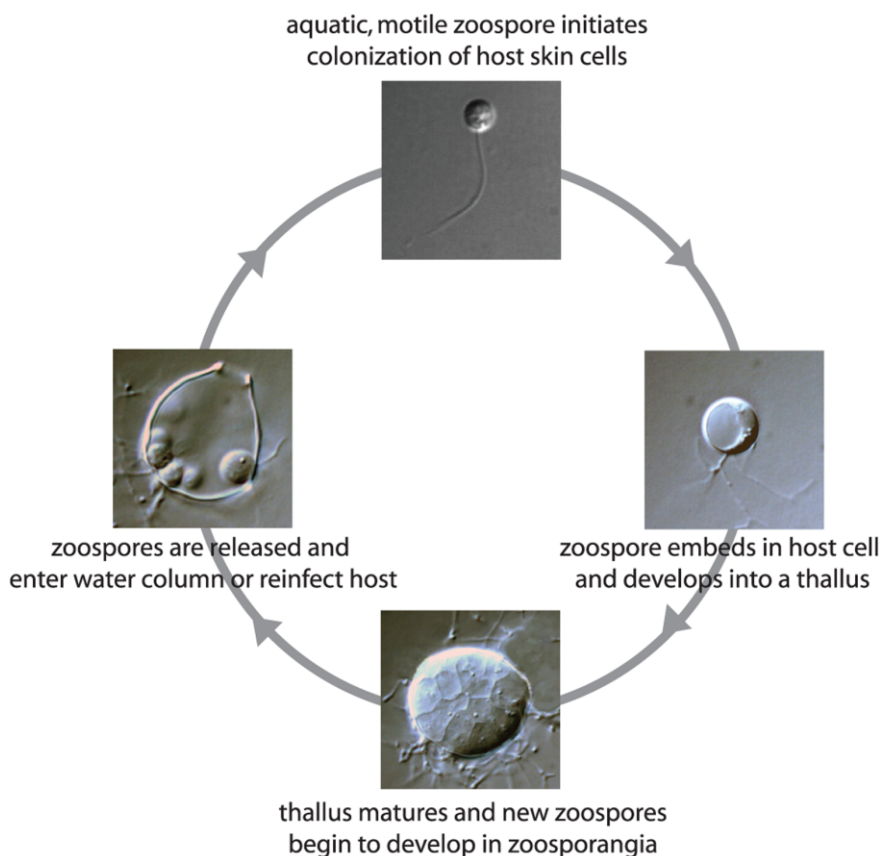


Figure 2: The life cycle of Bd. The motile zoospores use their posterior flagellum to reach new hosts via chemotaxis, initiating the colonization of the keratinized tissue. After the host cells are infected, new zoospores undergo maturation and development inside a sessile thallus and are released back to the environment or reinfect the host (Figure from Rosenblum et al., 2010)).

The impact of the fungal pathogen on the amphibian species is, however, variable and dependent on many different factors (biotic, abiotic, host and/or population associated and intrinsic from *Bd*) (Figure 3). Abiotic factors, such as climate related variables and ultra violet exposure (Walker et al., 2010), can act together in areas with *Bd* reservoirs and high density of host species (Briggs et al., 2010), adding significant factors that may drive the population to the edge of extinction. The presence of *Bd* zoospore predators (such as rotifers and Parameciidae) in the environment is also a significant factor in defining the infection probability (Schmeller et al., 2014). *Bd*-intrinsic variables, such as lineage, also can affect *Bd* virulence and cause more severe outbreaks, especially when colonizing a susceptible species and/or population (due to their skin microbiome composition, life stage and host immune response) (Fisher and Garner, 2020). All of this combined with temperature and precipitation variables (Ruthsatz et al., 2020) may cause a diverse range of disease outcomes. Recently, more importance has been given to environmental and climatic factors and their interaction with host immune defense in the context of disease dynamics.

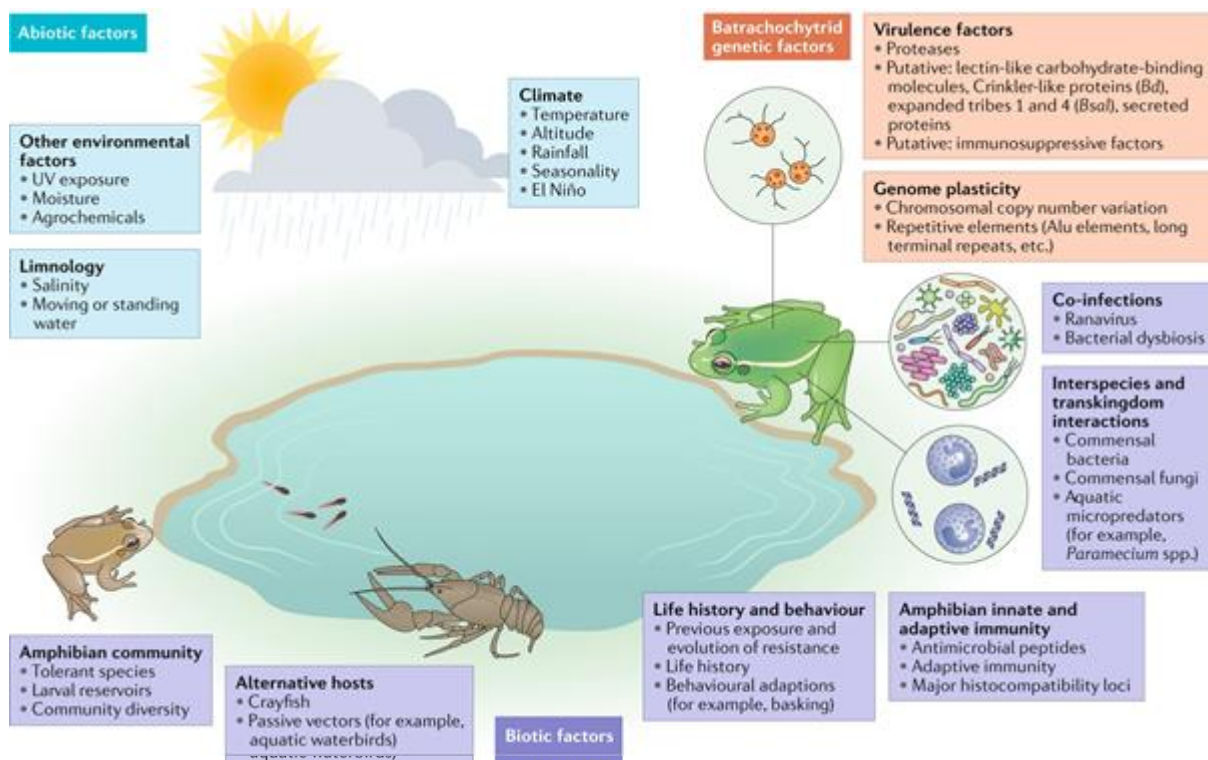


Figure 3: Biotic, abiotic and *Bd*-specific factors impacting the severity and virulence of *Bd* outbreaks (from Fisher and Garner, 2020).

One of the most important climatic factors impacting Bd spread is temperature, due to its optimal temperature range growth (Bradley et al., 2019). Bd can grow and reproduce between 4°C and 25°C, but growth can be maximal under mild temperatures (17-25°C) (Piotrowski et al., 2004). However, in the case of temperature shifts, pathogens in general are more capable to acclimate faster than ectothermic hosts, causing a delay in host immune defense (Raffel et al., 2013). For amphibian larvae, a single shift of 5°C upwards (15°C to 20°C) or downwards (20°C to 15°C) is already a significant factor in increasing or decreasing Bd infection, respectively (Bradley et al., 2019), indicating the sensitiveness of this host-pathogen interaction. With more variable climate and increase in mean temperatures, amphibians might be more susceptible to Bd infections due to different combinations of factors (e.g. larger areas with Bd optimal temperatures, delay in host defense to acclimatize to new conditions, temperature and precipitation influence on amphibian physiology, habitat destruction), acting as a combination of stressors to amphibians.

Bd - host dynamics

After the establishment of Bd, different species and even different populations of the same species exhibit distinct reactions to the infection, ranging from tolerance or resistance (enzootic populations), to massive local declines (epizootic populations) (Figure 4). In general, tolerance mechanisms allow individuals to survive and to maintain their fitness in the presence of a pathogen, even with high pathogen loads, while resistant hosts and/or populations (=enzootic) show a decrease in pathogen load, via neutralization or killing (King and Divangahi, 2019). Sensitive hosts and/or populations undergo a severe decline (=epizootic), potentially leading to rapid local extinctions.

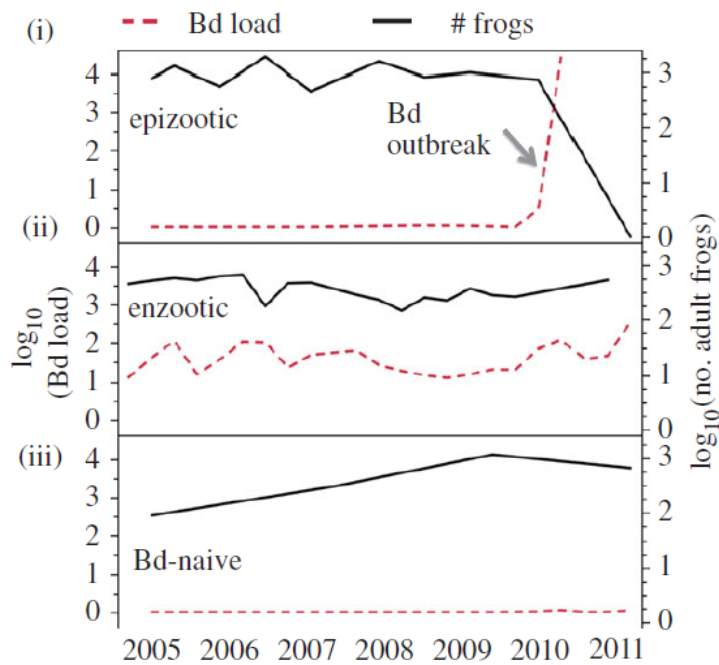


Figure 4: Dynamics of (i) epizootic, (ii) enzootic and (iii) uninfected populations in comparison to Bd infection severity (Bd load) over a 7-year period. Population (i) is stable in numbers until the arrival of Bd, which caused rapid population decline and local extinction. Population (ii) is numerically stable over time and co-exist with Bd, which also does not increase in severity. Finally, population (iii) has never had contact with Bd and maintains a stable population over the years (Figure from Jani et al., 2017).

Many factors can be responsible for the population response to Bd, as shown in Figure 3 and discussed in chapters 2 and 4. Besides pathogen growth and virulence, in order to cause a significant decline and even the extinction of a population in an epizootic event, Bd or any pathogen needs an alternative transmission dynamic which is not compromised when hosts are dead (Smith et al., 2006). This can happen when infected hosts have unlimited contact with other individuals and when there is a pathogen reservoir in the same habitat (Wilber et al., 2017). In fact, it has been found that transmission depending on host density and contact was a more accurate model in describing Bd-infection dynamic and outcome, with host individuals being infected over a large range of temperatures (Wilber et al., 2017). Additional significantly enhancing factors in Bd spread can include the environmental zoospore reservoir and the host resistance to Bd (Wilber et al., 2017).

One of the specific host factors impacting on host resistance is the skin microbiome, due to its role as part of the amphibian immunity (Rollins-Smith, 2020). The amphibian skin microbiome has been largely studied in regard to its composition across

life stages (Prest et al., 2018), time (Bletz et al., 2017b), seasons (Longo et al., 2015) and species (Medina et al., 2019) and under different conditions, including elevation (Muletz Wolz et al., 2018), temperature and precipitation (Ruthsatz et al., 2020), habitat disturbance (Jiménez et al., 2020), presence of contaminants (Costa et al., 2016), captivity (Sabino-Pinto et al., 2016), disease (Jani et al., 2021) and even global bioclimate patterns (Kueneman et al., 2019).

Recently, the body of studies connecting chytridiomycosis and amphibian skin microbiome has significantly increased. However, it is still uncertain which direction of impact is stronger, if it is chytridiomycosis altering microbiome dynamics (Jani and Briggs, 2014; Bates et al., 2019; Becker et al., 2019), if it is microbiome dynamics altering host susceptibility to Bd (Woodhams et al., 2007; Harris et al., 2009; Walke et al., 2015) or if both events can happen simultaneously at the same scale.

Since some populations and even individuals of declining species can show resistance or tolerance in the face of Bd, it has been explored how the initial skin microbiome composition can facilitate or hinder chytridiomycosis. Harris and colleagues (2009) found out that hosts enriched with the bacterial species *Janthinobacterium lividum* were more effectively protected against Bd due to the production of the anti-chytrid metabolite violacein. The initial microbiome composition was, therefore, essential in determining the individual disease outcome (Harris et al., 2009), a pattern also found in Panamanian species (Becker et al., 2015). The amphibian skin composition can also determine the coexistence with Bd in populations which are surrounded by extinct populations that underwent an epizootic event, an outcome explained by the proportion of individuals with antifungal bacteria (Lam et al., 2010). These results indicate that, just like in humans, amphibians may also benefit from herd immunity (Woodhams et al., 2007; Lam et al., 2010), when a minimum of 80% of the individuals (Lam et al., 2010) have, at least, one bacterial species with antifungal properties (Woodhams et al., 2007; Lam et al., 2010). Such findings point again for the interactions between host characteristics (such as density) and skin associated microbiome functions. However, dysbiosis, the alteration of the host microbiome, after Bd establishment can also be observed. In a study conducted in Brazil, spillover of Bd from infected aquatic populations to terrestrial ones was responsible for a lethal infection that drastically changed skin microbiome composition of the newly infected individuals (Becker et al., 2019). The same result was found in a combined set of field and laboratory experiments,

where an epizootic Bd event caused 100% mortality of post-metamorphs and altered significantly the skin microbiome in both conditions (field and laboratory), classifying Bd as a driver for bacterial dysbiosis (Jani and Briggs, 2014). The same has been reported with chytridiomycosis affecting salamanders (Bates et al., 2019).

While most of the research on this topic has focused on the skin microbiome of metamorphs and adults, the literature on pre-metamorphs is not so extent (Kueneman et al., 2014; Ellison et al., 2019a; Jimenez et al., 2019; Jervis et al., 2021). How tadpoles respond to different environmental conditions and pathogenic impact is, however, extremely relevant to understand if similar changes can cause the same impact on amphibian populations of different life stages or if tadpoles will respond in a different way considering their pre-metamorphic microbiome (Bataille et al., 2018).

Bd in mountain regions

Mountains are one of the areas under stronger climatic and anthropogenic pressures (Schmeller et al., 2018), as the warming in these regions had already increased by one degree by 2001, in comparison to 1970 (Epstein, 2001), as well as the frequency of insect-borne diseases due to higher mean temperatures and the urbanization in highlands (Epstein, 2001). Extreme events (such as strong rainfalls), more common due to climate change, may be important mechanisms of pathogen spread (Smith et al., 2011), which are then able to reallocate growing and reproductive defense resources and, therefore, increase their virulence (Frenken et al., 2017).

Due to climate change, temperatures in highland areas are shifting towards the growth optimum of Bd (Pounds et al., 2006), allowing Bd to colonize areas with naive amphibian populations. Under these circumstances, pathogens are more able to shift from cold to warmer conditions faster than hosts, causing a higher sensitivity to disease when amphibians are confronted with warmer temperatures in infected habitats (Bradley et al., 2019). Consequently, Bd has been already found infecting amphibians across a wide variety of elevations, with more severe outbreaks in higher altitudes (Walker et al., 2010; Medina et al., 2017). In the Pyrenees Mountains, for example, Bd was detected in the years 2000 (O'Hanlon et al., 2018) and amphibian populations have experienced declines, mainly of *A. obstetricans*, due to Bd. Future projections indicate a

significant change in the timing of spring thaw in the Pyrenees, and this has been shown to increase the prevalence and severity of the outbreaks (Clare et al., 2016). In this region, a single introduction and subsequent spread (or multiple introductions of an identical Bd clone) was responsible for the Bd range observed today (Walker et al., 2010; Clare et al., 2016; Courtois et al., 2017; Bates et al., 2018). Montane amphibian populations have also been affected in many other mountain ranges across the world (Muths et al., 2008; Hirschfeld et al., 2016; LaBumbard et al., 2020).

Amphibian ecology

Amphibians are known to play different roles in ecosystem dynamics, with significant consequences when their populations are under threat. Tadpoles, for example, are important grazers in streams, and an almost complete loss of their populations (98%) can cause a significant increase (twice as normal) in algae and detritus biomass and 50% reduction in nitrogen uptake (Whiles et al., 2013). For the ecosystem, it means a significant reduction in the stream respiration and less biological activity in the sediments. Additionally, amphibians aid in the control of mosquito-borne disease through predation, in ecosystem structure via soil burrowing and aquatic bioturbation, and also decomposition, providing additional phosphorus through waste excretion (Hocking and Babbitt, 2014), among others, such as impact on primary consumers in food web and energy flow in streams (Ranvestel et al., 2004). Due to their essential role in freshwater ecosystems, amphibians are also known as ‘ecosystem engineers’ (Wood and Richardson, 2010).

Amphibians are ectothermic animals that require a high level of humidity in their habitat to perform their physiological needs, such as functional performance (Greenberg and Palen, 2021) and exchange of gas and fluids through the permeable skin (Beebee, 1996), an essential organ for their homeostasis. Right temperature and humidity conditions are also necessary for their immune protection, which starts on the amphibian skin (more details in chapter 2).

After the tadpole stage, individuals go through a metamorphosis, when they move from an aquatic environment to a terrestrial one. The process usually occurs in a short period of time, accompanied by significant changes in the whole body, such as the

development of internal organs and the keratinization and increase in epidermal cells of the skin (Vitt and Cardwell, 2013). Just like the rest of the amphibian body, their skin microbiome also undergoes a shift during this period, with the post-metamorph skin microbiome differing from the skin composition of tadpoles (Kueneman et al., 2014).

Questions driving the work

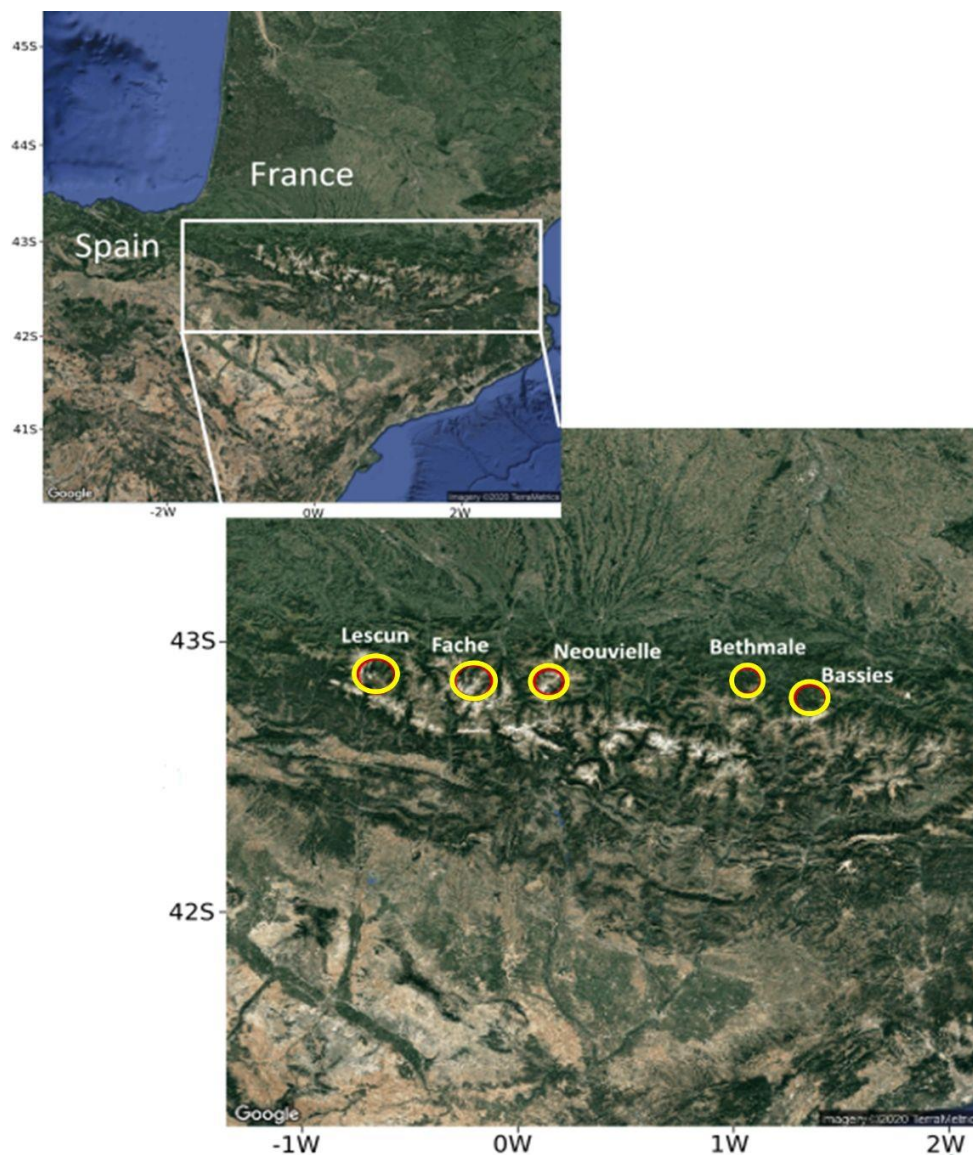
The amphibian skin microbiome has been the focus of many studies considering its vital role in amphibian homeostasis and immune protection. However, the symbiotic relationship between amphibian hosts and skin microbiome has been affected by diverse environmental changes (e.g. climate variation, precipitation, habitat fragmentation, salinity) (more details in chapter 2) and the spread of the pathogen *Batrachochytrium dendrobatidis*. Different studies have tried to disentangle the relationships between climate, altitude, habitat disturbance and host susceptibility to disease. Here, we contribute to this body of knowledge by working in an altitudinal mountain range on the bacterial microbiome composition of three amphibian species that are under distinct estimated anthropogenic pressures (livestock presence, tourism pressure and fish introduction) and climatic conditions. I aimed to first explore the variations in bacterial skin microbiome of the amphibian species considering only anthropogenic and climatic factors, and second, to further consider the pathogen disturbance in the environment and on the skin. The work aimed to answer the following questions:

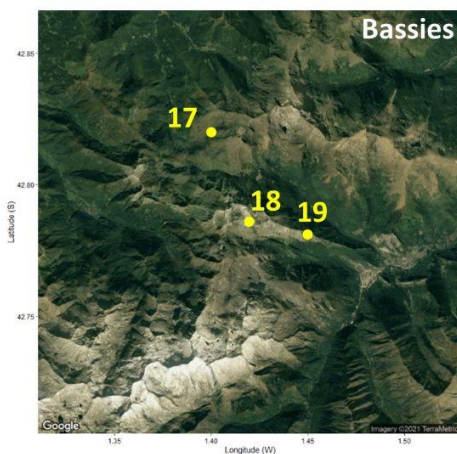
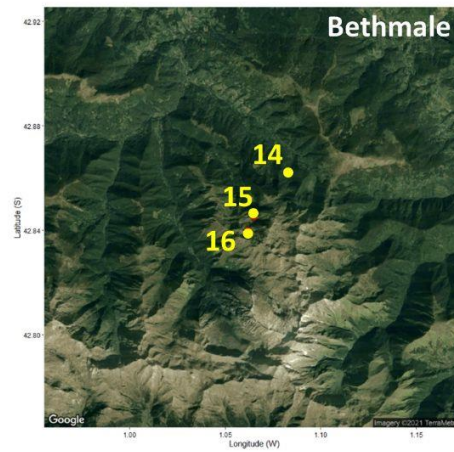
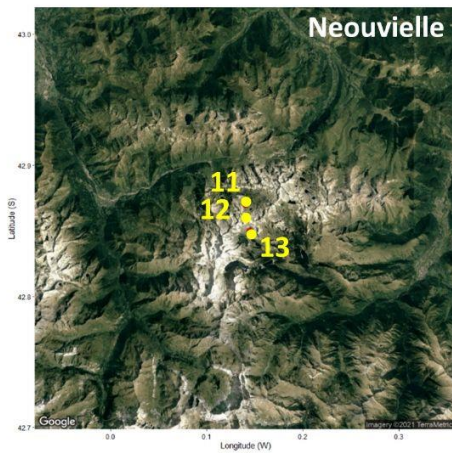
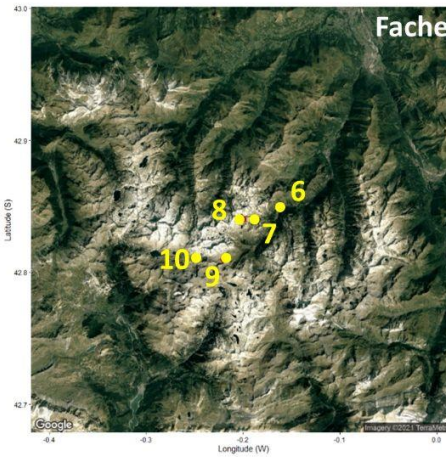
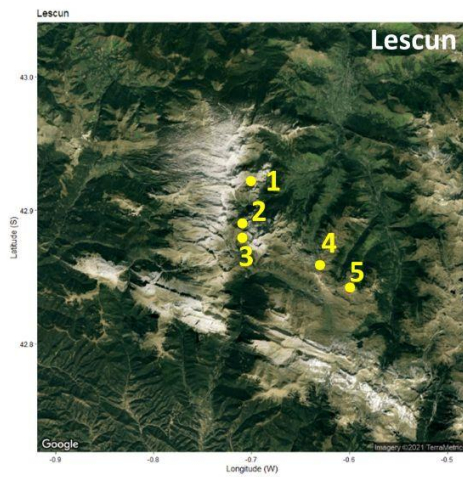
- 1) Do anthropogenic factors affect the bacterial skin composition of tadpoles from the species *A. obstetricans*, *R. temporaria* and *B. bufo*?
- 2) Do climatic variables, such as length of growing season, precipitation index and consecutive dry days, impact the bacterial skin composition of *A. obstetricans*, *R. temporaria* and *B. bufo* tadpoles in a mountain range?
- 3) Is the host species a relevant factor influencing the skin community of *A. obstetricans*, *R. temporaria* and *B. bufo* in the tadpole stage as well?
- 4)
 - a) Do *A. obstetricans* tadpoles show different bacterial and micro eukaryotic communities dynamics in Bd-infected and uninfected populations?
 - b) Do *A. obstetricans* tadpoles show different bacterial and micro eukaryotic communities dynamics in enzootic and epizootic populations?

c) Do Bd presence and load at an individual scale correlate to bacterial and micro eukaryotic communities composition?

Site locations

In total, tadpoles from *A. obstetricans*, *R. temporaria* and *B. bufo* were sampled in 19 lakes. All sites were located in the Pyrenees Mountains (Figure 5) and all the sampling was done in 2016, 2017 and 2018. As described in chapters 3 and 4, different numbers of lakes were visited for the analysis of each chapter. More details on sampling design and sample sizes can be found in the following chapters.





1. Lhurs (1691)	11. Pecheur (2307)
2. Ansabere (1876)	12. Madamete Haut (2379)
3. Acherito (1858)	13. Gourg de Rabat (2407)
4. Puits (1891)	14. Bethmale (1039)
5. Arlet (1974)	15. Ayes (1681)
6. Paradis (1619)	16. Bellonguere (1864)
7. Vallon Amont (2215)	17. Arbu (1729)
8. Embarrat (2180)	18. Alate (1865)
9. Grand Fache (2430)	19. Labant (1548)
10. Fache Espagne (2522)	

Figure 5: Site locations across the Pyrenees. The lakes are in five distinct regions, indicated with the yellow circles in the top image. Lake names are shown in the black square in the bottom image, accompanied by their legend numbers and their altitude, in meters, between brackets.

Studied species

1. *Alytes obstetricans*

Also known as the Common midwife toad, *A. obstetricans* is commonly found across Europe with three sub-species present on the continent. It is more frequently found in areas with sparse vegetation and permanent waters. Tadpoles can overwinter and undergo metamorphosis only during the following year (or even later), especially in mountains, where the growing season length is usually shorter. The male is responsible for the parental care behaviour, carrying the eggs on the back until they hatch, when they are released, as tadpoles, into the water.

The species is especially susceptible to Bd, and mass mortalities and population declines due to Bd have been registered in Spain more than 20 years ago (Bosch et al., 2001), and later in the Pyrenees (Walker et al. 2010). Considering the long stay of the tadpoles in the lakes over the winter, they can aid in the maintenance of Bd in the population over time (Clare et al., 2016). Other factors, such as fish introduction and habitat loss are also known threats for this species (AmphibiaWeb 2021). The species is under legal protection in France, Belgium, Switzerland, Spain, Germany and Netherlands, and its habitat is protected in France and Luxembourg (EU Species Action Plan, 2012). The general population trend is decreasing, according to IUCN (Bosch et al., 2009).



Figure 6: *A. obstetricans* male carrying the eggs until they hatch and tadpole. Photos by: Matthieu Berroneau (www.matthieu-berroneau.fr) and Bernard Dupont (<https://www.flickr.com/people/65695019@N07>)

2. *Rana temporaria*

The European common frog, *Rana temporaria*, is present in more European countries than *A. obstetricans*, reaching up to West Siberia, with adults carrying out period migrations between winter habitat, breeding sites and summer habitats (Reh and Seitz, 1990). Opposite to *A. obstetricans*, this species does not overwinter in their habitat. It inhabits a range of habitats: forest, bushlands, both dry and swampy meadows and different kinds of anthropogenic landscape, such as fields, gardens and parks. *R. temporaria* is generally not threatened, except in highly disturbed areas with the destruction of breeding sites (AmphibiaWeb 2021). In general, it is not considered to be susceptible to Bd, but changes in spring thaw have been connected to infection dynamics in *R. temporaria* in the Pyrenees (Clare et al., 2016). The general population trend is stable, according to IUCN (Kuzmin et al., 2009).



Figure 7: Adult and tadpole of *R. temporaria*. Photos by: Holger Krisp (own work) and Neil Phillips (<http://www.uk-wildlife.co.uk/>)

3. *Bufo bufo*

With the popular name of Common toad, *Bufo bufo* occupies a broad area, from Denmark and Finland until the limits of Serbia and Kazakhstan. Similar to *R. temporaria*, this species also does not overwinter in lakes and undergo metamorphosis in the same year. It can be found in forest regions, associated with wet sites and dense vegetation, but usually in a low abundance. Many parasites and predators can affect the species populations, but the impact has not been quantified so far. The current threats to *B. bufo* are deforestation and artificial drying of wetlands (AmphibianWeb 2021). *B. bufo* is also

not considered susceptible to Bd, but just like *R. temporaria*, spring thaw timing has been linked to Bd dynamics in this species, in the Pyrenees (Clare et al., 2016). The general population trend is stable, according to IUCN (Aram et al., 2009).



Figure 8: Adult and tadpole *B. bufo*. Photo by: Karamell, own work

Content and student contributions to each chapter

The general introduction - chapter 1 - as well as the general conclusion - chapter 5 - were based on the work and results discussed in chapter 2, 3 and 4. In chapter 1, I introduce the general concepts and the current knowledge on global changes, biodiversity loss, wildlife diseases, amphibian conservation and the amphibian skin microbiome and describe the guiding questions, followed by a description of the sampling sites and model species.

Chapter 2 is a review and concept article (published in *Trends in Parasitology*) on the interactions between the environment, the host, the host microbiome and the pathogens, where a new approach was proposed to the traditional *disease triangle*, a concept used to explore the outcome of a disease considering the simultaneous interactions between host, environment and pathogen. This concept, however, does not take into account the microorganisms which are associated with the host and their own interactions with the three factors defining the *disease triangle*. We recommended the transition from the *disease triangle* to a *disease pyramid*, where the host microbiome is one of 4 connecting edges with specific responses according to its associated species, the environmental conditions and the pathogen variables. The paper was published in July 2020, in the Journal *Trends in Parasitology*, under the title “Environmental Factors and

Host Microbiomes Shape Host – Pathogen Dynamics”. My contribution, as the main author, was to (1) gather all the recent available information on the topics covered in the paper, (2) describe them through the paper and summarize in the main table, (3) write the first version of the manuscript, (4) incorporate the comments and changes suggested by the co-authors and (5) follow the review and submission process. The work of the co-authors was essential to include the *disease pyramid* approach and to sustain its validity in the view of the interactions between host, host microbiome, pathogens and environment, as well as to structure the manuscript and provide me with the first contact with the scientific writing and the submission process.

In chapter 3, entitled “Climate and fish impact tadpole skin microbiome in mountain lakes”, I analyzed the bacterial skin microbiome of the three amphibian species described above (*A. obstetricans*, *B. bufo* and *R. temporaria*) present in our lakes under (a) different climatic variables (length of the growing season, number of frost days and simple precipitation index), (b) estimated anthropogenic factors (tourism impact, fish stocking and livestock presence) and (c) lake characteristics (longitude, pH, lake area and altitude). I analyzed if and how the microbiome composition would be affected by changes in the environmental conditions and discussed the results of the climatic and anthropogenic variables in the *disease pyramid* context. I contributed to this chapter in all the steps of the work. I was present in the field work in 2018 to collect the samples during the three sampling seasons, I extracted the samples from 2018 for microbiome sequencing, sequenced the samples from 2016, 2017 and 2018, pre-processed them in the microbial analysis pipeline, carried out the statistical analysis, wrote the first version of the manuscript and added the changes and comments suggested.

Chapter 4 - “Interactions between *Batrachochytrium dendrobatidis* and amphibian skin microbiome in montane common midwife toads” - focuses on the bacterial and microeukaryotic communities on the skin of *A. obstetricans* in (1) populations with and without Bd, (2) epizootic and enzootic populations, (3) as well as infected and uninfected individuals from Bd-positive sites. First, I analyzed if Bd presence in a lake would be correlated to changes in the skin microbiome of tadpoles, both in composition and diversity. I also analyzed if in enzootic and epizootic populations, the skin microbiome (both bacteria and microeukaryotes) would change over time, during the time period of 3 years (2016, 2017, 2018), which could indicate a long-term alteration of the microbiome related to the infection dynamic on the population. And lastly, I examined, in

infected populations, the skin microbiome at the individual level, considering infected and uninfected tadpoles and their bacterial and micro eukaryotic communities composition as well as diversity. My contribution to this chapter was similar to that of chapter 3 (it is the same dataset). I was part of the three sampling campaigns in 2018, extracted the samples for microbiome analysis for the additional micro eukaryotic dataset, performed Q-PCR analysis for Bd detection (which included 30 samples/ lake/ sampling time to confirm the lake infection status into Bd-positive and Bd-negative), carried out the statistics analysis - followed by frequent discussions with my supervisors - wrote the first version of the manuscript and incorporated the changes suggested. The samples from 2016 and 2017 were the same samples as the ones in chapter 3, but they were processed and analyzed for micro eukaryotes as well. The chapter was also significantly improved after corrections made by the supervisors, but it is also waiting for further alterations and comments from the other co-authors before submission.

In chapter 5, first I draw general conclusions about the climatic and anthropogenic impacts (chapter 3) on the bacterial skin microbiome of the three sampled species in the Pyrenees. Later on, I add the pathogenic factor in the framework, taking into account both the individual severity of infection (based on Bd load) and the infection dynamic of each population (epizootic and enzootic) on the bacterial and the micro eukaryotic skin communities (chapter 4). As described in chapter 2, I place my conclusions in the context of the disease pyramid and discuss how the host-host microbiome-environment-pathogen interactions occur considering the ongoing environmental changes and amphibian conservation threats posed by Bd.

Chapter 2: Environmental Factors and Host Microbiomes Shape Host – Pathogen Dynamics

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Keywords: parasite, fungi, Anthropocene, disease triangle, habitat degradation, disease pyramid

Abstract

Microorganisms are increasingly recognized as ecosystem-relevant components because they affect population dynamics of hosts. Functioning at the interface of the host and pathogen, skin and gut microbiomes are vital components of immunity. Recent work reveals a strong influence of biotic and abiotic environmental factors (including the environmental microbiome) on disease dynamics, yet the importance of the host-host microbiome-pathogen-environment interaction has been poorly reflected in theory. We use amphibians and the disease chytridiomycosis caused by the fungal pathogen *Batrachochytrium dendrobatidis* to show how interactions between host, host microbiome, pathogen and the environment all affect disease outcome. Our review

provides new perspectives that improve our understanding of disease dynamics and ecology by incorporating environmental factors and microbiomes into disease theory.

From Disease Triangle to Disease Pyramid

Directly transmissible and vector-borne pathogens, which are affected by global change, are increasingly seen as risks to humans and wildlife (Altizer et al., 2013; Cavicchioli et al., 2019). Despite considerable research effort, human malaria continues to be a major health issue for millions of people and predicting future range expansion with expected climate changes has proved problematic; yet, for other diseases, predictions of how climate changes may alter dynamics is improving (Altizer et al., 2013; Cavicchioli et al., 2019). For example, the tiger mosquito *Aedes albopictus*, transmitting the Chikungunya viral disease, is currently expanding its geographic range across Europe and the Americas, putting millions of humans at risk. In livestock, the vector-borne bluetongue disease has emerged in northern Europe in response to climate change, and is causing the death of millions of animals at massive financial costs (Jones et al., 2019).

A clear indication that host-pathogen interactions are responding to global change is supported by the global increase of **emerging and re-emerging infectious diseases** (see Glossary) of human, wildlife and plant hosts posing threats to biodiversity and public health (Fisher et al., 2012). Impacts of the environment on host-pathogen interactions can be subtle and can vary across years. In 2010, a thermal anomaly occurred in Curaçao, causing a dramatic increase in white plague disease and ciliate infection in Caribbean coral *Diploria labyrinthiformis* (Altizer et al., 2013). In a vastly different system, Clare and colleagues demonstrated that seasonality, in particular the timing of spring ice-thaw of a Pyrenean mountain lake, affects the susceptibility of host amphibians to infection by the emerging pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) (Clare et al., 2016). These examples illustrate how disease risk and dynamics may result from (at least) three factors: the host, the pathogen and the environment.

In disease ecological theory, a 'disease triangle' is a concept that illustrates the outcome of the dynamic interactions consisting of hosts, pathogens, and the environment (Stevens, 1960) (see also (Snieszko, 1974)) (Section 1). It was formalized in plant

pathology because plants cannot move to escape unfavorable environmental conditions. We believe the concept is useful to better understand disease dynamics in human and wildlife diseases in a rapidly changing world. It considers the environment in its large sense, including abiotic conditions (e.g. temperature, moisture, pH, soil composition, solar radiation, seasonality, water chemistry), as well as biotic conditions (e.g. social environment, community composition, sex ratio, vegetation cover, abundance and distribution of vectors and intermediate hosts).

Section 1: The Disease Triangle and Former Disease Concepts

The disease triangle concept was developed by Stevens (1960) in the context of plant pathology. Disease is the outcome of a dynamic interaction between the host, the pathogen and the environment, and is illustrated using an equilateral triangle (Figure 9) where the gradient of host susceptibility (resistance and tolerance), pathogen pathogenicity (infectivity and virulence), and environmental conditions affect the disease outcome at the center of the triangle. The effect of disease is most intense at the center, where susceptibility, pathogenicity, and environmental conditions favor the pathogen over the host. Disease outcome can vary dramatically if environmental conditions, host resistance or tolerance, or the pathogen pathogenicity change. Thus, a potentially infectious microorganism does not always invade a host, and a 'pathogen' can be neutral or even a beneficial mutualist under other conditions. The disease triangle is quite similar to the concept developed by Snieszko for fish diseases, in which infectious disease occurs when a susceptible host is exposed to a virulent pathogen under proper environmental conditions (Snieszko, 1974) (Figure 9). While the environmental microbiome is embedded in the 'environment' component of these models, they lack an important component of disease dynamics: the host microbiome. Recently, the importance of microorganisms in shaping disease dynamics gained more attention and has been reflected in new theoretical models. Brucker and colleagues proposed to illustrate the interactions between the host, the host microbiome and the pathogen with a triangle of interactions (response or causation) (Brucker et al., 2008) (Figure 9). In this model, the environment experienced by the host, the pathogen and the host microbiome was not considered. Finally, the concept by Snieszko (1974) between the host, the pathogen and the environment was extended to include the microbial community of the pathogen, as the pathobiome concept arose (Vayssier-Taussat et al., 2014) (Figure 9). However, in this

model, a clear distinction between the host microbiome, the pathogen microbiome, and the environmental microbiome was not made. Interestingly, in this model, the theories applying to hosts can also be applied to vectors of diseases, e.g. mosquitos. In this case, the disease is the consequence of an interaction between the vector, the pathogen, microbiome and the environment (Figure 9).

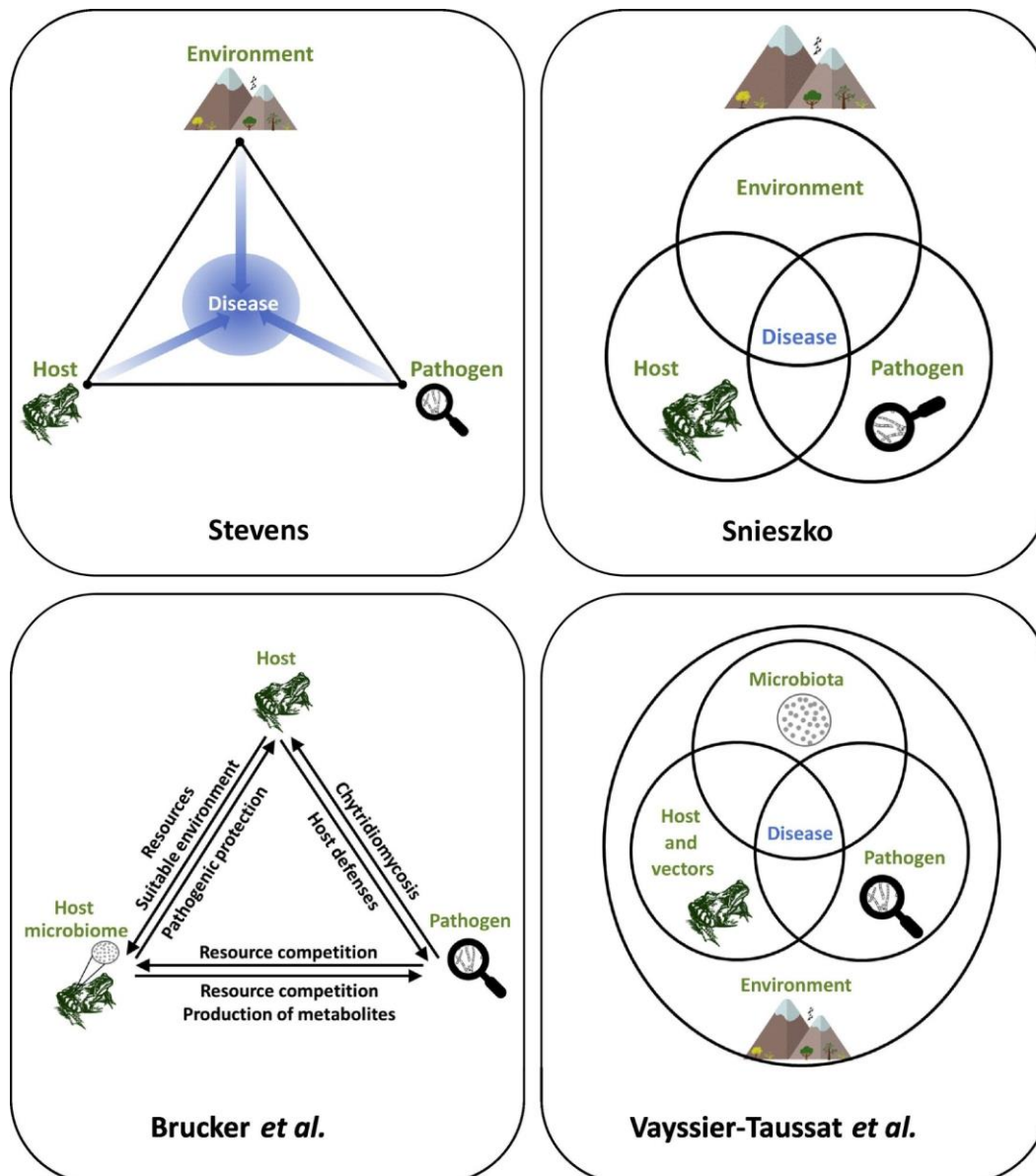


Figure 9. Illustration of Former Models.

In spite of its explanatory power, the disease triangle represents an oversimplified view of the dynamics of infectious diseases; and thus, we must broaden the original concept to include an additional and often neglected aspect of the complex system: the diverse **microbiomes** of the environment and the host. The advent of next generation

sequencing has provided cost-effective ways to describe the highly diverse world of **microorganisms**. We are only beginning to appreciate the distribution, abundance, and diversity of microbial communities, the full extent of which, however, remains highly controversial (Louca et al., 2019). The total number of bacteria and archaea are estimated as $\sim 10^{30}$, the atmosphere contains $\sim 10^{22}$ microbial cells, and terrestrial and marine environments each comprise $\sim 10^{29}$ microorganisms (Cavicchioli et al., 2019). A human body harbors at least 10^{14} microbial cells and 10^{15} viruses (Clemente et al., 2012), with the human gut microbiome alone containing ~ 1000 bacterial species, but only 150 to 170 predominating in any one person (Qin et al., 2010). New data are helping to describe their functional and ecological capacities, as well as the nature of their physiological interactions with hosts (Clemente et al., 2012; Cavicchioli et al., 2019) (Figure 10).







Host	<p style="text-align: center;">Adaptation to the environment</p>  <ul style="list-style-type: none"> • Temperature tolerance • Short time adaptation to environmental changes 	<p style="text-align: center;">Host health</p>  <ul style="list-style-type: none"> • Biological barrier, part of the immune system • Competition with pathogens • Disease mitigation 	<p style="text-align: center;">Physiological functions</p>  <ul style="list-style-type: none"> • Synthesis of vital nutrients • Energy uptake and growth • Influence on host behavior and reproduction
Ecosystem	<p style="text-align: center;">Ecosystem stability</p>  <ul style="list-style-type: none"> • Buffering against change • Maintenance of biodiversity • Maintenance of ecosystem processes 	<p style="text-align: center;">Ecosystem health</p>  <ul style="list-style-type: none"> • Environmental barrier against alien species • Competition with opportunists • Resistance, resilience or tolerance 	<p style="text-align: center;">Ecological functions</p>  <ul style="list-style-type: none"> • Nutrient cycling • Energy flux • CO₂ sequestration • Nitrogen retention

Figure 10. Importance of the Microbiome to Hosts and Ecosystems. The host microbiome allows the host to adjust to its environment, provides protection against pathogens, and contributes to physiological functions (nutrition, growth and reproduction). Similarly, the environmental microbiome promotes ecosystem stability and the maintenance of biodiversity by preserving ecosystem health and contributing to important ecological functions (e.g. nutrient cycling, energy uptake, carbon sequestration and nitrogen retention). Host and environmental microbiomes are interconnected and regularly exchange microorganisms.

Microbiota and microbiome research have identified microbial communities as important contributors to ecosystem, wildlife and human health (Clemente et al., 2012; Nicholson et al., 2012; Cavicchioli et al., 2019). We now understand that microbial communities maintain ecosystem health by influencing the global food web, including agriculture, nutrient cycling, nitrogen retention and CO₂ sequestration (Cavicchioli et al., 2019)(Figure 10). Further, by maintaining ecosystem health, they also contribute to plant, wildlife and human health. Host microbial communities (i.e. **host microbiome**) can be considered part of the extended phenotype of host defenses. The gut microbiome is involved in energy harvest and storage, brain functioning, development of the intestine

and immune system and susceptibility to disease (Clemente et al., 2012; Nicholson et al., 2012). Not only does the gut microbiome act as a barrier to infection by defending the host against colonization by pathogens, it also interacts with the host immune system, providing signals to promote maturation of immune cells (Clemente et al., 2012). The properties of the host microbiome have led to the concept of **holobiont** in which the host and its microbial partners are merged into a symbiotic superorganism (Margulis, 1993), and later to the concept of **pathobiome** to further consider microbiome communities in disease dynamics (Vayssier-Taussat et al., 2014; Bass et al., 2019).

In amphibians, as well as in other vertebrates, the skin microbiome is an integral component of the immune system, acting as a barrier to infection (Section 2). For example, the amphibian chytridiomycosis, a disease caused by the chytrid fungi *Batrachochytrium dendrobatidis* (hereafter *Bd*) and *Batrachochytrium salamandrivorans*, is not simply occurring when the pathogen is present and infects the skin of susceptible species but depends also on the composition of skin microbial communities (Brucker et al., 2008; Kueneman et al., 2014; Jiménez and Sommer, 2017). Generally, a better understanding of microbiomes in disease dynamics and their appreciation in disease ecology will contribute to disease mitigation.

Section 2. The Amphibian Immune System

The immune system of amphibians is similar to other vertebrates (Rollins-Smith and Woodhams, 2012) and is complex. It includes three components: barriers (constitutive defenses), innate response and acquired (adaptive) response (Figure 11), whose functions partly overlap.

The first line of defense consists of (a) a physical barrier comprised of an epithelium, such as skin or gut surface, covered by a mucus (a matrix of mucopolysaccharides secreted by the mucous glands and complemented by antimicrobial peptides, AMPs, secreted by the granular glands), (b) a biological barrier (i.e. skin and gut resident microbiomes), and (c) a chemical barrier (e.g. the acidic pH of the gut, AMPs and lysozyme in the mucus, and antimicrobial metabolites produced by the resident microbiome). Amphibians use their skin to breathe (many species lack lungs) and to maintain homeostasis. Electrolytes and H₂O, O₂ and CO₂, both actively and passively cross the amphibian epithelium surface and the mucus that act together as a semi-porous physical barrier. This increases the

importance of its chemical and biological components when acting as a barrier against pathogens. Resident microbiomes, naturally living on the host epithelium, compete for space and resources with pathogens, presumably preventing them from adhering to the skin, proliferating and entering the host (Rollins-Smith et al., 2012). Host microbiomes actively secrete antimicrobial metabolites, contributing to the chemical barrier that the host creates.

Pathogens that cross those barriers face the innate and adaptive immune response. The innate system includes lysozyme, complement lytic system, and AMPs. It also involves natural killer cells (non-specifically attack and lyse infected cells), and dendritic and macrophage cells (phagocyte infected cells, produce cytokines, and activate B and T lymphocytes) (Rollins-Smith et al., 2012).

The most advanced response is the adaptive (acquired) response, preventing re-infection. After first infection, this system provides a fast secondary response during subsequent exposure. When stimulated by an antigen, B lymphocytes produce antibodies which neutralize pathogens by agglutination, inducing complement activation, and tagging antigen for destruction by phagocytes. T lymphocytes need direct contact with an infected cell to eliminate it. They do not produce antibodies but regulate their production (Rollins-Smith et al., 2012). Several kinds of T lymphocytes exist, including regulatory T cells, T helper cells (secrete cytokines and coordinate antibody production) and cytotoxic T cells (destroy pathogenic cells by physical and chemical lysis). However, some pathogens, including *Bd*, can suppress the adaptive response (Fites et al., 2013; McMahon et al., 2014).

In larval amphibians (e.g. tadpole stage), the immune system is not as developed as it is in fully metamorphosed individuals, but still competent (Rollins-Smith and Woodhams, 2012).

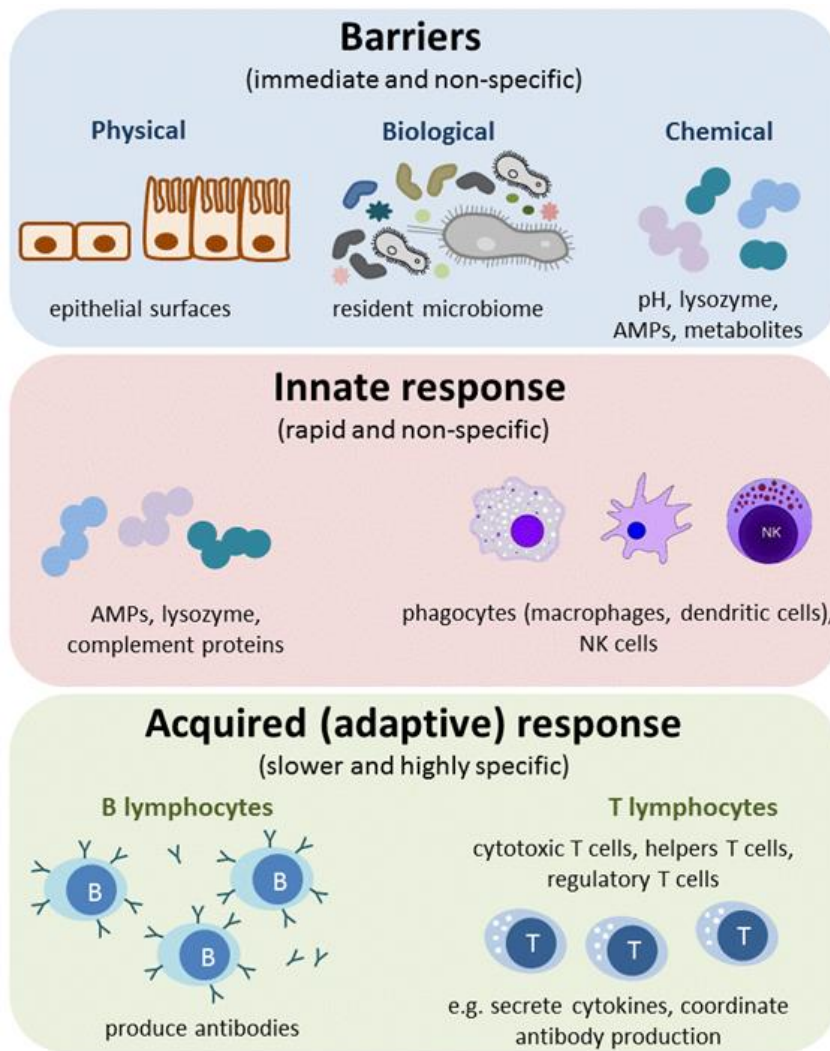


Figure 11. The Three Components of the Amphibian and Vertebrate Immune System - Barriers, Innate Response and Acquired (Adaptive) Response.

Here, we review and illustrate the dynamic interaction between the host, the host microbiome, the *Bd* pathogen, and the environment (including the environmental microbiome) (Figure 12, Key Figure) using the well-studied case of the disease chytridiomycosis caused by *Bd*. This four-way interaction highlights the need to adopt a holistic approach to understand disease dynamics and ecology. Only when considering microbiomes in such a holistic approach, will we be able to evaluate the impact of a changing environment on ecosystem, wildlife and human health and overcome limitations of current concepts.

Variety and Roles of Microorganisms

In 1546, Fracastorius suggested that infection is contagious and transmitted by 'particules'. Microscopic organisms were discovered a century later, by Hooke and van Leeuwenhoek who described respectively the microfungus *Mucor*, and 'animalcules' (i.e. protozoa and bacteria). In 1857, Pasteur postulated that infectious diseases are caused by a variety of 'germs' that are everywhere, even in the air. Microorganisms can be found in all habitats (soil, water, air), in the intestines of hosts, as well as on the exterior of organisms (Finlay and Esteban, 2001). Host-associated microorganisms can be pathogenic, but also can be commensal or beneficial symbionts.

Microorganisms play important ecological roles. Bacteria help controlling host populations as they can be pathogenic, benign, or beneficial to hosts (e.g. helping in the digestion of food and the protection of hosts against infections) (Grice and Segre, 2011). Archaea provide major pathways for ammonia oxidation in the environment, in methanogenesis, in sulphur oxidation and in nitrification (Jarrell et al., 2011). Many fungi are also microscopic and are important in nutrient cycling, decomposition and growth control of other organisms (Frenken et al., 2017; van der Linde et al., 2018). Parasitic and pathogenic fungi cause mycoses, which are increasingly seen as threats to biodiversity (Fisher et al., 2012).

The community of microorganisms is usually described by marker genes and in rarer cases their full genome, i.e. the microbiome, a term usually used when they are associated with a host individual or species (Marchesi and Ravel, 2015). The microbiome composition may vary between species, body areas and geographic regions. The microbiome performs some main functions, such as disease mitigation (Brucker et al., 2008; Kueneman et al., 2014; Jiménez and Sommer, 2017), digestion, and has an influence on host behavior, development and reproduction (Heijtz et al., 2011; Colombo et al., 2015; Waite and Taylor, 2015) (Figure 10). The microbial taxa shared between the majority of individuals within an animal species is called core microbiome, and it is probably the main responsible component for performing these essential functions (Turnbaugh and Gordon, 2009). Perturbations in the microbiome composition and function (known as dysbiosis) could interrupt its regular functions and are suggested as a cause for some diseases (Bass et al., 2019).

The Model System: Amphibian hosts and *Bd*

In a world where a quarter of all species are threatened with extinction (IPBES, 2019), amphibians are the most threatened vertebrates, with rapid population declines detected in 43% of the >8100 amphibians species described (<https://amphibiaweb.org>) (Stuart et al., 2004; Fisher et al., 2012). Loss of amphibians has cascading effects throughout food webs and may alter environmental balance (e.g. water quality and pest control) (DuRant and Hopkins, 2008; Whiles et al., 2013). In some systems (e.g. North American arboreal forests) amphibians are the most abundant terrestrial vertebrates and they facilitate important carbon cycle functions such as leaf litter retention and carbon capture (Best and Welsh, 2014), functions that are tied to global cycles.

Together with habitat loss and overexploitation, emerging infectious diseases are the major threats to amphibians. The fungus *Bd* is considered the most destructive pathogen for wildlife, implicated in the decline of > 500 frog species worldwide (Scheele et al., 2019), and the extirpation of populations and entire species (Fisher et al., 2009). *Bd* pathogenicity is based on its ability to infect a vital organ, amphibian skin, which is permeable and maintains homeostasis. *Bd* disrupts these fundamental functions, ultimately resulting in cardiac arrest (Voyles et al., 2011). It infects hosts as a motile zoospore form in water and colonizes a keratinized or keratinizing part of the host body (Berger et al., 2005). In the host skin, *Bd* zoosporangia produce new zoospores that are released in the environment and can either re-infect the same host or infect a new host. Many susceptible amphibians are infected by *Bd* at the host larval stage and die during host metamorphosis, when host immunity is suppressed and keratinized skin cells spread over the body. *Bd* can establish itself in various environments and has been detected on all the continents that amphibians are known to occupy (Fisher et al., 2009). Moreover, amphibians being ectotherms, their physiology, including their immunity, is environment-dependent. Finally, the amphibian skin microbiome has attracted considerable attention compared to other wildlife microbiomes, and therefore a large body of literature is available on the interactions between amphibian hosts, the pathogen *Bd*, skin microbiome and the environment.

Amphibian Microbiome

In amphibians, external (skin) and internal (gut) microbiomes have been described. The amphibian gut microbiome includes protists and bacteria which stimulate growth (Pryor and Bjorndal, 2005). The amphibian skin microbiome is composed of different groups, such as bacteria, fungi and other micro-eukaryotes (Kearns et al., 2017; Kueneman et al., 2017). The origin of the amphibian gut and skin microbiome is not yet clear. There is some evidence that the amphibian microbiomes may result from both species-specific self-acquisition of microbes in the habitat, as well as social transmission, followed by selection of rare environmental microbiome taxa (Loudon et al., 2014; Walke et al., 2014; Jiménez and Sommer, 2017; Abarca et al., 2018; Estrada et al., 2019) (Table 1). Transmission of microorganisms, in this case bacteria, between individual hosts can be vertical or horizontal (for fungi and other micro-eukaryotes the process is still unknown). Vertical transmission occurs especially in species with parental care, when part of their microbiome is transferred by hosts to eggs and protects them (Walke et al., 2011). Horizontal transmission is likely to occur during mating and during congregations of conspecifics or other species (Bletz et al., 2013). The environmental microbiome also contributes to the host microbiome. Individuals kept in enclosed habitats are exposed to a poorer environment compared to specimens that live in natural habitats, translating in poorer skin microbiomes (Becker et al., 2014; Sabino-Pinto et al., 2016) (Table 1). Salamander larvae experimentally transferred from stream to pond had a modified skin microbial community, likely due to microbes gathering in their habitat (Bletz et al., 2016). In addition to environmental impacts on the microbiome, species-specificity of microbiomes has also been described (McKenzie et al., 2012; Kueneman et al., 2014; Belden et al., 2015).

Gut and skin microbiomes can be influenced by many host factors, including genetics, sex, behavior and diet (Antwis et al., 2014; Belden et al., 2015; Jiménez and Sommer, 2017) (Table 1). Captive individuals of the Red-eyed tree frog (*Agalychnis callidryas*) that received a diet rich in carotenoid have greater species richness and abundance of skin bacteria compared to those with a carotenoid-free diet (Rebollar et al., 2016a). Host health status has been shown to influence host microbiome, which in turn can influence disease dynamics (Antwis et al., 2014; Jani and Briggs, 2018). Composition of the skin microbiome also varies with life stage: micro-eukaryotes are significantly

more diverse and abundant in adults and sub-adults as compared to metamorphs and tadpoles, with a dominance marked by fungal taxa (Kueneman et al., 2016b; Prest et al., 2018) (Table 1). During metamorphosis, amphibians undergo many changes: body shape, diet, habitat (from aquatic to more terrestrial) and skin cell composition. During that period, their immune system is suppressed, which apparently creates open niches, inducing a higher bacterial diversity (Kueneman et al., 2016b) and facilitating the proliferation of, and infection by, opportunistic pathogens.

Table 1: Overview of Recent Studies Focusing on Amphibian Skin Microbiome and Host-, Pathogen (*Bd*)-, Environment-Related Variables.

Host species	Sampling site	Molecular marker	Target microbial group	Interaction	Main findings	Ref.
Host-Microbiome						
<i>Anaxyrus boreas</i>	Colorado, USA	V4 region 16S rRNA gene	bacteria	life stages	skin communities changed across life stages	Prest et al., 2018
<i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i>	Panama	V4 region 16S rRNA gene	bacteria	year, date of sampling	skin communities differed across years on both species. Differences in relative abundance of key OTUs explained by rainfall	Estrada et al., 2019
Host-Microbiome-Environment						
Acquisition/maintenance of microbiome						

<i>Cymops pyrrhogaster</i>	breeding facility and pond in Japan	V4 region 16S rRNA gene	bacteria	wild/captive conditions	wild individuals had more diverse communities, but similar OTUs richness	Sabino-Pinto et al., 2016
<i>Atelopus zeteki</i>	Panama	V4 region 16S rRNA gene	bacteria	wild/captive conditions	wild population had 3 times more unique OTUs	Becker et al., 2014
<i>Plethodon cirineus</i>	controlled environment	V4 region 16S rRNA gene	bacteria	environmental soil vs. sterile media	diversity across treatments and decreased in the sterile media	Loudon et al., 2014
<i>Plethodon cirineus</i>	controlled environment	V4 region 16S rRNA gene	bacteria	cage with or without bacteria reservoir	bacteria related to antifungal isolates were more likely to persist on salamanders, regardless the environment	Loudon et al., 2016
<i>Plethodon jordani</i>	North Carolina, USA	V4 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	OTUs highly associated to salamanders tended to be absent/too rare in the environment	Fitzpatrick and Allison, 2014
<i>Rana catesbeiana</i> , <i>Notophthalmus viridescens</i>	Virginia, USA	V2 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	relative abundance of OTUs shared by amphibians and environment was inversely related	Walke et al., 2014

<i>Atelopus certus</i> , <i>Craugastor fitzingeri</i> , <i>Colostethus panamansis</i> , <i>Espadarana prosoblepon</i> , <i>Strabomantis</i> <i>bufoniformis</i>	Serrania del Sapo, Panama	V4 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	microbiome communities were enriched with rare environmental OTUs, and high percentage of OTUs shared between frogs and habitat	Rebollar et al., 2016b
<i>Cryptobranchus</i> <i>alleganiensis</i> <i>alleganiensis</i>	Indiana, West Virgiana, North California, Tennessee and Georgia, USA	V2 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	variation in community diversity among populations and in proportion of shared OTUs between animals and river	Hernández- Gómez et al., 2017
89 species of frogs	Madagascar	V4 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	host microbial communities were different and less diverse than environmental ones	Bletz et al., 2017
<i>Rana marina</i>	Puerto Rico and Costa Rica	V4 region 16S rRNA gene	bacteria	geographic location	significant environmental influence in composition, richness and abundance of microbiome taxa	Abarca et al., 2018
genera <i>Ensatina</i> and <i>Batrachoseps</i>	California, USA	V3-V4 region 16S rRNA gene	bacteria	geographic location	strong site differences in bacterial communities	Bird et al., 2018
<i>Rana pipiens</i> , <i>Pseudacris</i> <i>triseriata</i> , <i>Ambystoma</i> <i>tigrinum</i>	Colorado, USA	V2 region 16S rRNA gene	bacteria	altitudinal gradient	host species community similarity	McKenzie et al., 2012

<i>Eleutherodactylus coqui</i>	Puerto Rico	V4 region 16S rRNA gene	bacteria	altitudinal gradient, intact/disturbed forest	diversity changed with site, elevation and land use	Hughey et al., 2017
<i>Lissotriton boscai</i> , <i>Telmatobius marmoratus</i>	Galicia, Spain	V4 region 16S rRNA gene	bacteria	life stage, aquatic/terrestrial environment	terrestrial adults had more diverse and richer bacterial communities	Sabino-Pinto et al., 2017
<i>Anaxyrus boreas</i> , <i>Pseudacris regilla</i> , <i>Taricha torosa</i> , <i>Rana catesbeianus</i>	California, USA	V4 region 16S rRNA gene	bacteria	species, geographic location	amphibian skin identity was the strongest predictor of microbiome composition	Kueneman et al., 2014
<i>Plethodon glutinosus</i> , <i>P. cinereus</i> , <i>P. cylindraceus</i>	Central Appalachians US	V3-V5 region 16S rRNA gene	bacteria	species, altitudinal gradient	diversity changed with elevation and co-occurring salamanders had similar microbiome structure	Muletz Wolz et al., 2018
<i>Ensatina eschscholtzii xanthoptica</i>	San Francisco and Sierra Nevada, USA	V3-V4 region 16S rRNA gene	bacteria	life stage, sex, geographic location	isolated populations had similar communities, which were significantly different from the environment	Prado-Irwin et al., 2017
Possible environmental stressors						
<i>Pelophylax perezi</i>	Portugal	nearly full length of the 16S rRNA gene	bacteria	metal contamination, salinity	low diversity and density on individuals from metal contaminated population	Costa et al., 2016

<i>Pseudacris crucifer</i>	controlled environment	V4 region 16S rRNA gene	bacteria	coal combustion waste	little impact from acute exposure to fly ash on the bacterial communities	Hughey et al., 2016
205 amphibian species	13 countries	V4 region 16S rRNA gene	bacteria	thermal stability, habitat class (aquatic, terrestrial, arboreal), elevation	bacterial richness decreased in warmer and more stable environments, and in arboreal hosts	Kueneman et al., 2019
<i>Atelopus zeteki</i>	controlled environment	V4 region 16S rRNA gene	bacteria	microbiome, <i>Bd</i>	survival to <i>Bd</i> infection was related to initial composition of the skin bacterial community	Becker et al., 2015
12 amphibian species	Costa Rica	nearly full length of the 16S rRNA gene	bacteria	microbiome, <i>Bd</i>	11% of the bacterial isolates collected from the species exhibited <i>Bd</i> inhibition and 2,2% enhanced <i>Bd</i> growth	Madison et al., 2017
<i>Dendrobates sp.</i>	aquarium and animal care facility, USA	V4 region 16S rRNA gene + ITS	bacteria and fungi	<i>Bd</i> inhibition and enhancement	abundance of cutaneous fungi contributed more to <i>Bd</i> defense than bacteria; different	Kearns et al., 2017
<i>Bombina orientalis</i>	controlled environment	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria and micro-eukaryotes	life stage, microbiome, <i>Bd</i>	major change in community until 15 days after metamorphosis; richness diverged between aquatic and terrestrial stages	Bataille et al., 2018

<i>Anaxyrus boreas</i>	Colorado, USA	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria	life stage, microbiome, <i>Bd</i>	life stage had the largest effect on microbiome; diversity of micro eukaryotes was lowest in tadpoles	Kueneman et al., 2016
<i>Rana sierra</i>	California, USA	V3-V4 region 16S rRNA gene	bacteria	life stage, microbiome, <i>Bd</i>	skin microbiome of highly infected juveniles had reduced richness and lower variation between individuals	Ellison et al., 2019
<i>Rana cascadae</i>	Northern California, USA	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria and micro-eukaryotes	life stage, microbiome, <i>Bd</i>	<i>Bd</i> was significantly lower on tadpoles and highest on subadults	Kueneman et al., 2017
<i>Lithobates yavapaiensis</i> , <i>Eleutherodactylus coqui</i>	Arizona, USA and Puerto Rico	V4 region 16S rRNA	bacteria	life stage, season, <i>Bd</i>	winter-sampled individuals exhibited higher diversity; hosts with higher bacterial diversity carried lower <i>Bd</i> loads	Longo et al., 2015
<i>Craugastor fitzingeri</i> , <i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i>	Panama	V4 region 16S rRNA gene	bacteria	different species, <i>Bd</i>	treefrogs had a significantly higher number of culturable <i>Bd</i> -inhibitory OTUs than terrestrial species	Rebollar et al., 2019

<i>Rana catesbeiana</i> , <i>Notophthalmus viridescens</i> , <i>Pseudacris crucifer</i> , <i>Anaxyrus americanus</i>	Virginia, USA	V4 region 16S rRNA gene	bacteria	different species, <i>Bd</i>	dominant bacteria had higher <i>Bd</i> inhibition in bullfrog and newt. Dominant and rare bacteria did not differ in inhibition in spring peeper and toad, in which <i>Bd</i> was lower	Walke et al., 2017
<i>Rana sierra</i>	California, USA	V1-V2 region 16S rRNA gene	bacteria	resistant/non-resistant population to <i>Bd</i> , <i>Bd</i>	different bacteria richness between resistant/non-resistant populations	Jani et al., 2017
<i>Rana sierra</i>	controlled environment	V1-V2 region 16S rRNA gene	bacteria	resistant/non-resistant population to <i>Bd</i> , <i>Bd</i>	frogs housed in water from resistant populations had greater bacterial richness than those housed in non-resistant population water	Jani and Briggs, 2018
<i>Rana sierra</i>	California, USA	V1-V2 region 16S rRNA gene	bacteria	<i>Bd</i> epizootic/enzootic	100% mortality of post metamorphic frogs during <i>Bd</i> epizootic; several bacteria taxa showed the same response to <i>Bd</i> across multiple field populations	Walke et al., 2015
<i>Rana catesbeiana</i>	controlled environment	V4 region 16S rRNA gene	bacteria	before/after exposure to <i>Bd</i>	microbial community of frogs prior to <i>Bd</i> exposure influenced infection intensity	Kueneman et al., 2016a
<i>Anaxyrus boreas</i>	controlled environment	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria and micro eukaryotes	four probiotic treatments, <i>Bd</i>	amphibians in captivity lost the <i>Bd</i> -inhibitory bacteria; inoculations of the <i>Bd</i> -inhibitory probiotic increased survival	Bataille et al., 2016

Host-Microbiome-Pathogen-Environment						
<i>Bombina orientalis</i>	South Korea	V3 region 16S rRNA gene	bacteria	wild/captive conditions, <i>Bd</i>	<i>Bd</i> infection intensity was correlated neither with richness nor diversity indices; diversity was greater, and microbiome structure more complex in wild toads	Medina et al., 2019
<i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i> , <i>Craugastor fitzingeri</i>	Panama and USA	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , geographic location	no clustering of OTUs based on <i>Bd</i> infection status	Belden et al., 2015
<i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i> , <i>Silverstoneia flotator</i> , <i>Craugastor fitzingeri</i> , <i>Rana catesbeiana</i> , <i>Pseudacris crucifer</i> , <i>Notophthalmus viridescens</i> , <i>Anaxyrus americanus</i>	Panama and USA	16S rRNA gene database and ITS1	bacteria and fungi	species, <i>Bd</i> , geographic location	the host species was more important in determining microbiome composition than geographic location or <i>Bd</i> load	Rebollar et al., 2018
<i>Silverstoneia flotator</i>	Panama	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , elevational gradient	similar skin communities across elevations; richness varied with <i>Bd</i> presence; severe outbreaks occurred at high elevation	Medina et al., 2017

<i>Rana sphenocephala</i>	controlled environment	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , controlled mesocosm	efforts to maintain a normal skin community using semi-natural mesocosms failed to provide long term protection	Holden et al., 2015
<i>Bolitoglossa</i> (3 spp.), <i>Pseudoeurycea</i> (3 spp.), <i>Plectrohyla</i> (4 spp.)	Mexico and Guatemala	V3-V4 region 16S rRNA	bacteria	<i>Bd</i> , forest type	phylogeny influence of diversity and structure of microbiome at higher taxonomic levels; the habitat predominated on lower scales	Ellison et al., 2019b
<i>Dendropsophus. minutus</i>	São Paulo and Rio Grande do Sul, Brazil	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , land cover, forest connectivity	bacterial diversity and <i>Bd</i> loads increased towards natural vegetation	Becker et al., 2017
<i>Ichthyosaura alpestris</i> , <i>Lissotriton vulgaris</i> , <i>Triturus cristatus</i>	Kleiwiesen and Elm, Germany	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , water temperature	skin microbe fluctuations not correlated with fluctuations of pond microbiota; significant correlation between water temperature and newt bacterial community structure	Bletz et al., 2017b
<i>Acris blanchardi</i>	Ohio and Michigan, USA	V3 region 16S rRNA gene	bacteria	<i>Bd</i> , pH, CaCO ³ , conductivity, natural/managed habitat	microbiome composition associated with water conductivity, ratio of natural to managed land, and latitude	Krynak et al., 2016
<i>Craugastor fitzingeri</i>	Panama	metagenome	bacteria	<i>Bd</i> positive and negative sites	bacterial communities in positive sites were less diverse than in negative ones	Rebollar et al., 2018

Environmental Impacts on Amphibian Immunity

In amphibians, as in all vertebrates, the first protection against pathogens is the physical, chemical and biological barriers of the epithelium (Section 2). Amphibians have a permeable skin that allows exchange of gases, water and electrolytes. The resident microbiome present on the skin is a dynamic community that competes with exogenous microorganisms for space and resources, and can prevent pathogens from adhering to the skin (Section 2). The host microbiome can also actively defend the host by producing antimicrobial peptides or metabolites. For example, *Janthinobacterium* spp., a symbiotic skin bacterium, produces the anti-fungal metabolite violacein (Brucker et al., 2008). Violacein was isolated from amphibian skin and transferred to the *Bd*-susceptible Mountain yellow-legged frog (*Rana muscosa*), resulting in a successful bio-augmentation that increased host survival (Harris et al., 2009).

Many factors are known to affect amphibian immunity and thus host-pathogen interactions (e.g. Table 1). Temperature is likely the most important environmental factor that affects microbiomes. Since amphibians are ectotherms, all of their physiological responses are temperature-dependent, including immunity. Several studies have found that *Bd* infection probability and impacts of chytridiomycosis are higher at lower temperatures (e.g. (Berger et al., 2005; Walker et al., 2010; Forrest and Schlaepfer, 2011)). For that reason it could be suggested that climate change may be beneficial for hosts that suffer from *Bd* infections. However, the interaction between the host, the pathogen and the environment is complex. Climate change predictions suggest large increases in the variability of climatic conditions that may favor chytridiomycosis (Rohr and Raffel, 2010; Raffel et al., 2013). Controlled-temperature experiments on the Cuban treefrog (*Osteopilus septentrionalis*), revealed that the temperature-dependent growth of *Bd* on hosts was greater at warmer temperatures than the pattern of *Bd* growth in culture, emphasizing the importance of accounting for the host-pathogen interaction when predicting climate-dependent disease dynamics (Raffel et al., 2013). Another striking result was the demonstration that global warming is predicted to cause an increase in susceptibility to *Bd* in some amphibian species due to earlier ice-thaw of montane lakes in spring (Clare et al., 2016).

Besides temperature, additional environmental parameters that may alter amphibian immune responses include pH and chemicals in the environment. Studies have also shown that the presence of heterospecifics, intra and inter-specific competition, predation, food availability, and pond drying, can all cause stress in hosts that may suppress immune response (Rollins-Smith and Woodhams, 2012). In the Pyrenees, presence of zooplankton in lake water also protects amphibians against *Bd*, since zooplankton predation diminishes the population of the infectious stage (zoospores) of the pathogenic fungus (Schmeller et al., 2014). The list of factors is not exhaustive, and we believe additional impacts of environmental conditions will be discovered as the impacts of human activities on the environment continue to increase.

Environmental Impacts on Host-Host Microbiome Interactions

The skin and gut microbiome can be seen as a miniature ecosystem at equilibrium. Just like any ecosystem, a stressor may alter the balance of the associated microbiome. As a response, the richness and composition of the microbiome may either resist, or shift to a new stage. It may stay permanently at this new equilibrium as an **acclimation response**, or it may exhibit resilience and return to the former equilibrium. From the perspective of the host, when environmental modifications occur, the microbiome may exhibit flexibility. This flexibility might increase the host adaptive capacity by providing the host with higher **phenotypic plasticity**, compared to the one acquired only through the host genome or through a static gut/skin microbiome (Feldhaar, 2011) (Figure 10). The bacteria *Serratia symbiotica*, for example, provides a reproductive advantage (higher fecundity) to the insect pea aphid after an event of heat stress, compared to control individuals (Russell and Moran, 2006). Hence, the environment has the potential to influence the outcome of host-microbiome interactions, because it can impact both the host and the host microbiome.

The composition, richness and diversity of host microbiome can be modified directly by biotic and abiotic environmental factors (Table 1). It can also be modified indirectly, through the host physiology, as the host-microbiome interactions create the environment in which the microbiome lives. A recent study analyzed samples from over 200 amphibian species (>2,300 individuals) across a broad biogeographic range to

investigate how climatic variables (temperature, precipitations and seasonality), elevation, latitude and microhabitat class relate to skin microbial communities (Kueneman et al., 2019). Cold winter temperatures and seasonality were the best predictors of richness and composition of skin bacterial communities at the global scale. Latitude (with a decreased richness at lower latitudes) was also important, likely due to an inter-correlation between latitude and low temperatures (Kueneman et al., 2019). Finally, skin bacterial richness was also influenced by microhabitat. Bacterial richness on amphibian hosts appeared higher in more seasonal environments, especially those with colder winters during which the amphibian immunity is suppressed for longer periods of time. Thus, there might be selection for low temperature resistance in the amphibian skin microbiome, counteracting low internal immunity of amphibians (Kueneman et al., 2019).

Additional environmental stressors of importance for the host-microbiome interaction include habitat degradation, ultraviolet radiation, environmental pollution, invasive species and climate change (Blaustein and Kiesecker, 2002; Blaustein et al., 2003; Beebee and Griffiths, 2005). In particular, climate change is predicted to impact many terrestrial and aquatic host species and this may allow pathogens to spread into new geographic areas under optimal temperatures (Epstein, 2001; Walther et al., 2002). Habitat degradation, such as deforestation and fragmentation, decreases biodiversity in macro-organisms, and this pattern extends to their associated microbiomes (Trevelline et al., 2019). Habitat degradation thus impairs vertical and horizontal transmission of microbiomes and induces lower microbiome skin diversity. Human activities can also modify the communities of environmental microorganisms (Trevelline et al., 2019). For example, livestock grazing provides copious amounts of livestock feces, which can enrich the environment for microorganisms, or, on the contrary, can deposit antibiotics and antifungal compounds that degrade the habitat for natural microorganisms. Finally, other pathogens in the environment may also change the outcome of host-microbiome-pathogen interactions through alteration of the host immunity or condition, or through pathogen-pathogen competition for hosts (Rollins-Smith and Woodhams, 2012).

Host-Host Microbiome-Pathogen Interactions

Microorganisms in the environment, including potentially pathogenic microorganisms, represent an important source of disturbance to the natural skin and gut microbiomes of hosts. These potential pathogenic microorganisms may also be favored or inhibited by the microbiome composition of the host and/or vector (Bahrndorff et al., 2016). For example, the susceptibility of the mosquito *Anopheles gambiae* to *Plasmodium falciparum* infection is inversely correlated to the abundance of the bacteria *Enterobacteriaceae* in their gut (Boissière et al., 2012). In the *Bd*-amphibian system, there is a significant relationship between *Bd* infection and the microbiome composition of amphibian skin (Becker et al., 2015; Madison et al., 2017; Ellison et al., 2019a) (Table 1). Hosts with greater microbiome diversity are more resistant to this invasive pathogen, while populations that coexist with *Bd* for a long time have a higher proportion of anti-*Bd* skin bacteria than populations which are declining due to the disease and never had contact with *Bd* before (Lam et al., 2010; Ellison et al., 2019a). This evidence confirms that pathogens can influence and be influenced by the amphibian skin microbial community.

Most research conducted so far on amphibian skin microbiome focused on bacteria (Table 1), even though skin microbial communities also comprise eukaryotic microorganisms such as protists and fungi (Kearns et al., 2017; Kueneman et al., 2017). Two recent studies suggest that fungi might be more efficient or have a higher competition capacity against other invasive fungi (Kueneman et al., 2016b; Kearns et al., 2017), opening new avenues on the role played by fungal communities in protecting amphibians against *Bd*. Bacterial viruses (phage), as key drivers for mortality and diversity of bacterial communities, represent another so far unexplored frontier in disease mitigation.

To our knowledge, research conducted on amphibian-host microbiome-pathogen interactions has been almost exclusively conducted on *Bd*. So far, only a few studies explored this interaction on different pathogens. A study on FV3-like ranavirus unravels the importance of habitat-microbiome interactions on disease outcome in the amphibian host *Rana temporaria*, and supports the idea that the amphibian skin microbiome likely also protects amphibians against pathogens other than *Bd* (Harrison et al., 2019). In

contrast, research on the newly discovered fungus *B. salamandrivorans* suggests that skin microbiome of salamanders contains inhibitory bacteria at numbers too low to confer sufficient protection against the pathogen and even constitutes a source of opportunistic pathogens contributing to pathogenesis (Bletz et al., 2018; Smith et al., 2018; Bates et al., 2019).

Environmental Impacts on Host-Host Microbiome-Pathogen Interaction

Due to recent technological breakthroughs, research on wildlife microbiomes is relatively new, and thus, it is not surprising that the number of studies available to address the four-way-interaction (the host-microbiome-pathogen-environment interaction) in disease ecology is very limited. Our review reveals that most studies have only considered the geographic location as a potential source of variation in the outcome of the host-microbiome-pathogen interaction (Table 1). Regarding abiotic factors, studies have investigated the effects of elevational gradient (Medina et al., 2017), water temperature (Bletz et al., 2017b), and pH, CaCO₃ and conductivity (Krynak et al., 2016) on the skin microbiome of amphibians. Few studies take biotic factors, such as land cover, forest type, connectivity and habitat management into account when comparing skin microbiomes of amphibians (Holden et al., 2015; Krynak et al., 2016; Becker et al., 2017; Ellison et al., 2019b). We expect future studies will include climatic variables, elevation, latitude, and microhabitat characteristics at a large geographic scale as is seen in Kueneman et al. (2019). Interestingly, to our knowledge, no study has considered the potential role stochasticity may play in shaping the relationship between the host, the host microbiome, the pathogen and the environment.

There is now substantial evidence that the host microbiome plays an important role in modulating pathogen invasion and disease outcome. This is likely because host microbiomes are located precisely at the interplay between host, pathogen, and environment. In some cases, the microbiome is considered as an extension of the innate immune system of amphibian hosts (Rollins-Smith and Woodhams, 2012), and the host and its microbiome are fused into a holobiont (Margulis, 1993). As such the host microbiome is considered a part of the host/holobiont when implementing the disease

triangle concept. Such a representation is restrictive, since it undermines the fundamental interaction between two different entities (the host and its microbiome) and the fact that the environment can affect both separately and independently, with various outcomes on disease dynamics. Growing evidence from multiple studies demonstrates the importance of environmental conditions in modulating the host-microbiome-pathogen interaction. Any environmental condition is imposed simultaneously on the host, the microbiome and the pathogen; however, all three factors may respond in different ways, independently of each other. We propose that a holistic approach, in which the disease triangle is extended to a disease pyramid, can best capture the complex nature of these interactions (Figure 12).

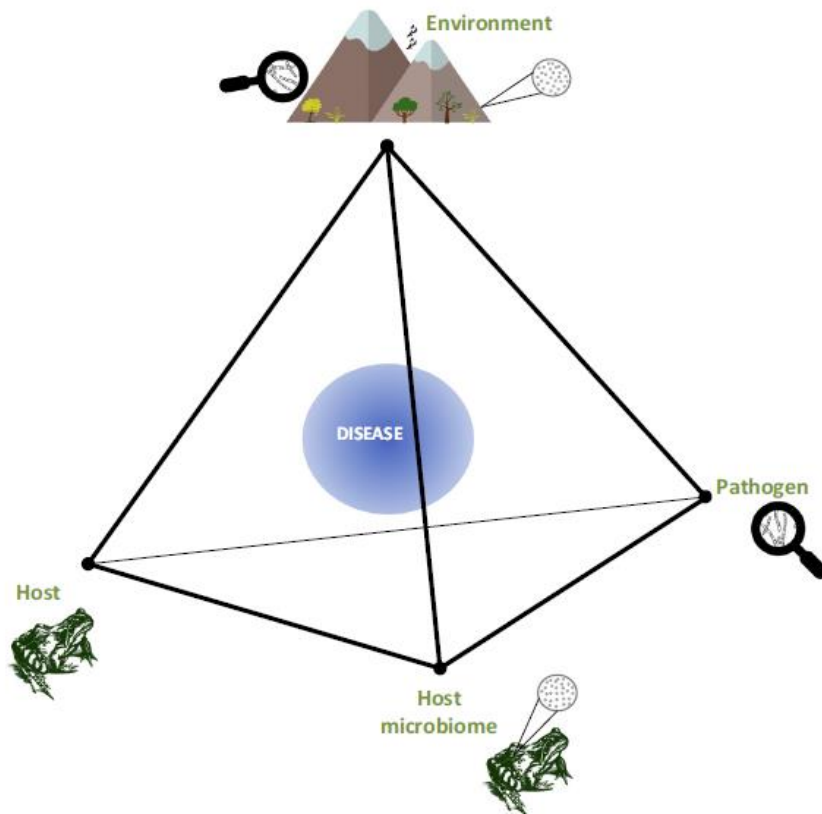


Figure 12, Key Figure. The Disease Pyramid: A Four-Way Interaction between Host, Microbiome, Pathogen and Environment. The pyramid contains a gradient of host susceptibility (from resistance and tolerance to high susceptibility), host microbiome permeability to the pathogen (from a complete barrier to a fully porous barrier), pathogen pathogenicity (from low to high infectivity and virulence), and environmental conditions (from poorly to highly favorable to the disease) that each feed towards the center of the pyramid to affect disease outcome. A permeable/porous host microbiome does not act as a barrier to pathogens, it allows pathogen establishment, proliferation and invasion of the host. A given environmental condition interacts simultaneously and independently with the host, the host microbiome and the pathogen in various ways, modifying each as well as their interactions with each other. The disease is more intense at the center, where the strongest susceptibility, permeability, pathogenicity, as well as the most favorable environmental conditions for the disease are found. Environmental conditions include biotic (e.g. environmental microbiome) and abiotic factors.

A disease triangle may allow the eventual linking of both single and multiple pathogen systems as co-infections become more widespread. *Bd* and ranaviruses can affect the same host population, requiring the immune system to act against two pathogenic targets simultaneously. The pathobiome concept, representing a consortium

of microbes acting altogether as a pathogenic entity, is meant to capture the idea of co-infection (Vayssier-Taussat et al., 2014; Bass et al., 2019). In this concept, the pathogen and either environmental or host microbe are assembled, leading to similar issues raised for the holobiont. Because environmental microbiome and host microbiome have different community composition, and different physiological and ecological functions (Figure 10), we recommend considering them in a disease pyramid as two separated factors, yet connected and interdependent, with microbes potentially moving from one community to another. In our model, additional microbes acting with the pathogen can either come from the environment (environmental microbiome), or already live on the host (host microbiome). Generally speaking, the pathobiome concept makes it more difficult to apprehend the pathogen at all its life stages. When the pathogen is colonizing the host, the pathobiome is what we call the host microbiome (as at this stage, the pathogen is living on the host). However, many pathogens have free life stages, outside the host, and it is unclear what is the pathobiome at that moment (environmental microbiome or host microbiome?). Our pyramid does consider the life of pathogens outside of the host and the various interactions it might have.

We need to move from the reductive vision of a three-edged disease triangle to the broader vision of a four-edged disease pyramid that considers the host, the microbiome, the pathogen, and the environment as interdependent components that affect disease dynamics. Any modification in any one of the vertices means a change in all other relationships; thus, the disease pyramid takes into account the role of the environment without isolating the other entities (host, microbiome, pathogen) from the whole system of which all are essential parts. In addition, the theory developed for and applied to hosts in the disease pyramid is also entirely valid for disease vectors such as, for example, mosquitos (see also Vayssier-Taussat et al (2014) and Section 1).

Concluding Remarks

Human activities are profoundly changing the environment in numerous ways. Therefore, if we want to maintain host health and control wildlife diseases, it is important that we develop a research approach that includes both biotic and abiotic factors in

studies of host-microbiome-pathogen interactions (see Outstanding Questions). Research on the amphibian-*Bd* system provides an opportunity to better understand the complex relationships between the host, the microbiome, the pathogen, and the environment, depicted by the suggested disease pyramid. Given the current threats pathogens pose to biodiversity, it is necessary to extend this research to other wildlife pathogens. For amphibians, it means for example *B. salamandrivorans*, ranaviruses, and herpesviruses. Harrison and colleagues studied the impact of different habitat characteristics of garden ponds on the *Rana temporaria* microbiome-ranavirus interaction (Harrison et al., 2019). They found that more diverse microbiomes were more resistant to the virus and that individuals living in more complex habitats had lower rates of mortality.

Future research on wildlife pathogens should consider the disease pyramid as a foundation by which to yield the necessary insights to understand and mitigate disease impacts and their different components. The disease pyramid provides an overarching comprehensive framework that can be applied to many hosts (animals, plants, humans), their microbiomes, their pathogens, and can theoretically incorporate environmental conditions as well as varied responses to environmental changes. Such a comprehensive framework will be increasingly necessary in medical sciences and disease ecology in a rapidly changing world.

Acknowledgments

The project People, Pollution, and Pathogens (P³) is financed through the call "Mountains as Sentinels of Change" by the Belmont-Forum (ANR-15-MASC-0001-P3, DFG-SCHM 3059/6-1, NERC-1633948, NSFC-41661144004, NSF-1633948). Information on ongoing activities can be found on p3mountains.org. AdC is funded by the *National Council for Scientific and Technological Development* - CNPq (Brazil). DSS holds the AXA Chair for Functional Mountain Ecology funded by the AXA Research Fund through the project GLoMEC. We thank H. Sentenac for scientific discussions.

Résumé du chapitre 2

Nous présentons ici un chapitre qui est à la fois une synthèse littéraire et la présentation d'un nouveau concept basé sur les interactions entre l'environnement, l'hôte, le microbiome de l'hôte et les pathogènes. Nous avons exploré toutes les directions des relations entre ces facteurs pour proposer une nouvelle approche de la dynamique des maladies, en contraste avec le triangle des maladies déjà établi. Dans ce système traditionnel, seules les interactions entre l'hôte, l'agent pathogène et l'environnement sont prises en compte. Le concept traditionnel ne tient pas compte, cependant, d'un acteur important de la dynamique de la maladie : le microbiome associé à l'hôte. Dans ce chapitre, nous passons en revue des articles et des études récents qui montrent la pertinence du microbiome de l'hôte, en particulier le microbiome cutané des amphibiens, dans la réponse de l'hôte aux infections pathogènes. Nous examinons non seulement comment le microbiome cutané peut agir contre les agents pathogènes et, par conséquent, aider le système immunitaire, mais nous montrons également comment il peut être directement affecté par (1) Bd (par exemple), dans le cas des amphibiens ; (2) les facteurs environnementaux, tels que les variables climatiques et l'occupation des sols ; (3) les espèces hôtes ; (4) le temps ; (5) le lieu ; (6) les facteurs de stress environnementaux, tels que les polluants et (7) l'altitude, entre autres. Tous ces facteurs ont été confirmés comme affectant la composition et/ou la diversité du microbiome cutané des amphibiens et, par conséquent, pourraient potentiellement affecter la capacité du microbiome à aider le système immunitaire des amphibiens.

Dans ce chapitre, nous avons ajouté les facteurs qui font partie de la dynamique de la maladie (environnement, hôte, microbiome de l'hôte et pathogène) un par un, et à la fin avons construit ce que nous avons appelé la pyramide des maladies, comme une représentation de toutes les relations intrinsèques qui pourraient se produire dans ce système. Sur cette base, nous avons recommandé la transition du triangle des maladies à la pyramide des maladies, afin de prendre en compte une plus grande variation dans la réponse de l'hôte aux agents pathogènes, en raison de la contribution du microbiome de l'hôte à la santé de ce dernier.

Chapter 3: Climate and fish impact tadpole skin microbiome in mountain lakes

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Abstract

Every multicellular host possesses an associated microbiome, for example on its skin or in its gut. It is still unclear how this host microbiome can be affected by environmental disturbances and climatic factors, with possible consequences for the health of wildlife and ecosystems. Here we examined the impacts of climatic, temporal, spatial, and anthropogenic factors on bacterial skin microbiome of tadpoles, for three amphibian host species (*Alytes obstetricans*, *Rana temporaria* and *Bufo bufo*). We targeted the V3-V4 region of the 16S rRNA gene of 516 tadpole samples from 19 mountain freshwater lakes across the French Pyrenees. The sites were situated along 5 altitudinal gradients, and differed in their level of anthropogenic activities, such as tourism, pastoralism and fish stocking, as well as in regard to climatic conditions. Our results show that climatic variables (number of frost days, length of growing season and precipitation) and the presence of fish were the most important factors driving the variation of the most abundant genera on the skin and, therefore, impacting the community composition in general. We conclude that the combined effect of climate change and fish stocking on the tadpole skin microbiome will likely increase the susceptibility to external pressures,

including pathogens, with important consequences for amphibian populations and mountain freshwater ecosystems.

Keywords: skin microbiome, tadpole, host-microbiome association, amphibians, anthropogenic factors, livestock

Introduction

Every multicellular host possesses an associated microbiome, i.e. a community of microorganisms (bacteria, yeast, fungi, protists, and archaea) in combination with their genomes. This microbiome occurs, for example, on the skin or in the gut of a host. The host microbiome can be associated with plants (Prado et al., 2020), insects (González-Serrano et al., 2020), as well as vertebrates (Wang et al., 2018; Rebollar and Harris, 2019). The host microbiome helps to maintain essential functions, such as synthesis of vital nutrients, temperature tolerance and energy uptake and therefore impacts host growth. It also acts as an external part of the immune system and a first barrier against exogenous microorganisms, including pathogens (Bernardo-Cravo et al., 2020).

Composition and diversity of the host microbiome can be affected directly and indirectly by host and environmental factors. For example, host physiology, life stage, health status, sex, genetics and diet have been shown to influence microbiome composition (Prado-Irwin et al., 2017; Muletz Wolz et al., 2018; Prest et al., 2018; Tabrett and Horton, 2020; Tong et al., 2020). Biotic and abiotic environmental factors also influence the community of microorganisms (Costa et al., 2016; Rebollar et al., 2019; Ruthsatz et al., 2020). All those factors are connected in the concept of a host-microbiome-pathogen-environment pyramid, the disease pyramid, where changes in the surrounding environment can lead to new living conditions for the host and for the microbiome, and a more permissive context for invasive species and pathogens (Bernardo-Cravo et al., 2020).

Identifying drivers of host microbiome diversity and composition is vital for the detection of dysbiosis, to understand the physiological and ecological functions of the host microbiome, and to predict future changes (Kueneman et al., 2019). For example, the

indirect impact of warming temperatures can cause gut microbiome dysbiosis in amphibian tadpoles, associated with a slower body growth (Greenspan et al., 2020). While the environmental drivers of free-living microbes are becoming better known (Liu et al., 2020), they are still little understood in the case of the microbial community forming the host microbiome, particularly under changing environmental conditions or in association with different species.

The amphibian skin microbiome is an ideal model to explore environmental drivers of host microbiome composition. Amphibians are ectotherms, consequently they are more sensitive to thermal changes and do not offer protection from seasonal temperature changes to their microbiome (Kueneman et al., 2019). Amphibians also occupy a large range of geographic areas which are subject to various environmental and climatic conditions, as well as important anthropogenic pressures (Stuart et al., 2004). Finally, amphibians are currently facing global declines due to different factors, including the disease chytridiomycosis caused by the chytrid pathogenic fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) (Scheele et al., 2019). The skin microbiome has been shown to be able to modulate infection in salamanders (Brucker et al., 2008) and in some species of frogs (Woodhams et al., 2015; Rebollar et al., 2016b), however, this cannot be generalized (Medina et al., 2019).

A number of possible drivers for the skin microbiome composition of adult amphibians have been identified to date, including geographic location (Abarca et al., 2018; Bird et al., 2018), latitude (Kueneman et al., 2019), altitude (Hughey et al., 2017; Medina et al., 2017; Muletz Wolz et al., 2018), pH (Krynak et al., 2016), as well as habitat disturbance. Indeed, habitat fragmentation, farming practices and pastoralism have been shown to impact the amphibian skin microbiome (Becker et al., 2017; Jiménez et al., 2020; Preuss et al., 2020). Recently, key climatic variables, such as temperature parameters and precipitation seasonality, were also discovered to have an impact on the diversity of amphibian skin microbiomes on a global scale (Woodhams et al., 2020).

So far, most of the amphibian microbiome research has focused on the adult or metamorph stage, while we know little on how tadpole microbiomes respond to climatic, anthropogenic or biotic variables. Results obtained at the adult stage cannot be generalized to earlier stages as, during metamorphosis, a significant shift in the skin

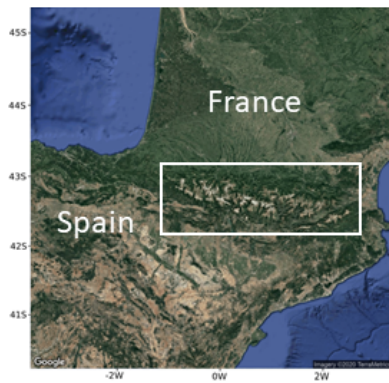
microbiome occurs, with increased phylogenetic diversity and an inverse pattern for many abundant taxa on the later life stage (Bataille et al., 2018).

We here explored remaining gaps about the external variables driving the skin microbiome composition of three montane species during the tadpole stage (*Alytes obstetricans*, *Rana temporaria* and *Bufo bufo*) in the Pyrenean Mountains. We investigated 19 lakes located along five altitudinal gradients and subject to various climatic conditions and anthropogenic pressures (tourism, fish stocking and pastoralism), across three consecutive years. Here, we elucidate how the amphibian skin microbiome is affected by (1) host species, (2) geography, (3) habitat characteristics, (4) climatic conditions, and (5) anthropogenic factors.

Material and Methods

Study area and sampling design

Nineteen lakes across the Pyrenean Mountains were chosen along five altitudinal gradients (between 1063 and 2522 meter above sea level) (Figure 13). The maximum distance between lakes was 173 km from West to East, and 16 km from North to South. Tadpoles of three species, the Common midwife toad *A. obstetricans* (13 lakes), the Common frog *R. temporaria* (15 lakes) and the Common toad *B. bufo* (3 lakes), were captured by dip-netting around the edge of the lakes, in 2016, 2017 and 2018. We covered the full growth season by sampling three times per year: (1) end of June - beginning of July, (2) August, and (3) end of September - beginning of October. Tadpoles of *A. obstetricans* were sampled in 2016, 2017 and 2018, *R. temporaria* individuals in 2017 and 2018, while *B. bufo* was sampled in 2018 only (Suppl. Mat. Table 4). The skin microbiome was sampled over the whole body with sterile dry swabs (MW100, MWE Medical Wire, Corsham, UK). After swabbing, the individuals were immediately released back to the lake. The swabs were immediately frozen on dry ice at -79°C before their transfer to a freezer (-20°C) until DNA extraction. We aimed to collect microbiome samples from up to ten individuals for each species per lake and time point. In total, we sequenced and analyzed microbiome samples from 516 individuals (341 *A. obstetricans*, 155 *R. temporaria*, 20 *B. bufo*) (Suppl. Mat. Table 4).



WEST

Fache Espagne
 2016: Ao (4) – ASVs: 174 +/- 20.5
 2017: Ao (15) – ASVs: 196 +/- 66.5
 Rt (5) – ASVs: 81 +/- 20.7
 2018: Ao (10) – ASVs: 188 +/- 50.4

Grand Fache
 2016: Ao (4) – ASVs: 228 +/- 106.2
 2017: Ao (10) – ASVs: 167 +/- 69.2
 2018: Ao (5) – ASVs: 92 +/- 33.3

Embarrat
 2017: Ao (10) – ASVs: 94 +/- 57.6
 2018: Ao (15) – ASVs: 92 +/- 49.5
 Rt (9) – ASVs: 82 +/- 43.7

Vallon Amont
 2016: Ao (5) – ASVs: 122 +/- 14.18
 2017: Ao (5) – ASVs: 80 +/- 39
 2018: Ao (28) – ASVs: 113 +/- 69.6
 Rt (10) – ASVs: 153 +/- 25.2

Paradis
 2016: Ao (5) – ASVs: 127 +/- 18.5
 2017: Ao (10) – ASVs: 192 +/- 34.5
 Rt (5) – ASVs: 146 +/- 22.4
 2018: Ao (10) – ASVs: 91 +/- 19.4
 Rt (10) – ASVs: 113 +/- 42.5

Arlet
 2016: Ao (5) – ASVs: 193 +/- 55.6
 2017: Ao (5) – ASVs: 163 +/- 32.7
 2018: Ao (6) – ASVs: 184 +/- 79.2

Puits
 2016: Ao (10) – ASVs: 238 +/- 84.6
 2017: Ao (10) – ASVs: 238 +/- 93.4
 2018: Ao (23) – ASVs: 198 +/- 58.5
 Rt (5) – ASVs: 139 +/- 32.7

Lhurs
 2016: Ao (9) – ASVs: 170 +/- 92
 2017: Ao (10) – ASVs: 237 +/- 84.7
 Rt (5) – ASVs: 290 +/- 22.4
 2018: Ao (13) – ASVs: 154 +/- 58.2
 Rt (5) – ASVs: 205 +/- 41

Acherito
 2016: Ao (5) – ASVs: 304 +/- 70.4
 2017: Ao (10) – ASVs: 314 +/- 89.7
 2018: Ao (20) – ASVs: 208 +/- 63
 Bb (5) – ASVs: 88 +/- 35

Ansabere
 2016: Ao (10) – ASVs: 153 +/- 52.5
 2017: Ao (5) – ASVs : 270 +/- 101.8
 Rt (5) – ASVs: 195 +/- 49.6
 2018: Ao (10) – ASVs: 284 +/- 41.6
 Rt (5) – ASVs: 261 +/- 27.4
 Bb (10) – ASVs: 234 +/- 64.8

EAST

Alate
 2017: Rt (5) – ASVs: 226 +/- 44
 2018: Rt (10) – ASVs: 147 +/- 43

Labant
 2017: Rt (5) – ASVs: 264 +/- 47.7
 2018: Rt (5) – ASVs: 149 +/- 24.2

Arbu
 2017: Rt (5) – ASVs: 161 +/- 9.8
 2018: Rt (5) – ASVs: 168 +/- 17.5

Bellonguere
 2016: Ao (5) – ASVs: 104 +/- 28.6
 2017: Ao (5) – ASVs: 117 +/- 41.6
 Rt (9) – ASVs: 136 +/- 30
 2018: Ao (3) – ASVs: 160 +/- 19.2
 Rt (14) – ASVs: 161 +/- 34.7

Ayes
 2016: Ao (5) – ASVs: 129 +/- 54.6
 2017: Ao (10) – ASVs: 138 +/- 46.5
 Rt (5) – ASVs: 179 +/- 29.1
 2018: Ao (20) – ASVs: 134 +/- 59.4
 Rt (10) – ASVs: 217 +/- 63

Bethmale
 2018: Bb (5) – ASVs: 100 +/- 25.2

Gourg de Rabas
 2018: Ao (7) – ASVs: 125 +/- 37.9
 Rt (3) – ASVs: 148 +/- 13.1

Madamete Haut
 2018: Rt (10) – ASVs: 150 +/- 28.2

Pecheur
 2018: Rt (5) – ASVs: 131 +/- 26.1

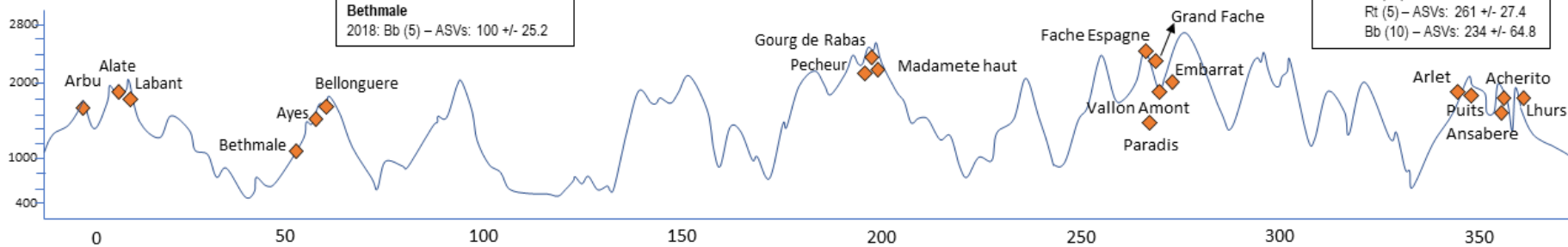


Figure 13: Altitude of the lakes used in the study (y axis) and distance (km) between them (x axis). The mean numbers of ASVs found in each host species per year are in the black boxes. Ao=*A. obstetricans*, Rt=*R. temporaria*, Bb=*B. bufo*. Number of samples collected are between parentheses

Environmental data

For each of our lakes, we obtained latitude, longitude, altitude, lake surface area, pH, relative O₂ and salinity. We used E-obs gridded climatic data with daily resolution (Version 20.0e, Cornes et al., 2018) and the R-package `climindex.pcic` (David Bronaugh for the Pacific Climate Impacts Consortium, 2020) for calculation of climindex climatic extreme indices (<https://www.climindex.org/>) for the years 2016, 2017 and 2018. We calculated the following indices: number of icing days ($T_{\max} < 0^{\circ}\text{C}$), length of the growing season ($T_{\text{mean}} > 5^{\circ}\text{C}$), number of summer days ($T_{\max} > 25^{\circ}\text{C}$), number of frost days ($T_{\min} < 0^{\circ}\text{C}$), annual total precipitation and simple precipitation index (daily precipitation $\geq 1\text{mm}$) per study site (Suppl. Mat. Table 5).

We coded a fish index based on fish presence, abundance and frequency of fish stocking events. We used fish stocking data provided by the Fédérations de Pêche of the French Départements Ariège, Haute Garonne, Hautes Pyrénées and Pyrénées Atlantiques, and visually confirmed on sites. The fish index varied from 0 (no stocking and no fish detection), 1 (stocking done less frequently than every two years and verified fish presence) to 2 (regular stocking every two years officially and verified fish presence). All the fish accounted for have been introduced in the lakes, since there are no native fish in the Pyrenean lakes.

The tourism index was calculated considering the following factors: (a) proximity of a mountain touristic lodge (absence = 0 points, refuge more than 500m away from the lake = 1 point, if less than 500m away = 2 points), (b) proximity of the GR10, which is the most popular hiking trail in the Pyrenees (no GR 10 passes by the sample site = 0 points, GR 10 passes = 1 point), (c) proximity of secondary hiking trails to the lakes (0 = absence, 1 = presence), (d) the distance to the closest parking lot (distance $> 7.1\text{km}$ = 1 point, 5.1 to 7km = 2 points, 3.1 to 5km = 3 points, 1.1 to 3 km = 4 points, between 0 and 1km = 5 points), (e) the presence or signs of camping material and/or tents close to the lakes (absence = 0, presence = 1 point). All factors were assessed visually during each sampling campaign. The overall classification for the tourism index varied from 1 to 10 (the sum of points of each factor above). Lastly, the livestock index was estimated on the presence of sheep, cow and horse/donkey on the surroundings and/or the presence of shepherd huts close to the lakes. The rank ranged from 0 = no animal and no shepherd hut, 1 = one type

of animal seen or the presence of a known shepherd, and 2 = two types of animals present, with one of them being sheep, where there was a shepherd hut around. No lake was classified as 3 (presence of all three kinds of livestock).

Skin microbiome sequencing

DNA was extracted from all the 516 swabs using the Macherey-Nagel™ NucleoSpin™ Soil kit (Düren, Germany). The DNA concentration of each sample was quantified using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) to prepare a PCR plate with equalized DNA samples around 1µM. Negative controls from non-used sterile swabs were included and processed together with the other samples.

For the sequence analysis of the skin bacterial community, the V3-V4 region of the 16S rRNA gene was amplified using Illumina primers and adaptors (F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (Klindworth et al., 2013). Our PCR mix contained 12.5µL MyTaq™ Mix (Bioline), 1µL of forward and reverse primer (at 1µM concentration), 0.5µL of BSA, and 10µL of sample DNA at a concentration of 1µM, summing up to 25µL per sample. The PCR conditions were as follows: 95°C for 3 minutes, 35 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s, followed by a final extension step at 72°C for 5 minutes. PCR products were visualized on a 1.5% agarose gel and we checked that the extraction controls showed no amplification. The amplification product was cleaned up using Agencourt AMPure XP beads to remove non-target DNA and again visualized on a 1.5% agarose gel, to confirm the presence of the product and to ensure no posterior contamination on the control samples. Post clean up products were re-amplified for ten cycles according to the Illumina protocol using sample-specific Illumina Nextera Index primers. Indexed products were cleaned up again with Agencourt AMPure XP and quantified using the Qubit® 2.0 fluorometer previously described. After quantification, the samples were diluted to 4nM, pooled, and the concentration once again quantified. The pool was then sequenced on an Illumina MiSeq, using a v2 kit of Illumina.

Sequence data were analyzed with QIIME2-2019.1 (Bolyen et al., 2019). The default protocol was used, including filtration and assignment of sequences to samples, quality check control, correction of possible amplicon sequence data errors and

taxonomy classification to the amplicon sequence variant (ASV) table. Forward and reverse reads were joined and sequences were filtered using a quality score of Q20 (removing reads with <99% base call accuracy). Sequences were taxonomically classified at 99% similarity according to the SILVA132 reference database (Yilmaz et al., 2014). We excluded all taxa with a mean relative abundance < 0.001%, leaving 4697 microbial taxa for subsequent analyses.

The data was not rarefied at any step, since rarefaction implies loss of information and precision when trying to define a common read depth (McMurdie and Holmes, 2014). However, the rarefaction curve was plotted to confirm that the richness has been fully covered for all samples (Suppl. Mat. Figure 19).

Statistical analysis

Statistical analyses were conducted in RStudio (R Core Team, 2019), XLSTAT (version 2016), and SAS® Studio 3.8.

We selected the 20 most abundant genera with the R package *microbiome* (Lahti 2019), resulting in a threshold of 0.7% mean abundance across all samples. With this selection, we captured the variation of the genera composing the skin microbial community. The taxa classified as ‘Unknown/Uncultured’ were numerous with their sum of frequencies above 0.7%. We removed those taxa due to the limited information available. Our subsequent analyses were therefore based on data from 17 taxa. When subsetting the dataset for specific analysis only for *A. obstetricans* and *R. temporaria*, we ended up with 16 ($\geq 0.6\%$ mean abundance across all *A. obstetricans* samples) and 17 taxa ($\geq 0.8\%$ mean abundance across all *R. temporaria* samples), respectively, after removing unclassified genera. We visualized the relative abundance with the *compositional* approach from the R package *vegan* (Oksanen et al. 2019). We did a core microbiome analysis at both the ASV and genus levels with the R package *microbiome* using a threshold of 80% of prevalence.

Pairwise Spearman correlations were conducted on relative abundances of the specific abundant taxa for *A. obstetricans* (n=16) and *R. temporaria* (n=17), and significant correlations were used to draw microbial networks with the R package *qgraph* (Epskamp S et al. 2012). The ‘Other’ node was kept in the network, it represents all known taxa with

a frequency of <0.001%. To examine a species effect, we used XLSTAT to run a cluster analysis (agglomerative hierarchical clustering with dissimilarities proximity type, Euclidean distance and complete linkage agglomeration method) on the relative abundance data to determine the number of clusters. We then used the R package *dendextend* (Galili 2015) to draw the radial dendrogram. For the spatial analysis, we conducted Mantel tests (with the *vegan* package) between the matrix of lake-lake geographic distance (Haversine distance based on latitude and longitude) and the matrix of Bray-Curtis dissimilarity based on the relative abundance of the 17 genera. A Mantel test was done for each year (2016, 2017 and 2018), and either for *A. obstetricans* alone, or all available species.

To explanatory variables for the microbial presence-absence and relative abundance, we used generalized linear mixed models (GLMMs, proc glimmix SAS Studio®). To avoid redundancy and reduce the number of models, we first examined intercorrelations between the selected abundant genera across the *A. obstetricans* and *R. temporaria* datasets (Suppl. Mat. Tables 6 and 7, respectively), based on species networks, pairwise Kendall's Tau correlations and principal component analyses (PCA) (R packages *psych*, *factoMineR* and *factoextra*). As a result of the correlation analysis and the PCA, we retained the following six genera: *Acinetobacter*, *Desulfovibrio*, *Limnohabitans*, *Polynucleobacter*, *Pseudomonas* and *Rhodoferrax*, which were not significantly correlated to each other ($p > 0.05$).

We tested for collinearity between the explanatory variables with Kendall's Tau correlations. Because pH was correlated to both O₂ and salinity ($n=19$, $T=0.52$, $p=0.02$; $T=0.57$, $p=0.01$, respectively), O₂ and salinity were not included in the models. Similarly, most climatic variables were correlated to altitude, so we discarded number of icing days ($n=19$, $T=0.53$, $p<0.001$), number of summer days ($n=19$, $T=-0.46$, $p<0.001$), and total precipitation (which was correlated to length of growing season and to the simple precipitation index, $n=19$, $T=-0.44$, $p<0.001$ and $n=19$, $T=0.75$, $p<0.001$, respectively).

The models were built as follows: the dependent variables were either alpha diversity indices (number of observed ASVs and inverse Simpson index) across all the samples (with a log-normal distribution of error terms and the log link function), or presence-absence of each six genera retained (with a binomial distribution of error terms

and logit link function), or their relative abundance (with a log-normal distribution of error terms and identity link function). Time (sampling date), represented as Julian days (since 01/01/2016), and species were included as covariates in all models. Lake was included as a random factor to account for repeated sampling. The fixed factors were: longitude, altitude, lake surface, pH, length of growing season, number of frost days, simple precipitation index, as well as tourism, fish and livestock indices. We selected models on Akaike Information Criteria (AIC), starting with the full model and sequentially dropping non-significant fixed factors. For the binomial models, the AIC values for the presence-absence models were calculated considering model selection based on maximum likelihood data, while the AIC value was automatically generated for the relative abundance models. Competing best models were ranked according to the smallest AIC and the highest cumulative model weight.

In case of a significant effect of any of the anthropogenic factors (tourism, livestock or fish indices) in more than one genus, a permutational multivariate analysis of variance (permanova) was performed on the whole community. This allowed us to identify if the anthropogenic influence was specific for the selected genera or for the whole microbial community. For the permanova analysis, the abundance was transformed to compositional data and calculated based on the Bray-Curtis dissimilarity distance with the *vegan* package. The coefficients from the most important taxa responsible for the difference between the groups were extracted and the taxa identified.

Results

Community composition and core microbiome

In all three amphibian species, *Limnohabitans* was the most abundant bacterial genus (all species: mean relative abundance 28.5%; *A. obstetricans*: 31%; *R. temporaria*: 25%; *B. bufo*: 20%), followed by *Tyzzellerella 3* (all species: 4.5%; *A. obstetricans*: 4.8%; *R. temporaria*: 4.3%; *B. bufo*: 3%) and *Rhodoferrax* (all species: 3.4%; *A. obstetricans*: 4.3%; *R. temporaria*: 1.8%; *B. bufo*: 0.3%). The microbiome of all three amphibian species was mainly composed of the same genera: *Acinetobacter*, *Aeromonas*, *Cyanobium PCC-6307*, *Desulfovibrio*, *Limnohabitans*, *Polynucleobacter*, *Pseudomonas*, *Rhodoferrax*,

Sphingorhabdus and *Tyzzereella 3* (Figure 14), accounting for 46% of the skin community. However, a wide range of rare but known species (termed ‘Other’) had a high relative frequency with 231 genera (50.6%) in *B. bufo*, 393 genera (30.6%) in *R. temporaria* and 279 genera (21.4%) in *A. obstetricans* (Figure 14).

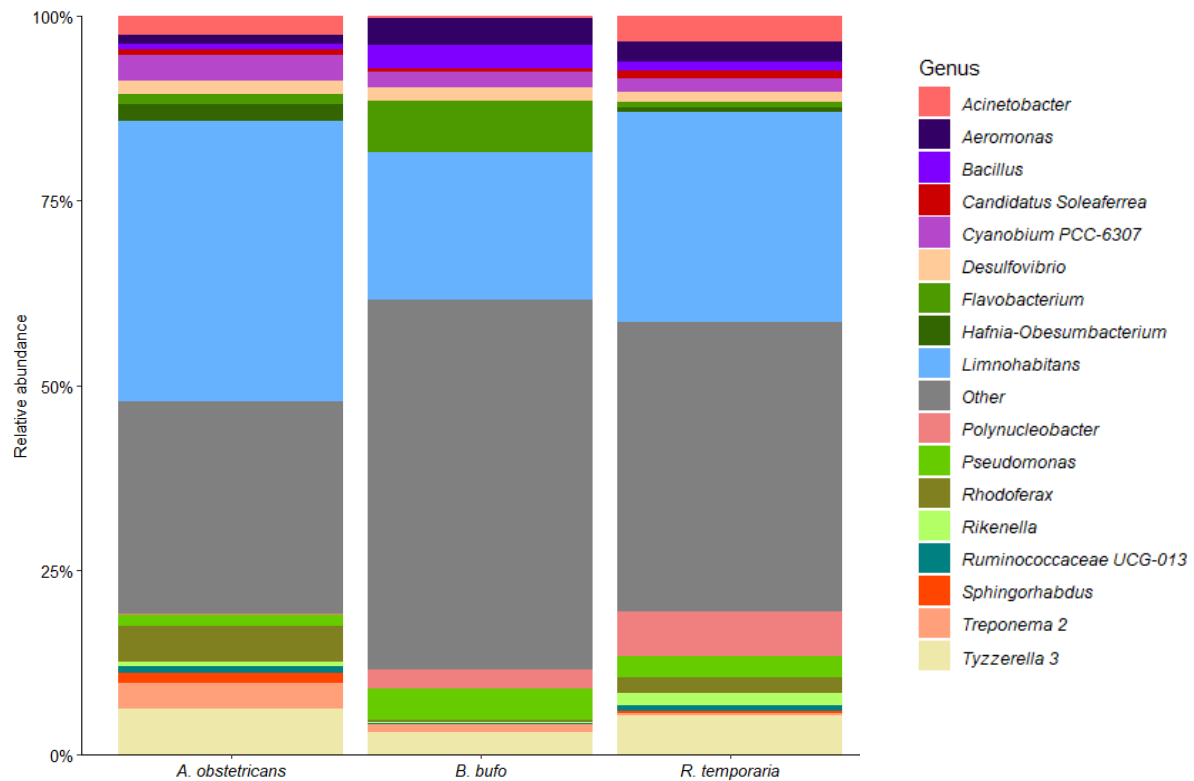


Figure 14. Relative abundance of the most abundant genera for each host species. The ‘Other’ represents the sum of taxa with an abundance <0.001%.

We did not observe a common core microbiome on the ASV level across all samples (Figure 15). The most abundant unique ASV was detected in 66% of all the samples and belonged to the *Limnohabitans* genus. In *A. obstetricans*, an ASV from the genus *Aeromonas* was shared between 71% of samples, while in *R. temporaria*, an ASV from the genus *Limnohabitans* was present in 68% of the samples. For *B. bufo*, the ASVs from *Dechloromonas* and from an unclassified genus of the *Sphingomonadaceae* family were observed in 99% of the samples. On the genus level, the core microbiome was composed of *Limnohabitans*, *Tyzzereella 3* and *Pseudomonas* across all amphibian species (Figure 15). The genus-level core microbiome of *A. obstetricans* comprised *Limnohabitans*, *Tyzzereella 3*, *Pseudomonas* *Aeromonas*, *Desulfovibrio* and *Cyanobium* PCC-

6307. *Limnohabitans*, *Tyzzzeria 3*, *Pseudomonas*, *Flavobacterium*, *Aeromonas*, *Bacillus* and *Desulfovibrio* made the genus-level core microbiome of *B. bufo*. In *R. temporaria*, the genus-level core microbiome was formed by *Limnohabitans*, *Tyzzzeria 3*, *Polynucleobacter* and *Rikenella*.

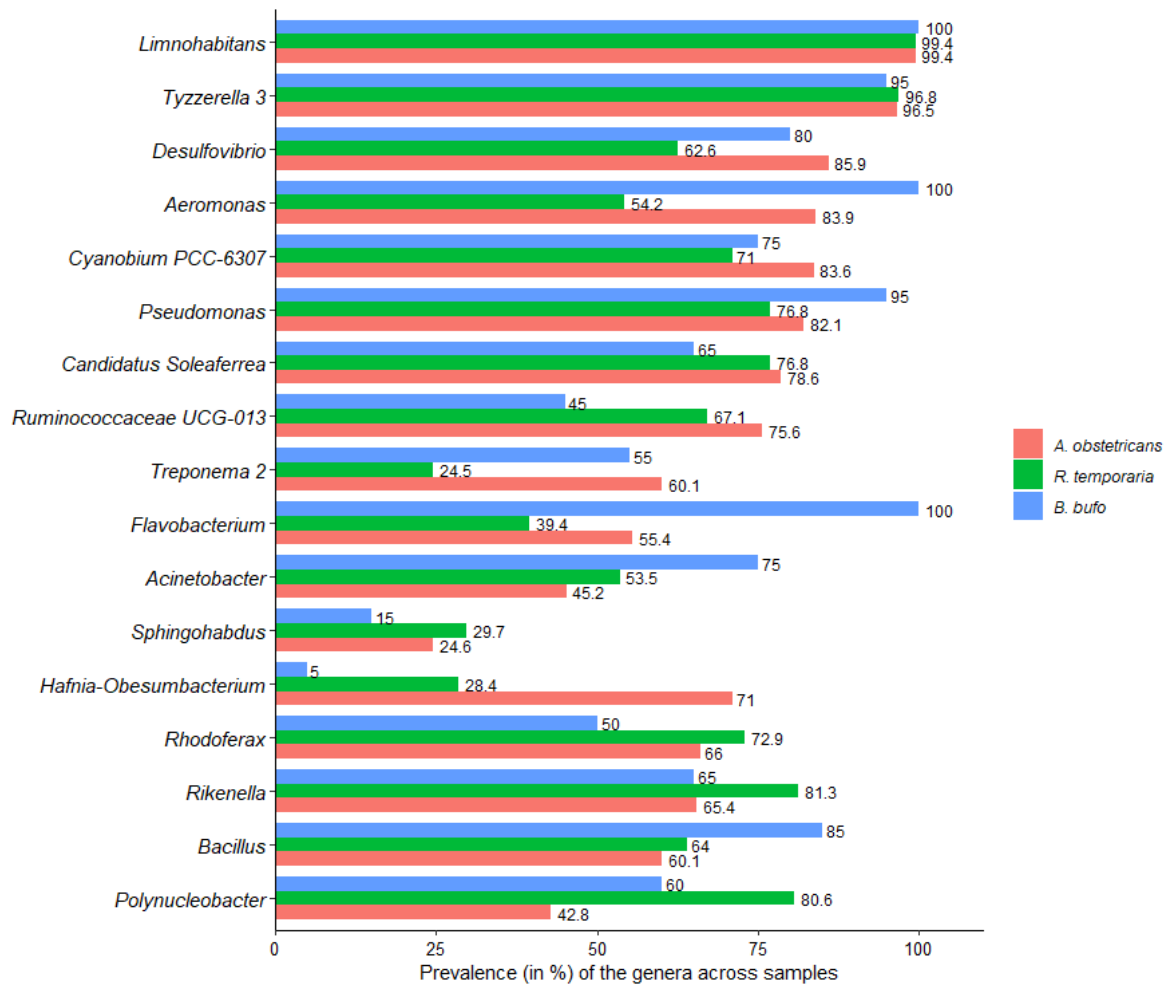


Figure 15. Prevalence of the most abundant genera across all samples and for each host species.

The microbiome networks of *A. obstetricans* and *R. temporaria* (Figure 16) showed some similar associations between specific taxa, such as between *Tyzzzeria 3* and *Desulfovibrio*. However, other associations had different directions and intensities. *Limnohabitans* and *Aeromonas* were positively and strongly associated in *R. temporaria*, but negatively and weakly associated in *A. obstetricans*. Unique connections were also created, due to the presence of species-specific taxa for each species, such as the

connections between *Treponema*, *Flavobacterium* and *Helicobacter*, all strong and positive, and only present in *A. obstetricans* individuals (Figure 16).

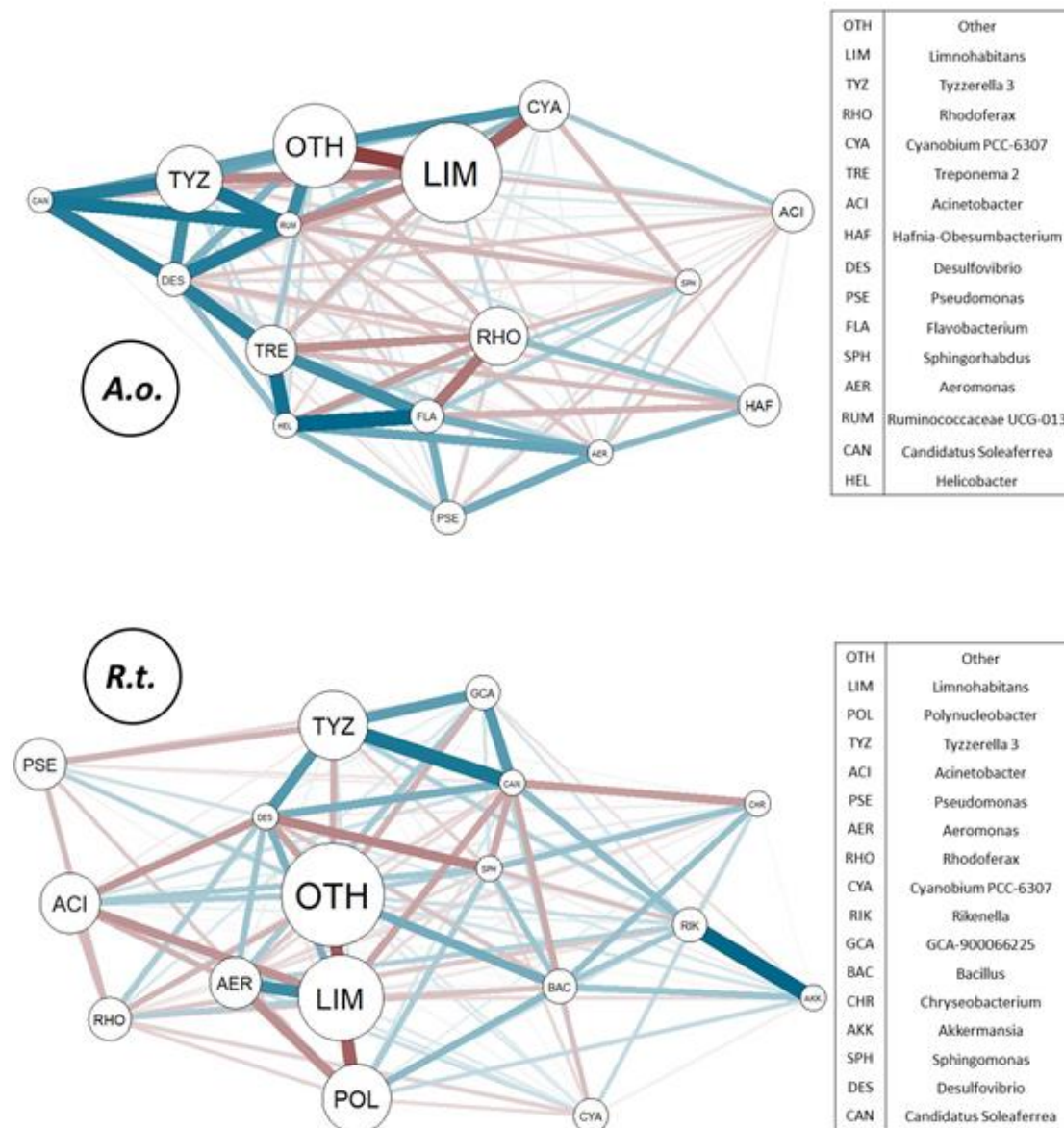


Figure 16. *A. obstetricans* (*A.o.*) and *R. temporaria* (*R.t.*) microbiome networks based on pairwise Spearman correlations of abundance. The genera in the legend are classified in descending order of abundance. Blue lines represent positive correlations, while red represent negative ones. The thicker the lines, the stronger the correlation. The size of the node (circle) reflects the relative abundance of the genus. The selected most abundant genera are represented. The less abundant genera are grouped in the category 'Other'.

Generally, however, we observed clusters that were not species-specific (Figure 17). In all four clusters, we found samples from the three different species, showing their overlap in regard to the skin microbiome. Cluster 1 (yellow, n=67) was characterized by a high amount of *Limnohabitans* (76.3%) and comprised samples from 12 populations located mainly in the western part of the French Pyrenees. Cluster 2 (light blue, n=331) combined samples from 16 populations with a lower amount of *Limnohabitans* (26.7%) and a medium abundance of 'Other' and 'Unknown' taxa (42.6%). In Cluster 3 (dark blue, n=110, 13 populations) a higher amount of 'Other' and 'Unknown' taxa than in all other samples was observed (77.3%). The smallest Cluster 4 (red, n=8) unites specimens with a higher relative abundance of *Polynucleobacter* (53.5%) of the same population (lake Alate). The cluster analysis did not support a clear species nor a clear geographic effect on the microbiome composition.

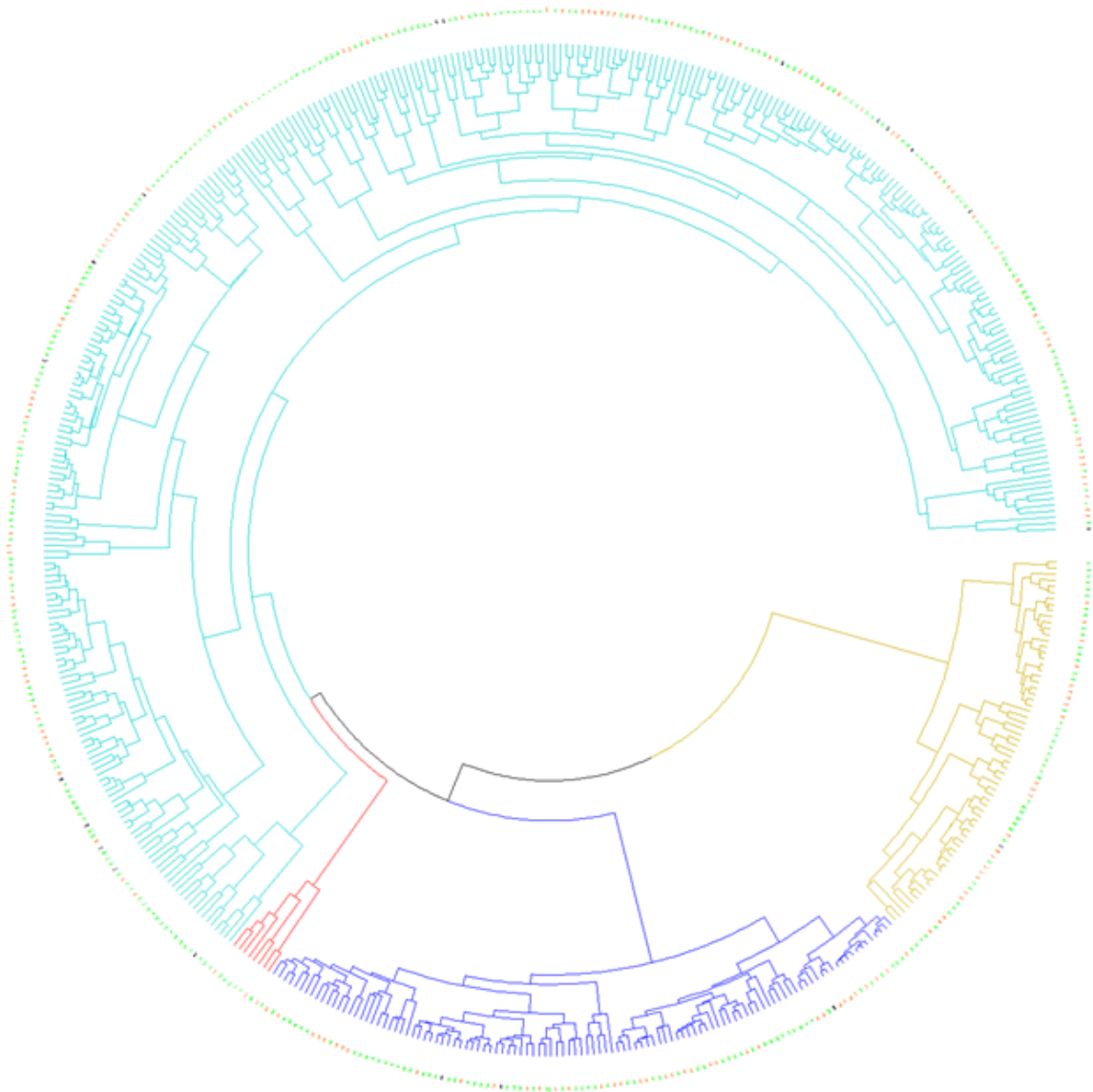


Figure 17. Dendrogram considering the relative abundance of the most abundant taxa across all samples of the three host species *A. obstetricans* (green), *R. temporaria* (orange) and *B. bufo* (black), identified as the dots on the external layer of the dendrogram. Cluster 1 = yellow; cluster 2 = light blue; cluster 3 = dark blue; cluster 4 = red.

We did not observe an isolation-by-distance of our lakes when considering only *A. obstetricans*, only *R. temporaria*, or when species were combined, in most cases (Mantel test, 2016: *A. obstetricans* alone, $R_{195}=0.17$, $p=0.13$; 2017: *A. obstetricans* alone, $R_{285}=0.05$, $p=0.29$, *R. temporaria* alone, $R_{135}=0.16$, $p=0.13$; both species combined, $R_{360}=0.15$, $p=0.05$; 2018: *A. obstetricans* alone, $R_{390}=0.13$, $p=0.1$, *R. temporaria* alone, $R_{300}=0.14$, $p=0.05$).

Only when the three amphibian species were combined in 2018, we observed a signal for an isolation-by-distance (all three species, $R_{540}=0.27$, $p=0.001$).

Impact of environmental factors on the community composition

Our GLMMs support that the host species was important to determine the presence of *Acinetobacter*, *Polynucleobacter* and *Desulfovibrio* (Table 2) and that the relative abundance of *Limnohabitans*, *Polynucleobacter* and *Pseudomonas* was driven by the host species (Table 3). Over time, the presence of the main genera did not change significantly (Table 2), however, the relative abundance of *Limnohabitans* significantly increased, while that of *Rhodoferrax* and *Polynucleobacter* significantly decreased. Generally, alpha diversity, expressed as the number of observed ASVs, changed over time ($F_{1,509}=6.59$, $p=0.016$) with a decreased tendency, and was also influenced by the host species ($F_{2,468}=3.51$, $p=0.031$).

Table 2: Best models explaining the presence of a selection of microbial genera. Only models with the lowest AIC are displayed. Time and species were always included as covariate in the final models. Significant values are marked in bold. The direction of the relationship (+/-) is only shown for significant variables.

Presence					
Genera	Explanatory variables	F-values	p-values	Direction of relationship	
AIC = 257.59	Time	$F_{1,508}=0.66$	0.416		
	Species	$F_{2,508}=0.97$	0.381		
	Rhodoferrax	Tourism index	$F_{1,13.37}=5.05$	0.042	-
		pH	$F_{1,12.33}=5.17$	0.042	-
		Precipitation index	$F_{1,508}=7.03$	0.008	+
		Number of frost days	$F_{1,19.38}=5.36$	0.032	-

	Time	$F_{1,508}=3.72$	0.054	
	Species	$F_{2,508}=3.42$	0.033	
Acinetobacter	Altitude	$F_{1,18.40}=5.92$	0.025	-
AIC = 2423.35	Length of growing season	$F_{1,508}=33.82$	<0.001	+
	Precipitation index	$F_{1,508}=6.27$	0.013	-
	Number of frost days	$F_{1,15.47}=10.08$	0.006	+
	Time	$F_{1,509}=0.21$	0.650	
	Species	$F_{2,509}=27.13$	<0.001	
Polynucleobacter	Altitude	$F_{1,17.81}=11.19$	0.004	-
AIC = 2510.06	Length of growing season	$F_{1,509}=5.39$	0.021	-
	Number of frost days	$F_{1,21.01}=8.39$	0.009	+
Pseudomonas	Time	$F_{1,512}=0.96$	0.327	
AIC = 2515.50	Species	$F_{2,512}=1.50$	0.224	
Desulfovibrio	Time	$F_{1,512}=0.04$	0.840	
AIC = 2564.16	Species	$F_{2,512}=9.35$	<0.001	

Among the environmental data, pH was a poor predictor of microbiome composition, it only contributed to explain the presence of *Rhodoferrax* and the relative abundance of *Limnohabitans*, as was longitude (only contributing to explain the abundance of *Acinetobacter* and *Desulfovibrio*), while lake surface was never retained in any model (Tables 2 and 3). Altitude partly explains the presence of *Acinetobacter* and *Polynucleobacter*, and abundance of *Limnohabitans*, *Polynucleobacter* and *Desulfovibrio*.

Table 3. Best models explaining the relative abundance of a selection of microbial genera. Only models with the lowest AIC are displayed. Time and species were always included as covariate in the final models. Significant values are marked in bold. The direction of the relationship (+/-) is only shown for significant variables.

Relative abundance				
Genera	Explanatory variables	F-values	p-values	Direction of relationship
Limnohabitans AIC = 1642.47	Time	F _{1,496.4} =22.52	<0.001	+
	Species	F _{2,504.9} =16.86	<0.001	
	Altitude	F _{1,27.53} =4.44	0.044	+
	pH	F _{1,17.31} =7.10	0.016	+
	Length of growing season	F _{1,496.9} =7.38	0.007	-
Rhodoferrax AIC = 1210.23	Time	F _{1,334.9} =7.7	0.006	-
	Species	F _{2,336.7} =0.83	0.436	
	pH	F _{1,16.04} =11.88	0.003	-
	Fish index	F _{1,17.88} =5.69	0.0283	-
	Length of growing season	F _{1,339.4} =4.48	0.035	-
Acinetobacter AIC = 1006.52	Time	F _{1,241.5} =0.24	0.626	
	Species	F _{2,235.6} =1.04	0.356	
	Longitude	F _{1,22.2} =8.87	0.007	+
	pH	F _{1,15.66} =24.37	<0.001	-
	Fish index	F _{1,14.57} =7.26	0.017	+
	Length of growing season	F _{1,243} =68.48	<0.001	+
	Precipitation index	F _{1,234.4} =14.93	<0.001	-

	Time	$F_{1,268}=6.63$	0.011	-
Polynucleobacter	Species	$F_{2,276}=36.63$	<0.001	
AIC = 936.25	Altitude	$F_{1,30.41}=8.61$	0.006	-
	Length of growing season	$F_{1,265.7}=11$	0.001	-
	Time	$F_{1,410.7}=0.75$	0.386	
Pseudomonas	Species	$F_{2,357.4}=21$	<0.001	
AIC = 1515.13	Length of growing season	$F_{1,412}=40.12$	<0.001	-
	Precipitation index	$F_{1,394.7}=11.36$	<0.001	-
	Time	$F_{1,399.9}=1.33$	0.249	
Desulfovibrio	Species	$F_{2,345.8}=2.31$	0.101	
AIC = 1453.75	Longitude	$F_{1,14.92}=8.8$	0.010	-
	Altitude	$F_{1,17.93}=10.39$	0.005	-

Climatic factors explained the presence of microbial genera (Table 2) and/or their relative abundance (Table 3), with the exception of *Desulfovibrio*. Neither its presence, nor its relative abundance, was affected by any climatic variable, but was driven by the location (longitude and altitude) of the host habitat and the host species (Tables 2 and 3). Particularly, length of growing season explained the presence of *Acinetobacter* and *Polynucleobacter*. In addition, longer growing seasons translated into higher relative abundance of *Acinetobacter* and lower abundance of *Limnohabitans*, *Rhodoferrax*, *Polynucleobacter* and *Pseudomonas*. *Polynucleobacter* apparently did not cope well with low temperatures as longer periods of frost days reduced its occurrence probability. We also observed that the presence of *Rhodoferrax*, and *Acinetobacter* showed opposite patterns regarding the numbers of frost days (Table 2). Presence of *Rhodoferrax* and *Acinetobacter* was affected by precipitation (Table 2), and *Acinetobacter* and *Pseudomonas* were less abundant when the precipitation level was higher, showing a preference for habitats with lower precipitation levels (Table 3).

Our anthropogenic variables (tourism, fish stocking and livestock) explained little of the presence-absence and abundance of genera on tadpole skin. Presence of livestock was never retained as a significant explanatory variable of bacterial presence or abundance in our models (Tables 2 and 3). Tourism activity only explained the presence of *Rhodoferrax* (Table 2), and *Rhodoferrax* relative abundance was negatively influenced by fish presence. Fish presence, however, positively influenced the relative abundance of *Acinetobacter* (Table 3). Fish presence may therefore drive important changes in the microbial community (Permanova: $F=14.12$, $R^2=0.027$, $p<0.001$). These differences were driven by five different ASVs from *Limnohabitans*, one ASV from the *Burkholderiaceae* family, one from the *Sphingomonadaceae* family as well as one ASV from the genera *Polynucleobacter*, *Sphingorhabdus* and *Rhodoferrax*, each (Figure 18). These top 10 ASVs were more abundant in samples from lakes with fish than without fish. The total number of ASVs, however, did not significantly differ between samples from fishless lakes and lakes with fish.

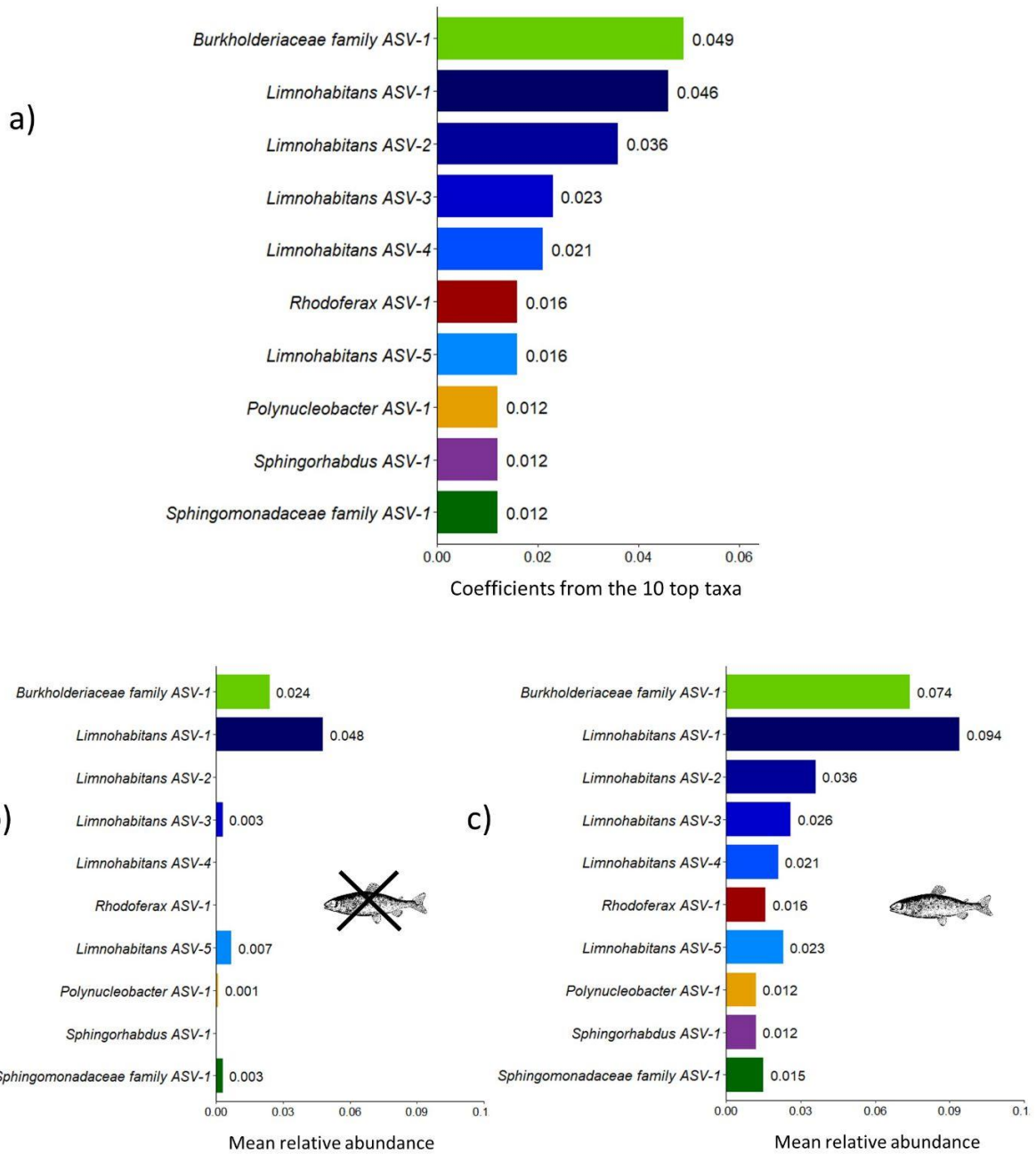


Figure 18. a) Top 10 taxa responsible for the difference in microbiome community composition between groups of samples free and under fish presence; b) Mean relative abundance of the top 10 taxa in samples from lakes free of fish and c) Mean relative abundance of the top 10 taxa in samples from lakes with fish.

Discussion

Recent studies have tried to disentangle the interactions between amphibian skin microbiome, amphibian host and environmental variables (Longo et al., 2015; Krynak et al., 2016; Estrada et al., 2019; Jiménez et al., 2020). Here, we analyzed the impact of climatic, temporal, spatial, and anthropogenic factors on bacterial skin microbiome of tadpoles, for three amphibian host species. Our study shows that changes in the skin microbiome composition are partly driven by the presence of fish in mountain lakes, suggesting that fish stocking has the potential to alter the capacities of tadpoles to deal with external pressures, assuming these changes have a functional consequence. Such alterations might be further enhanced by changing climate, as the number of frost days, length of growing season and precipitation strongly influenced the presence and abundance of the most common bacterial genera found on amphibian tadpole skin.

Many of the abundant genera found in this work have been found already in other studies as part of the amphibian skin microbiome, in adults of *R. temporaria* (Campbell et al., 2019), tadpoles (Jiménez et al., 2020) and adults of other species not covered in this work (Walke et al., 2017; Jimenez et al., 2019; Jiménez et al., 2020). *Limnohabitans* and *Desulfovibrio*, for example, have been found to be more abundant in tadpoles from disturbed habitats, while different bacterial species of *Acinetobacter* and *Pseudomonas* were more abundant in adults, also from disturbed habitats (Jiménez et al., 2020). *Pseudomonas*, *Flavobacterium* and *Acinetobacter* have been found to be more abundant in healthy individuals, in comparison to adults infected by *Batrachochytrium dendrobatidis* (Jimenez et al., 2019), indicating the range of species and conditions under which they are associated with amphibian hosts.

Fish are known to affect amphibian larvae through predation, which can cause an accentuated decline of frog populations (Vredenburg, 2004). Fish presence induces an important stress on tadpoles, altering their foraging behavior, time to metamorphosis, and morphology (Buskirk and Mccollum, 2000; Polo-Cavia et al., 2020). This stress may alter the interactions between hosts, host microbiome and pathogens (Bernardo-Cravo et al., 2020). In addition, fish presence may also more directly alter the environmental microbiome from which tadpoles recruit their skin microbiome. In our study, an effect of fish presence on the microbial community was observed on the relative abundance of the

most abundant genera (*Limnohabitans*, *Rhodoferrax*, *Acinetobacter*, *Polynucleobacter*, *Sphingorhabdus*). Among them, *Acinetobacter* is a known genus with emerging pathogenic species that affect fish, such as *A. johnsonii* and *A. lwoffii* (Kozińska et al., 2014). The presence of this genus was positively correlated with the fish index, indicating that it was more abundant where there were more fish, suggesting that the high abundance identified on the amphibian skin could come from the fish present in the lake. With our data resolution, it was not possible to determine to which species the *Acinetobacter* ASVs belonged to, but if the fish were infected with the pathogenic *Acinetobacter* species, they could be responsible for the introduction of new pathogens into the habitat, with possible transfer to amphibians (Kiesecker et al., 2001; Price et al., 2017). We observed that individuals from lakes with fish showed an altered microbiome in regard to 10 of the most differentially abundant ASVs between lakes with and without fish. In short, the skin microbiome of tadpoles is altered directly and/or indirectly by fish, potentially allowing parasites and pathogens to bypass the protective role of the host microbiome (Hernández-Gómez, 2020).

Among the anthropogenic factors analyzed (presence of livestock, fish introduction and tourism pressure), we found no effect of livestock on the amphibian skin microbiome. Other studies found that livestock increases the probability of run-offs of fecal coliforms in agricultural pond systems, leading to skin-microbiome changes towards *Bd*-facilitative bacteria (Preuss et al., 2020). The opposite results seen in our work can be probably explained by low livestock unit numbers, the type of livestock (usually cows and sheep) and higher hydrological turnover in our lakes, all likely leading to a dilution of bacteria from livestock, not detectable on the tadpole skin. Tourism significantly impacted the presence (and fish stocking the relative abundance) of the genus *Rhodoferrax*, which consists of four confirmed species (*R. antarcticus*, *R. ferrireducens*, *R. fermentans*, *R. saidenbachensis* (Park et al., 2019) and several candidate species. *Rhodoferrax* species are frequently found in standing aquatic environments, and some species have been isolated from sewage, sludge and sediments (Jin et al., 2020). An introduction to mountain lakes via fish stocking is possible and the effect of tourism may be due to anglers. Given the missing impact of livestock, we do not think that sewage produced by tourists would be introducing *Rhodoferrax* to our lakes. While there are a few

limitations to our measure of the anthropogenic factors, our results suggest that, for tadpoles, the human impact on the overall diversity of the skin bacterial genera was small.

We observed impacts from non-anthropogenic factors. Most of the abundant genera responded to climatic data, particularly temperature-driven variables. Temperature is a well-known driver for microbial communities in all kinds of habitats (Cavicchioli et al., 2019) and for amphibian survival (Alford and Richards, 1999; Ficetola and Maiorano, 2016). For amphibians, both environmental temperature and humidity can be equally important to keep the body under the thermal functioning limit and the critical evaporative water loss level (Lertzman-Lepofsky et al., 2020). The genera *Limnohabitans*, *Rhodoferrax*, *Polynucleobacter* and *Pseudomonas* all had lower relative abundance when the growing season was longer. *Acinetobacter*, on the contrary, increased in abundance with longer growing seasons. In a previous study, the Betaprotobacteria of the genus *Polynucleobacter* were found to be positively correlated to water temperature, but negatively correlated with chlorophyll a (Wu and Hahn, 2006). As the selection of skin microorganisms is affected by the availability of the bacteria in the surrounding environment (Kueneman et al., 2019), the pattern we observed may be related to increased algal growth in years with longer growing seasons, negatively impacting on *Polynucleobacter* abundances. Generally, temperature-driven variables have the potential to change skin microbiomes of amphibians (Rebollar et al., 2020) and may influence disease dynamics of chytridiomycosis. In a Pyrenean montane lake, earlier ice-melt (translating in a longer growing season) increased infection probability in three amphibian species (Clare et al., 2016). As several *Pseudomonas* species are known to have anti-*Bd* effects (Harris et al., 2009) our study suggests that the findings of Clare *et al.* (2016) might be explained by the reduction of *Pseudomonas* species in years of longer growing seasons (but see Muletz-Wolz et al., 2019). However, more detailed studies are needed to shed more light on those complex interrelationships.

Because mountain ecosystems are highly sensitive and vulnerable to climate change, they are considered as sentinels of change (Schmeller et al., 2018). In the Pyrenees, in the last 50 years, the mean annual temperatures have significantly increased by 1.2°C (0.2°C per decade), that is 30% more than the world average (0.85°C) (OPCC-CTP, 2018), a warming that is comparable to that of the Alps (Einhorn et al., 2015), and is predicted to continue. Climate change is modifying and will continue to modify the

occurrence of extreme events, the amount of precipitation (rain and snow), as well as freeze and thaw cycles, with impacts on onset of snow melt (and thus length of growing season) and water temperatures. Our results suggest that these profound climate modifications will impact the amphibian skin microbiome, and thus impact the host's ability to cope with infectious diseases (such as chytridiomycosis or ranaviriosis) as the skin microbiome is part of the amphibian immune system (Bernardo-Cravo et al., 2020). With amphibians being keystone species in mountain freshwater ecosystems (Schmeller et al., 2018), our results point to cascading effects, potentially to the surrounding habitat as well.

Our results confirm the role played by the host on the microbiome composition (Kueneman et al., 2014; Belden et al., 2015). In *R. temporaria*, particularly *Aeromonas*, *Polynucleobacter* and *Pseudomonas* are more abundant than in *A. obstetricans*. There were also differences in microbial networks of *A. obstetricans* and *R. temporaria*. These networks differed in terms of intensity and direction of microbial interactions, but also in the connections themselves. Interestingly, *A. obstetricans* and *R. temporaria* have different life history traits. *R. temporaria* lays eggs early in the season and finishes the development from egg to froglet within the same year, even in mountain lakes. *A. obstetricans* lays eggs usually in mid-summer, tadpoles do not finish their metamorphosis in the same year and can hibernate up to several years in the lakes. Recruitment of microorganisms may therefore be very different in both species, linked to the availability of bacteria at the time of egg-hatching, availability which depends on lake temperature and other factors as shown above.

Our results show a consistent influence of the climatic factors on the tadpole skin bacterial community, together with a host influence on the network interactions, as well as impacts of fish presence. With our study, we contribute with important insights in three edges of the disease pyramid (Bernardo-Cravo et al., 2020), the host, the host microbiome and the environment, suggesting that interactions between all these three edges might explain pathogen occurrence (the fourth edge in the disease pyramid) in our study system. Our study suggests that ongoing and future climate change will have strong impacts on the amphibian skin microbiome, with likely consequences for disease dynamics of amphibians, the most threatened vertebrate group on the planet, and through cascade effects will even have consequences for whole ecosystems.

Acknowledgment

The project People, Pollution, and Pathogens (P³) is financed through the call 'Mountains as Sentinels of Change' by the Belmont-Forum (ANR-15-MASC-0001-P3, DFG-SCHM 3059/6-1, NERC-1633948, NSFC-41661144004, NSF-1633948). Information on ongoing activities can be found on p3mountains.org. A. B-C. is funded by the National Council for Scientific and Technological Development – CNPq (Brazil). D.S.S. holds the AXA Chair for Functional Mountain Ecology funded by the AXA Research Fund through the project GloMEc. We thank Nicole Steinbach, Stephan Schreiber and René Kallies for help in the lab. We also express our thanks to all student helpers, as well as Pilar Durantez, Sylvain Lamotte, Frederic Julien and others for support during and after fieldwork.

Supplementary Material for Chapter 3

Table 4: Number of sampled individuals per lake at each sampling time point and for each species. *A. obstetricans* = Ao; *R. temporaria* = Rt; *B. bufo* = Bb.

Lake name	Altitude (m)	Latitude-longitude	2016				2017				2018													
			June	July	Aug	Sept	June		July		Aug		June		July		Aug			Sept			Oct	
			Ao				Ao	Rt	Ao	Rt	Ao	Rt	Ao	Rt	Ao	Rt	Ao	Rt	Bb	Ao	Rt	Bb	Ao	
Acherito	1858	42.878; -0.709	5						5		5					10		5			5		5	
Ansabere	1876	42.888; -0.709	5			5				5	5					5	5	5	5			5		
Arlet	1974	42.84; -0.615	5						5						6									
Lhurs	1691	42.922; -0.702	5			4			5	5	5					5	5				8			
Puits	1891	42.864; -0.633	5			5			5		5				3	5	10				10			
Vallon Amount	2215	42.838; -0.187			5				5						10	5	7	5						10
Fache Espagne	2522	42.814; -0.246				4			5		10	5												10
Grand Fache	2430	42.813; -0.223				4			5		5					5								
Paradis	1619	42.849; -0.16				5			5	5	5				5	5	5	5						

Embarrat	2180	42.84; - 0.19							5		5				5		5	9					5
Gourg de Rabat	2407	42.852; 0.145															2	3		5			
Mad. Haut	2379	42.867; 0.144																5			5		
Pecheur	2307	42.863; 0.144											5										
Ayes	1681	42.844; 1.065		5			5	5			5		10	5			5	5		5			
Bellongue re	1864	42.84; 1.063		5			5	4			5	3	5					4			5		
Bethmale	1039	42.862; 1.084																	5				
Alate	1865	42.777; 1.406					5											5			5		
Arbu	1729	42.819; 1.437					5							5									
Labant	1548	42.775; 1.392					5							5									

Table 5: Description of the climatic data used for the analysis, directly taken from the Climdex indices

Index	Description
Number of icing days	Annual number of days with daily maximum temperature < 0 °C
Length of the growing season	Annual count between the first span of at least 6 days with daily mean temperature >5°C and the first span after July 1 st of 6 days with T<5°C
Number of summer days	Annual number of days with daily maximum temperature > 25°C
Warm spell	Annual count of days with at least 6 consecutive days when T> 90th percentile (above 90% of the temperatures detected on a 5-day window for the base period 1961-1990)
Number of frost days	Annual number of days with daily minimum temperature < 0°C
Consecutive dry days	Maximum number of consecutive days with precipitation level < 1mm
Consecutive wet days	Maximum number of consecutive days with precipitation level ≥ 1mm
Total precipitation	Annual total precipitation
Simple precipitation index	Daily precipitation amount on rainy days divided by the number of rainy days

Table 6: Correlation of the most abundant genera present in *A. obstetricans* samples. Values in bold are different from 0 with a significance level $\alpha \leq 0.05$

Variables	Rhodofera x	Limnohab ans	Hafnia- Obesumb acterium	Acinet obacter	Sphing orhabdus	Pseudo monas	Rikene lla	Aerom onas	Rumin ococcae ae UCG- 013	Cyanob ium PCC- 6307	Polynu cleobacte r	Tyzzere lla 3	Bacillu s	Other	Desulf ovibrio	Flavob acterium	Trepon ema 2
Rhodoferax	1																
Limnohabitans	0.158	1															
Hafnia- Obesumbacteriu m	0.274	-0.024	1														
Acinetobacter	-0.053	-0.193	0.05	1													
Sphingorhabdu s	-0.202	0.098	-0.067	0.062	1												
Pseudomonas	-0.151	-0.004	0.019	-0.162	0.095	1											
Rikenella	0.04	-0.256	0.112	-0.146	-0.134	0.087	1										
Aeromonas	-0.06	-0.12	0.29	-0.166	0.145	0.351	0.076	1									
Ruminococcae ae UCG-013	-0.075	-0.368	0.022	0.064	-0.238	-0.05	0.493	-0.185	1								
Cyanobium PCC-6307	-0.035	-0.492	-0.001	0.226	-0.208	0.064	0.075	0.05	0.286	1							

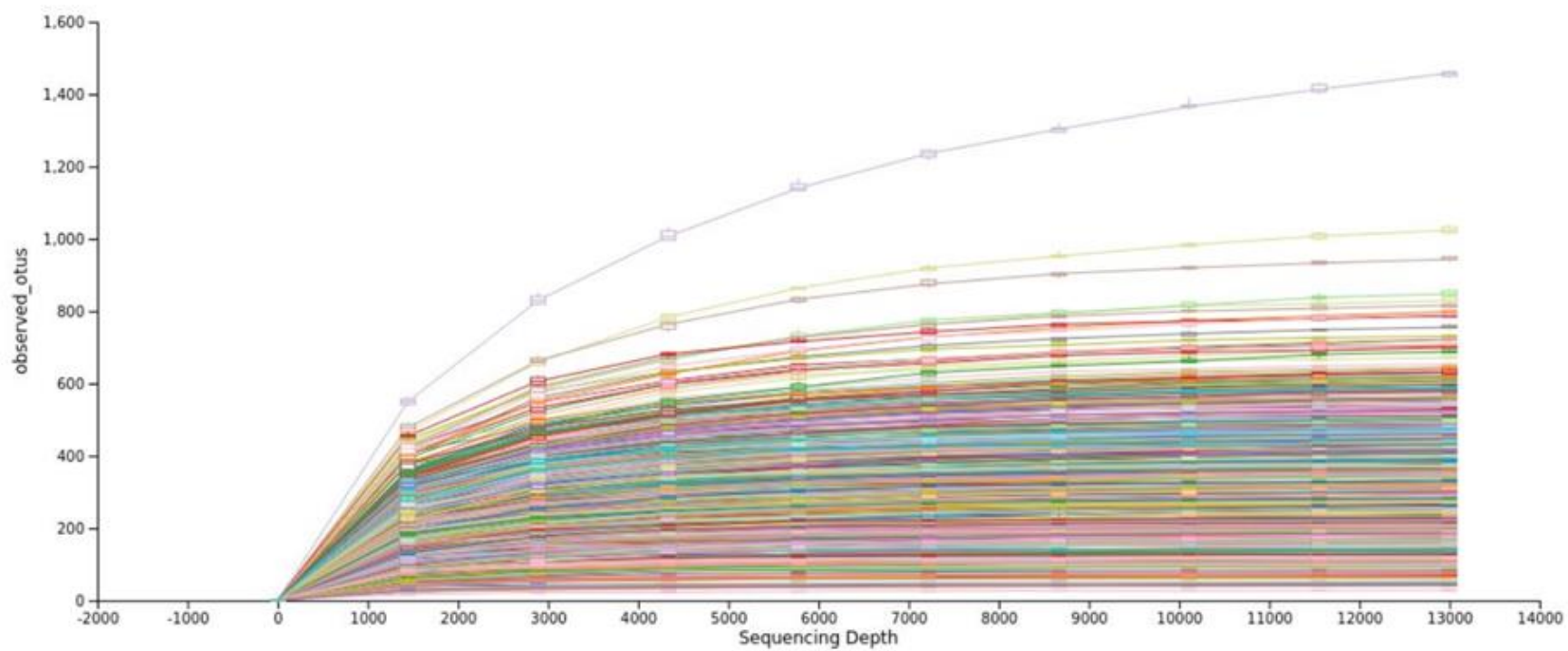
Polynucleobacter	-0.155	0.039	0.044	0.018	-0.054	0.195	-0.008	0.185	0.11	0.133	1						
Tyzzarella 3	-0.14	-0.38	0.119	0.105	-0.15	-0.056	0.453	0.05	0.564	0.255	0.261	1					
Bacillus	-0.297	-0.283	-0.031	0.222	0.134	0.06	-0.064	0.074	0.137	0.434	0.215	0.218	1				
Other	-0.21	-0.617	-0.033	0.117	-0.065	0.141	0.335	0.038	0.479	0.43	0.142	0.381	0.428	1			
Desulfovibrio	-0.21	-0.224	-0.148	-0.181	-0.124	-0.097	0.437	-0.065	0.539	0.055	0.184	0.468	0.091	0.343	1		
Flavobacterium	-0.437	0.041	-0.239	-0.079	0.214	0.341	-0.219	0.343	-0.141	0.086	0.428	0.12	0.242	0.07	0.135	1	
Treponema 2	-0.352	-0.22	-0.215	-0.081	-0.028	0.05	-0.07	0.176	0.083	0.132	0.273	0.131	0.261	0.246	0.527	0.47	1

Table 7: Correlation of the most abundant genera present in *R. temporaria* samples. Values in bold are different from 0 with a significance level $\alpha \leq 0.05$

Variables	Other	Aeromonas	Rhodferax	Treponema 2	Tyzzarella 3	Cyanobium PCC-6307	Limnohabitans	Polynucleobacter	Hafnia-Obesumbacterium	Ruminococcaceae UCG-013	Pseudomonas	Desulfovibratio	Bacillus	Acinetobacter	Sphingorhabdus	Rikenella	Flavobacterium
Other	1																
Aeromonas	0.25	1															
Rhodferax	0.205	0.001	1														
Treponema 2	-0.051	0.381	0.143	1													
Tyzzarella 3	-0.145	0.002	0.087	0.181	1												
Cyanobium PCC-6307	-0.078	-0.125	-0.198	-0.3	-0.142	1											
Limnohabitans	0.348	0.25	0.024	0.19	-0.328	-0.171	1										
Polynucleobacter	0.134	0.104	0.053	0.128	0.25	-0.157	0.204	1									
Hafnia-Obesumbacterium	0.083	-0.106	0.086	-0.253	0.258	-0.128	-0.177	0.067	1								

Ruminococcaceae UCG-013	0.096	-0.15	0.02	-0.023	-0.001	-0.19	-0.047	0.019	-0.034	1							
Pseudomonas	-0.059	0.059	-0.191	0.178	-0.103	0.224	0	-0.212	-0.156	-0.155	1						
Desulfovibrio	0.033	-0.009	0.104	0.223	0.524	-0.275	-0.011	0.142	0.117	-0.039	-0.018	1					
Bacillus	-0.266	-0.042	-0.153	0.158	-0.056	-0.155	-0.007	-0.316	-0.035	-0.126	0.194	0.149	1				
Acinetobacter	-0.409	-0.075	-0.291	0.047	0.345	-0.054	-0.164	0.086	0.07	-0.028	0.056	0.433	0.32	1			
Sphingorhabdus	-0.106	-0.127	-0.209	-0.152	-0.157	-0.128	0.063	0.041	0.042	0.095	-0.02	0.116	0.263	0.234	1		
Rikenella	-0.559	-0.44	-0.3	-0.339	-0.189	0.175	-0.141	-0.072	0.074	-0.004	0.049	-0.283	0.25	0.286	0.31	1	
Flavobacterium	-0.192	-0.281	-0.171	-0.003	-0.215	-0.197	0.344	0.12	-0.09	0.103	0.051	0.185	0.29	0.335	0.629	0.499	1

Figure 19: Rarefaction curve of all the samples included in the dataset, considering the minimum number of sequences (13000 sequences/ samples). Samples with less sequences were discarded (n=3). Maximum number of sequences/ sample was 187.590.



Résumé du chapitre 3

Dans ce chapitre – « Le climat et les poissons impactent les microbiome cutané des têtards dans les lacs de montagne » – j'ai basé mon travail sur trois des quatre arêtes de la pyramide des maladies : l'espèce hôte, l'environnement et le microbiome associé à l'hôte. Pour examiner les relations possibles entre ces facteurs, j'ai analysé le microbiome cutané bactérien de 516 échantillons de têtards de trois espèces d'amphibiens (*Alytes obstetricans*, *Rana temporaria* et *Bufo bufo*) qui ont été collectés au cours des années 2016, 2017 et 2018 dans 19 lacs des Pyrénées, le long d'un gradient altitudinal. Les lacs étaient sous l'influence de (1) différentes variables climatiques : longueur de la saison de croissance, nombre de jours de gel et indice simple de précipitation ; (2) d'impacts anthropiques : empoisonnement et pression touristique ; et (3) de caractéristiques spécifiques : longitude, pH et altitude.

J'ai analysé l'impact de tous ces facteurs sur le microbiome bactérien cutané des têtards, ainsi que l'influence de l'espèce hôte, dans le contexte de la pyramide des maladies.

J'ai pu identifier un impact de l'espèce hôte sur le réseau des taxons bactériens les plus abondants, ainsi que sur leur présence et leur abondance relative, ce qui indique que l'espèce hôte est l'un des facteurs pouvant affecter la composition du microbiome cutané, même à un stade précoce du développement. J'ai également détecté une influence des variables climatiques sur la présence et l'abondance relative des taxons bactériens cutanés les plus abondants, en particulier les variables longueur de la saison de croissance et indice de précipitation simple. La température et l'humidité sont déjà connues pour avoir un impact sur la survie des amphibiens, en raison de leur ectothermie, et cet impact pourrait être renforcé par les altérations possibles de la composition de leur microbiome cutané, ce qui montre un effet des facteurs climatiques sur l'hôte et le microbiome de l'hôte. Enfin, un effet de l'empoisonnement a été détecté sur la composition de l'ensemble de la communauté bactérienne. Il a été observé qu'un groupe de quelques taxons bactériens étaient tous plus abondants dans les têtards des lacs avec poissons, en comparaison avec les lacs sans poissons. Une telle dominance de seulement quelques taxons pourrait causer un déséquilibre de la communauté microbienne et, par conséquent, affecter ses fonctions régulières (aide au système immunitaire de l'hôte et adaptations à l'environnement, par exemple). La présence de poissons peut, normalement, provoquer une réponse de stress chez les têtards, puisque les têtards

peuvent servir de proies aux poissons. Une telle réponse peut, par conséquent, modifier la dynamique entre l'hôte et son microbiome. De plus, les amphibiens peuvent acquérir une partie de leur microbiome à partir du milieu environnant, et la présence de poissons peut modifier la disponibilité et la composition du microbiome de l'eau en raison d'effets en cascade sur la chaîne alimentaire. Ces deux facteurs, combinés, pourraient être responsables des altérations observées dans le microbiome de la peau des têtards.

Chapter 4: Interactions between *Batrachochytrium dendrobatidis* and amphibian skin microbiome in montane common midwife toads

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Abstract

Amphibians are one of the most threatened groups of vertebrates, and one of the main reasons is chytridiomycosis, a disease caused by the fungi *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal), which disrupt the skin of the infected individuals impacting the exchange of gas and liquids. While Bd infection has been linked to changes in the bacterial skin community of amphibians, how the microbiome changes over time in enzootic and epizootic populations is still unknown. We analyzed the links between (a) Bd-positive and negative populations, (b) enzootic and epizootic populations and (c) individual Bd infection loads of *A. obstetricans* tadpoles with both bacterial and micro eukaryotic skin communities. We sequenced the V3-V4 region of the 16S rRNA gene and the V8-V9 region of the 18S rRNA gene of tadpole skin microorganisms. Samples were taken across 11 lakes in the French Pyrenees, three times

per year in 2016, 2017 and 2018. Bd-positive and epizootic populations, as well as infected individuals, have a distinct microbiome structure (beta diversity) compared to Bd-negative and enzootic populations and uninfected individuals, respectively. Bd-positive populations also had a higher number of observed ASVs and Shannon index, for both bacterial and micro eukaryotic communities, while individual infection load was not significant in determining the skin diversity of individual tadpoles. Enzootic populations tended to become more similar to uninfected ones over time, with a decrease of bacterial diversity, in contrast to epizootic populations, which tended to increase their skin bacterial diversity over the same period. Our results indicate a selection process occurring on both microbiome composition and diversity in enzootic and epizootic populations in regard to a potentially more Bd-resistant microbiome in enzootic ones.

Keywords: endemic, epidemic, wildlife disease, pathogenic fungus, chytridiomycosis, amphibians

Introduction

Emerging infectious diseases (EIDs) of wildlife have shown a constant increase in the last decades, leading to further attrition of biodiversity (Cunningham et al., 2017; Fisher and Garner, 2020). EIDs are caused by different pathogenic agents, e.g. bacteria, viruses and fungi. The emergence of these diseases is the result of the complex interactions between the host, the pathogen, the environment and the host microbiome (Bernardo-Cravo et al. 2020).

In wildlife, several EIDs have gained attention for their severe impact on host populations, such as the white-nose syndrome in bats (Lankau et al., 2016), the cowpox disease in wild rodents (Hazel et al., 2000), and chytridiomycosis causing an amphibian pandemic (Kilpatrick et al., 2010; Scheele et al., 2019). Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd) or *B. salamandrivorans*, is recognized as the worst current wildlife disease, causing up to 90% decline and even extinction in amphibian populations across all the continents, except for Antarctica

(Scheele et al., 2019). The fungus establishes itself in keratinized tissue, present all over the body in adult frogs, and in mouthparts of tadpoles.

Bd may cause local outbreaks, both enzootic and epizootic, depending on the host species and locations (Briggs et al., 2010). When analyzing only amphibian survival and Bd infection in the environment, Bd can be facilitated or hindered according to temperature and humidity conditions (Berger et al., 2004; Knapp et al., 2011; Preuss et al., 2020; Lambertini et al., 2021; Walker et al., 2010), host density (Briggs et al., 2010), host life history traits, such as breeding behavior (Lambertini et al., 2021) and the presence of potential Bd reservoirs (Briggs et al., 2010).

Enzootic and epizootic dynamics, however, are not defined only by host, environment and pathogen virulence. The host associated microbiome is also relevant in this context (Bernardo-Cravo et al., 2020). Many studies already worked on the relationship between Bd and amphibian skin microbiome under different conditions, such as captivity (Medina et al., 2019), geographic locations (Belden et al., 2015), forest connectivity (Becker et al., 2017), pH (Krynak et al., 2016), host life stage (Kueneman et al., 2016), among others (Holden et al., 2015; Longo et al., 2015; Bletz et al., 2017), with different outcomes for each environmental condition. Pathogen disturbance, however, has been suggested to be the cause of reduced bacterial diversity (Longo et al., 2015; Ellison et al., 2019), but Bd infection intensity can also be previously determined by initial microbiome composition and metabolites on the skin (Becker et al., 2015), showing that Bd and skin microbiome have a bi-directional relationship.

Amphibians from epizootic and enzootic populations show different bacterial microbiome composition and richness (Jani et al., 2017; Bates et al., 2018). The temporal variation of these different populations is still unknown. A short-term follow-up of 2 weeks showed that the bacterial community can change differently in enzootic and epizootic populations (Jani and Briggs, 2014), indicating a possible evolution of chytridiomycosis over time, driving differences in amphibian skin communities. In the long-term, differences in microbiome response could be driven by different selection processes of bacterial taxa (Wilber et al., 2020), through an increase in abundance of specific ASVs with inhibitory-Bd properties and a decrease in abundance and richness of taxa with no Bd-properties.

Considering the relationship between skin microbiome communities and Bd , we analyzed skin microbiome samples from tadpoles of *A. obstetricans* in eleven lakes in the

Pyrenean Mountain range in regard to (1) population infection status (Bd-positive or Bd-negative), (2) disease dynamic (enzootic, epizootic and uninfected) over a time period of three years (2016, 2017 and 2018) and (3) Bd load from individuals in Bd-positive populations.

We work with two hypotheses for the skin community regarding disease dynamic. The first hypothesis assumes that individuals from enzootic populations have a higher microbial diversity, which is responsible for their better immune protection on a long-term basis (Bates et al., 2018; Ellison et al., 2019) and, therefore, for their survival. On the other hand, individuals from epizootic lakes would have a less diverse skin microbiome, making them more prone to disease populations crash. Our second hypothesis considers the beginning of the microbial selection process in epizootic lakes and its development in enzootic populations. In epizootic lakes, the selection would act on the individuals with higher diversity and, therefore, individuals with low microbial diversity would die in the beginning of the outbreak. In enzootic populations, we would find only individuals that survived Bd arrival, thus, with high microbial diversity. Once the taxa capable of providing a better protection to Bd remains, there is no need to invest on both diversity and composition, causing a decline in general diversity due to taxa which do not contribute to disease protection.

Material and methods

Study area and sampling design

We sampled a total of 309 *A. obstetricans* tadpoles, across enzootic (N=110 tadpoles and 3 populations), epizootic (N=76 tadpoles and 3 populations) and uninfected populations (N=123 and 5 populations), comprising eleven lakes (=populations) along three altitudinal gradients (between 1063 and 2522 meter above sea level) in the Pyrenees (Figure 20 and Suppl. Mat. Table 11). Individuals were captured by dip-netting in the years 2016, 2017 and 2018 for both Bd detection and skin microbiome analysis. We covered the full growth season by sampling at the end of June/beginning of July, August, and end of September/beginning of October.

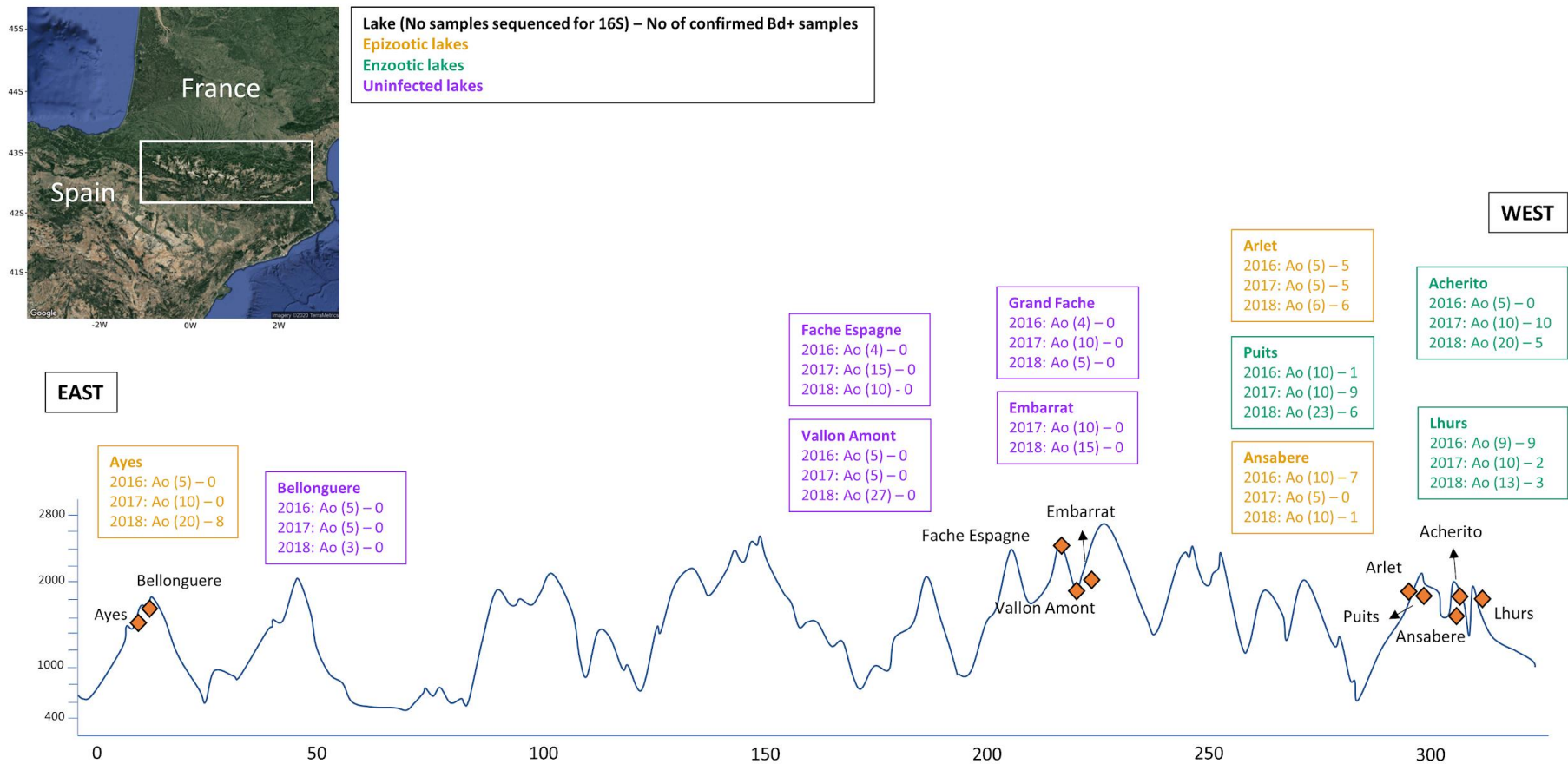


Figure 20: Altitude of the eleven lakes used in the study (y axis) and walking distance (in km) between them (x axis). The different colours represent the different disease classifications per population (orange: epizootic, green: enzootic and purple: uninfected). The number of *A. obstetricans* (Ao) samples sequenced for 16S rRNA gene in each year are between brackets and the number of confirmed infected individuals is written after the dash, as indicated in the legend. Only the bacterial dataset is displayed considering the overlap with the micro eukaryotic dataset

Skin microbiome and Bd data collection

Both the skin and the tadpole mouthparts were sampled with sterile dry swabs (MW100, MWE Medical Wire, Corsham, UK). Two swabs were used per individual: the first one was used on the whole body of the tadpole for microbiome analysis and a second one was used in the mouthparts for Bd detection. After the swabbing was complete, the individuals were immediately released back to the lake and the microbiome swabs were immediately frozen on dry ice at -79°C before their transfer to a freezer (-20°C) until DNA extraction. Bd swabs were stored in a cool box with ice packages and transferred to a fridge until DNA extraction. Where possible, we sampled 30 individuals per population for Bd detection (Boyle et al., 2004; Clare et al., 2016) (Suppl. Mat. Table 12), and of the 30 tadpoles, microbiome samples from up to ten individuals were taken.

Bd quantification

We applied the standard protocol for Bd quantification (Boyle et al., 2004). DNA from dry swabs (Medical Wire) was extracted using PrepMan Ultra reagents (Life Technologies). Cell disruption was done by adding microbeads to the reaction tube and shaking them in a FastPrep for 2x 45 seconds, with 30 second centrifugation in between. After a centrifugation for 3 minutes, we denatured the proteinase K in the reagent by heating it to 95°C for 10 min. After a final centrifugation of 3 minutes, the supernatant was transferred in 1.5 ml eppendorf tubes. Bd quantification was done with a diluted DNA sample (10x), using ITS1 and 5.8S regions of the Bd genome. The used primers were ITS1-3 Chytr (5'-CCTTGATATAATACAGTGTGCCATATGTC) and 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA) and the probe MGB2 (5'-6FAM CGAGTCGAACAAAAT MGBNFQ). The Q-PCR was run in duplex on a StepOnePlus™ Real-Time PCR System (Life Technologies). The Q-PCR master mix contained: 3.97µL of MiliQ water, 1µL of BSA, 12.5µL of TaqMan Fast Master Mix, 0.03µL of the MGB2 probe, 1.25µL of the ITS1-3 primer and 1.25µL of the 5.8S primer. We added 2.5µL of diluted DNA sample to 10 µL of the master mix. The Bd concentration was calculated based on the standard curve created with Bd standards from 0.1, 1, 10, and 100 Bd genomic equivalents (GE). We used the following Q-PCR run conditions: 50°C for 2 minutes, followed by 95°C for 10 minutes, 50 cycles at 95°C for 15s and 60°C for 1 minute. The samples were considered positive when

amplification above 0.1 genomic equivalents was detected in both replicates (Hughey et al., 2017).

Our Bd data, complemented by unpublished data since 2008, was used to classify (a) population infection status (Bd-positive populations n=6, Bd-negative n=5), (b) the disease classification per population (epizootic populations n=3, enzootic n=3) and (c) tadpoles into infected (Bd load>0) and uninfected (Bd load=0) individuals. Individual Bd loads were classified in low (0 - 20 GE), medium (21 - 100 GE), high (101 - 500 GE), and very high infection load (> 500 GE).

When comparing Bd-positive and Bd-negative populations, a dataset of 309 and 300 *A. obstetricans* samples was used, for bacteria and micro eukaryotes respectively (for bacteria: N=186 samples from Bd-positive populations and 123 from Bd-negative; for micro eukaryotes: N=185 samples from Bd-positive populations and N=115 from Bd-negative populations). In the comparison between enzootic and epizootic populations, we had 110 bacterial samples and 105 micro eukaryotic samples for enzootic populations, and 76 bacterial and 80 micro eukaryotic samples for epizootic populations (Table 11). Finally, in Bd-positive populations for the comparison between infected and non-infected individuals, 171 samples were used for 16S (78 infected and 93 uninfected individuals) and 164 (77 infected and 87 uninfected individuals) for 18S (Figure 20) (number of bacterial and micro eukaryotic samples per Bd load class can be seen in Figure 24).

Microbiome analysis

The DNA samples were extracted from swabs using the Macherey-Nagel™ NucleoSpin™ Soil kit (Düren, Germany). The extraction product was quantified using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) to determine the DNA concentration of each sample in order to prepare a PCR plate with DNA samples around 1µM for 16S and 18S rRNA genes, each. Negative controls from non-used sterile swabs were included and processed together with the regular samples.

For the profiling of the bacterial skin community, the V3-V4 region of the 16S rRNA gene was amplified using Illumina primers and adaptors (F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and R: 5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

(Klindworth et al., 2013).

For the micro-eukaryotic community, the V8-V9 region of the 18S rRNA gene was amplified, also with Illumina primers and adaptors (F:5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATAACAGGTCTGTGATGCCCT and R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTCYGCAGGTTACCTAC).

Our PCR mix contained 12.5µL MyTaq™ Mix, 1µL of 1µM forward and reverse primer, 0.5µL of BSA, and 10µL of sample DNA at a concentration of 1µM, summing up to 25µL per sample. The PCR conditions were as follows for the 16S rRNA gene protocol: 95°C for 3 minutes, 35 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s, followed by a final extension step at 72°C for 5 minutes. For the 18S rRNA gene, the conditions were as follows: 94°C for 3 minutes, 35 cycles at 94°C for 45s, 57°C for 60s and 72°C for 90s, with an extension step at 72°C for 3 minutes. PCR products and negative controls were visualized on a 1.5% agarose gel to confirm that no amplification of the control samples has occurred. The PCR products were cleaned up using Agencourt AMPure XP to remove non-target DNA and visualized once again on an agarose gel. Cleaned up products were submitted to a 10-cycle PCR according to the Illumina protocol with sample-specific Illumina Nextera Index primers. Indexed PCR products were cleaned up and quantified with the Qubit® 2.0 fluorometer. The samples were then diluted to 4nM, pooled, and the library was once again quantified to ensure the correct dilution for the sequencing. Finally, the library was sequenced on an Illumina MiSeq, using a MiSeq Reagent Kit v2.

Sequence data were pre-processed with QIIME2-2019.1 (Bolyen *et al.* 2019). The standard protocol was used, according to the following steps: filtration and assignment of sequences to the respective samples, quality control, correction of amplicon sequence errors, assignment of sequences to amplicon sequence variants (ASVs) and taxonomy classification. Forward and reverse reads were joined and sequences were filtered with a Q20 quality score. Sequences were clustered into ASVs at 99% similarity, according to the Silva132 reference database and a final ASV table and a taxonomic table were created (Yilmaz *et al.* 2014). Sequences belonging to Metazoa, Chloroplastida and Mitochondria groups, as well as Archaeae were removed from the dataset before analysis. We excluded all taxa with a sum across all samples < 0.01% for the micro-eukaryotic community (18S rRNA gene; n = 4180), and with a sum < 0.1% for the bacterial community (16S rRNA

gene; $n = 41980$), leaving 227 and 111 unique ASVs, respectively, for further analysis. The data was not rarefied at any step, since it can cause loss of precision and information when reaching a common read depth (McMurdie & Holmes 2014). However, the rarefaction curve was plotted to visualize the richness coverage for all the samples (Suppl. Mat. Figure 25).

Environmental data

For each of the eleven lakes, we obtained latitude, longitude, altitude, lake surface area, pH, relative O₂ and salinity. We used E-obs gridded climatic data with daily resolution (Version 20.0e, Cornes et al, 2018) and the R-package `climindex.pcic` (David Bronaugh for the Pacific Climate Impacts Consortium, 2020) for the calculation of climindex climatic extreme indices (<https://www.climdex.org/>) for 2016, 2017 and 2018. The calculation was done for the following indices: length of the growing season ($T_{\text{mean}} > 5^{\circ}\text{C}$), number of frost days ($T_{\text{min}} < 0^{\circ}\text{C}$), simple precipitation index (daily precipitation $\geq 1\text{mm}$) and number of consecutive dry days (daily precipitation $\leq 1\text{mm}$) per study site (Suppl. Mat. Table 13).

We created indices for fish, tourism and livestock impacts on amphibian skin microbial communities. The fish index was based on the frequency of stocking events. The fish stocking information was acquired through the Fédérations de Pêche of the French Départements Ariège, Haute Garonne, Hautes Pyrénées and Pyrénées Atlantiques, with visual confirmation on site. The index varied from 0 (the lake was neither stocked for fish, nor have we ever observed fish), 1 (fish stocking was less frequent than every two years, and fish presence was visually confirmed) to 2 (regular fish stocking every two years and confirmed fish presence). The tourism index varied from 1 to 10, considering the following parameters: (a) proximity of a mountain refuge (no refuge = 0 points, refuge more than 500m away from the lake = 1 point, if less than 500m away = 2 points), (b) proximity of the most popular hiking route in the Pyrenees, the GR10 (GR10 does not pass by the sampling location = 0 points, GR 10 passes by it = 1 point), (c) vicinity of other hiking routes to the sampling site (no route = 0, presence of route = 1), (d) distance to the closest parking lot (if $> 7.1\text{km}$ = 1 point, from 5.1 to 7km = 2 points, from 3.1 to 5km = 3 points, from 1.1 to 3 km = 4 points and between 0 and 1km = 5 points), and (e) the

indications of camping material and/or tents close to the lakes (no signs = 0, clear sign of camping = 1 point). Lastly, the livestock index was estimated based on the presence of sheep, cow, horse or donkey in the vicinity of the sampling sites and/or the presence of shepherd huts close to the lakes (0 = no livestock and no shepherd hut; 1 = one type of livestock was observed or there was a shepherd hut; 2 = two types of livestock were present).

Statistical analysis

Statistical analyses were conducted in RStudio (R Core Team, 2019), SAS® Studio 3.8 and the Galaxy page of the Huttenhower Lab (<http://huttenhower.sph.harvard.edu/galaxy/>). Different subsets of the dataset were used considering the different analysis.

We used permutational multivariate analyses of variance (permanovas) to compare the bacterial and the micro-eukaryotic communities between the same groups of samples (Bd-positive vs. Bd-negative populations, epizootic vs. enzootic populations, infected vs. uninfected individuals from Bd-positive populations). For the permanova analysis, the ASV abundance was transformed to compositional data and the Bray-Curtis dissimilarity distance was calculated with the *vegan* package. In order to identify potential bacterial and micro eukaryotic ASVs that were differentially abundant between the groups, we performed LDA Effect Size (LEfSe) analysis (LDA score ≥ 2 , $p \leq 0.01$) (Segata et al., 2010) on the Galaxy webpage of the Huttenhower Lab.

We performed a Mantel test (*vegan* R-package) between the matrix of the geographic distance between the lakes (Haversine distance based on latitude and longitude) and the matrix of Bray-Curtis dissimilarity based on the relative abundance of both the bacterial and the micro eukaryotic ASVs with the the whole available subset.

We used Kendall's Tau analysis to test for correlations among the annual climatic variables (number of frost days, growing season length, simple precipitation index and number of consecutive dry days) and among the anthropogenic indices (tourism, fish, livestock) and lake water pH. Because number of frost days and growing season length were correlated both in the Bd-positive and Bd-negative lakes dataset ($n=32$, $T_{30}=-0.41$, $p=0.02$) and in the enzootic and epizootic dataset ($n=18$, $T_{16}=-0.51$, $p=0.03$), we excluded frost days in our analysis (Suppl. Mat. Table 14) and kept growing season length, simple

precipitation index and number of consecutive dry days. We kept tourism pressure, fish introduction, livestock presence and pH in addition to our climate variables (Suppl. Mat. Table 15).

To explain alpha diversity indices (number of observed ASVs and Shannon index) for both bacterial and eukaryotic communities, we used generalized linear mixed models (GLMMs, *proc glimmix* SAS Studio®). The response variable was either the number of observed ASVs or Shannon index of bacteria and micro eukaryotes (with a log-normal distribution of error terms and log link function for number of observed ASVs and normal distribution for Shannon index and identity link function). For the models considering the population infection status (Bd-positive vs. Bd-negative populations) as a co-variable, we included the number of consecutive dry days, simple precipitation index, length of the growing season, tourism pressure, fish introduction, livestock presence and water pH in the first models, removing one variable at a time for the posterior models considering the highest p-value of each model. The final model was always the one with the lowest AIC. In the case of models with AIC differing only in decimals, the model that retained more significant variables was chosen.

Specific models considering the population infection status as response (response was either 0=Bd-negative population or 1=Bd-positive populations, with binomial distribution of error terms with logit link function, n=32 populations) were done with the same climatic variables (number of consecutive dry days, simple precipitation index, length of the growing season).

To explore if there are alterations in the alpha diversity indices for both bacteria and micro eukaryotes over time in enzootic and epizootic populations, we made specific GLMMs with the alpha diversity indices as response (same log-normal distribution of error terms and log link function for number of observed ASVs and normal distribution and identity link function for Shannon index), and only time (as julian days), disease classification per population (epizootic/enzootic/uninfected populations) and the interaction of time and disease classification as potential explanatory variables. Alpha diversity indices in the three population categories (epizootic/enzootic/uninfected) were compared with a Kruskal-Wallis test followed by a pairwise Dunn test with Benjamin-Hochberg adjustment (R-package *FSA*). The homogeneity of variance of the alpha diversity indices of the three groups was tested with a Fligner-Killeen test (R-package *car*). Lastly, we built GLMMs with alpha diversity indices of infected individuals,

considering only Bd load class and time as explanatory variables (n=6 populations, enzootic and epizootic populations combined).

Results

The skin microbiome in Bd-positive vs. Bd-negative populations

Bacterial communities showed a significant geographic signal with similarities decreasing with increasing spatial distance (Mantel test: $R_{5772}=0.46$; $p=0.001$), which was not the case in micro-eukaryotic communities ($R_{12204}=0.077$; $p=0.112$).

We observed significantly different bacterial and micro-eukaryotic communities in Bd-positive and Bd-negative populations (permanova: 16S: $F=27.41$, $R^2=0.08$, $p<0.001$; betadisper: $F=4.09$, $p=0.044$; 18S: $F=30.08$, $R^2=0.09$, $p<0.001$; betadisper: $F=45.81$, $p<0.001$) (Figure 21a and b). In the bacterial microbiome the differences were driven by the abundance of 79 ASVs (LefSe, LDA score ≥ 2 , $p\leq 0.01$): 42 ASVs from 26 taxa were more abundant in tadpoles from Bd-positive populations, and 37 ASVs (22 taxa) were more abundant in tadpoles from Bd-negative populations (Suppl. Mat. Figure 26). In the micro-eukaryotic community, differences were driven by abundance differences of 73 ASVs: 41 ASVs (26 taxa) were more abundant in individuals from Bd-positive populations and 32 ASVs (17 taxa) more abundant in Bd-negative ones (Suppl. Mat. Figure 27).

The skin bacterial diversity was mainly explained by the population infection status (Bd-positive or Bd-negative) and livestock, while micro eukaryotic diversity was explained by the population infection status, anthropogenic (livestock, tourism and fish) and climatic (growing season length, consecutive dry days and simple precipitation index) variables (Table 8). Both bacterial and micro eukaryotic communities had more ASVs and higher Shannon indices in Bd-positive populations, while micro eukaryotes had more ASVs and bacteria had a lower Shannon index when livestock pressure was higher. Specifically for the micro eukaryotes, the climatic variables had distinct impacts on its diversity: diversity was lower when the precipitation index was higher, and tadpoles had more ASVs with longer growing seasons and less consecutive dry days.

The population infection status has not been explained by any of the climatic variables tested (GLMM, growing season length: $F_{1,28}=0.03$, $p=0.863$; simple precipitation index: $F_{1,28}=0.01$, $p=0.921$; consecutive dry days: $F_{1,28}=0.26$, $p=0.617$).

Table 8: Best models explaining the alpha diversity indices of both bacteria and micro eukaryotic communities, considering the environmental variables and the population infection status (Bd-positive and Bd-negative). Only models with the lowest AIC are displayed. Significant values are marked in bold. The direction of the relationship (+/-) is only shown for significant variables.

Alpha diversity indices				
Response	Explanatory variables	F-value	p-value	Direction of relationship
Observed ASVs 16S AIC = 455.12	Livestock	$F_{1,7.88}=4.06$	0.079	
	Population infection status	$F_{1,8.53}=12.73$	0.007	Bd-pos > Bd-neg
Shannon 16S AIC = 855.71	pH	$F_{1,8.49}=3.48$	0.097	
	Livestock	$F_{1,6.95}=7.42$	0.030	-
	Population infection status	$F_{1,8.47}=9.0$	0.016	Bd-pos > Bd-neg
Observed ASVs 18S AIC = 593.81	Time	$F_{1,266.2}=16.59$	<0.001	-
	Tourism	$F_{1,7.59}=17.84$	0.003	+
	Livestock	$F_{1,8.01}=17.37$	0.003	+
	Growing season length	$F_{1,291}=16.09$	<0.001	+
	Simple precipitation index	$F_{1,261.4}=20.9$	<0.001	-
	Consecutive dry days	$F_{1,279.2}=11.74$	<0.001	-
	Population infection status	$F_{1,8.15}=8.37$	0.02	Bd-pos > Bd-neg
Shannon 18S AIC = 587.94	Time	$F_{1,290.8}=13.59$	<0.001	-
	Tourism	$F_{1,6.90}=20.29$	0.003	+
	Fish	$F_{1,7.13}=6.72$	0.035	+
	Livestock	$F_{1,6.66}=8.44$	0.024	+
	Simple precipitation index	$F_{1,292.6}=13.89$	<0.001	-
	Population infection status	$F_{1,6.39}=7.69$	0.030	Bd-pos > Bd-neg

The skin microbiome in enzootic vs. epizootic populations

We observed marked differences in the microbial communities on tadpole skin between epizootic and enzootic populations (permanova bacteria: $F=13.64$, $R^2=0.07$, $p<0.001$; betadisper $F=2.88$, $p=0.097$; permanova eukaryotes: $F=6.74$, $R^2=0.035$, $p<0.001$, betadisper: $F=1.79$, $p=0.18$) (Figure 21). We observed 18 bacterial ASVs (from 13 taxa) and 55 micro-eukaryotic ASVs (32 taxa) that were more abundant in tadpoles from epizootic populations, while 24 ASVs (18 bacterial taxa) and 14 ASVs (12 micro-eukaryotic taxa) were more abundant in tadpoles from enzootic populations (LefSe, LDA score ≥ 2 , $p\leq 0.01$) (Suppl. Mat. Figures 28 and 29).

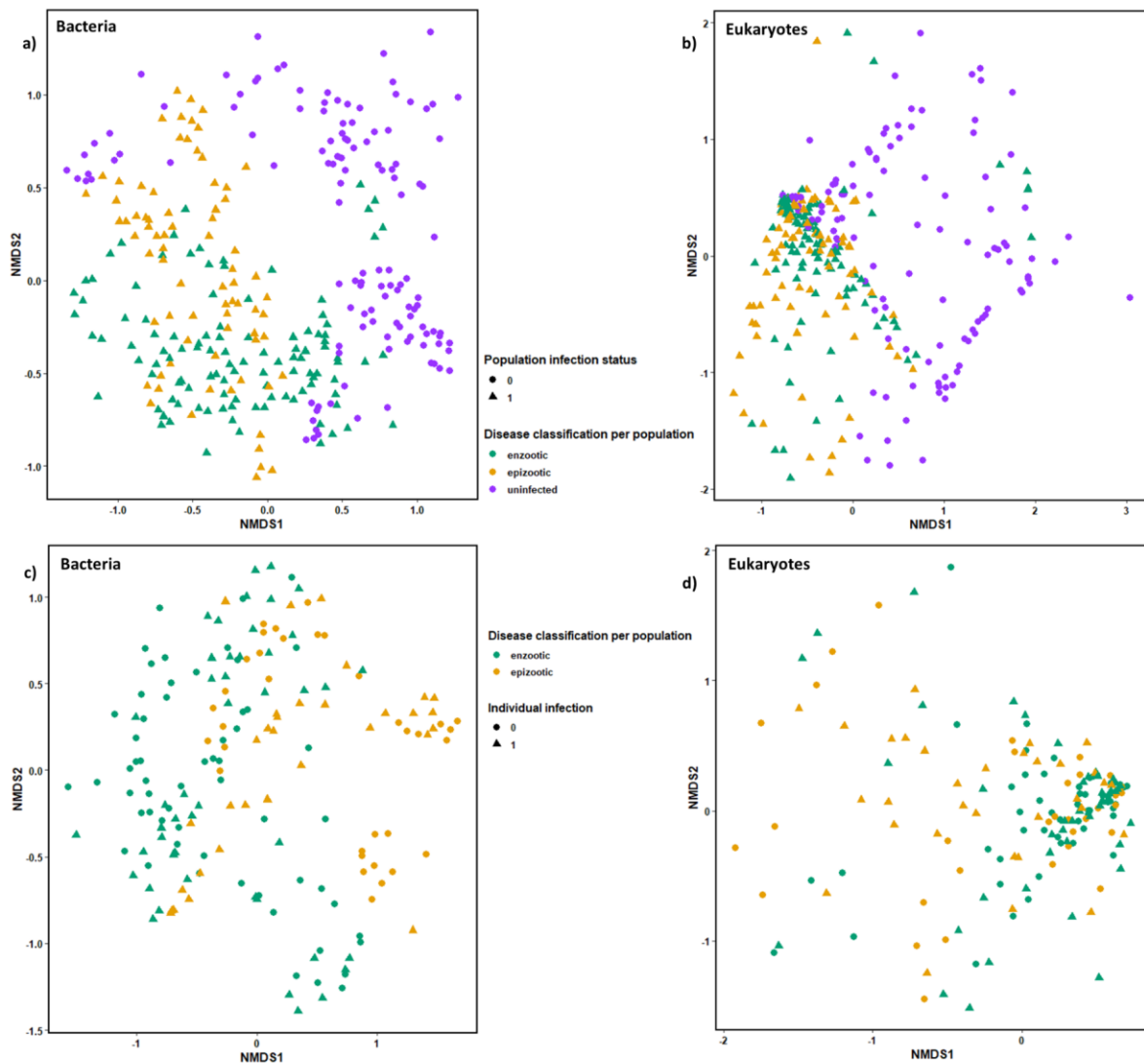


Figure 21. Non-metric multidimensional scaling (NMSD) plot of Bray–Curtis distances. Each point represents the skin bacterial community of a tadpole of *A. obstetricans*. A) Bacterial microbiome considering the population infection status (0=Bd-negative; 1=Bd-positive) and the disease classification per population (epizootic and enzootic). B) Micro eukaryotic microbiome considering Bd-positive and negative populations and disease classification per population. C) Bacterial microbiome considering infected (Individual infection=1) and uninfected individuals (Individual infection=0) from Bd-positive populations. D) Micro eukaryotic microbiome considering infected (Individual infection=1) and uninfected individuals (Individual infection=0) from Bd-positive populations.

The temporal trajectories of microbial diversity in epizootic, enzootic and uninfected lakes differed in their direction. In epizootic populations, alpha diversity increased over time, while it decreased in uninfected and enzootic populations (Table 9 and Figure 22).

Table 9: Single models explaining the alpha diversity indices of both bacteria and micro eukaryotic microbiomes, considering only the time, the disease classification per population (epizootic, enzootic and uninfected) and their interaction with time. Significant values ($p \geq 0.05$) are marked in bold.

Alpha diversity indices			
Response	Explanatory variables	F-value	p-value
Observed ASVs 16S AIC = 263.04	Time	$F_{1,29992}=0.09$	0.763
	Disease class	$F_{2,17.86}=4.12$	0.034
	Time*disease class	$F_{2,299.4}=5.1$	0.007
Shannon 16S AIC = 521.23	Time	$F_{1,299.1}=0.05$	0.822
	Disease class	$F_{2,15.43}=2.39$	0.124
	Time*disease class	$F_{2,298.8}=3.69$	0.026
Observed ASVs 18S AIC = 593.81	Time	$F_{1,288.9}=0.83$	0.363
	Disease class	$F_{2,13.17}=0.84$	0.452
	Time*disease class	$F_{2,288.7}=3.59$	0.029
Shannon 18S AIC=374.82	Time	$F_{1,289.3}=0.98$	0.322
	Disease class	$F_{2,14.12}=0.04$	0.958
	Time*disease class	$F_{2,289}=0.48$	0.618

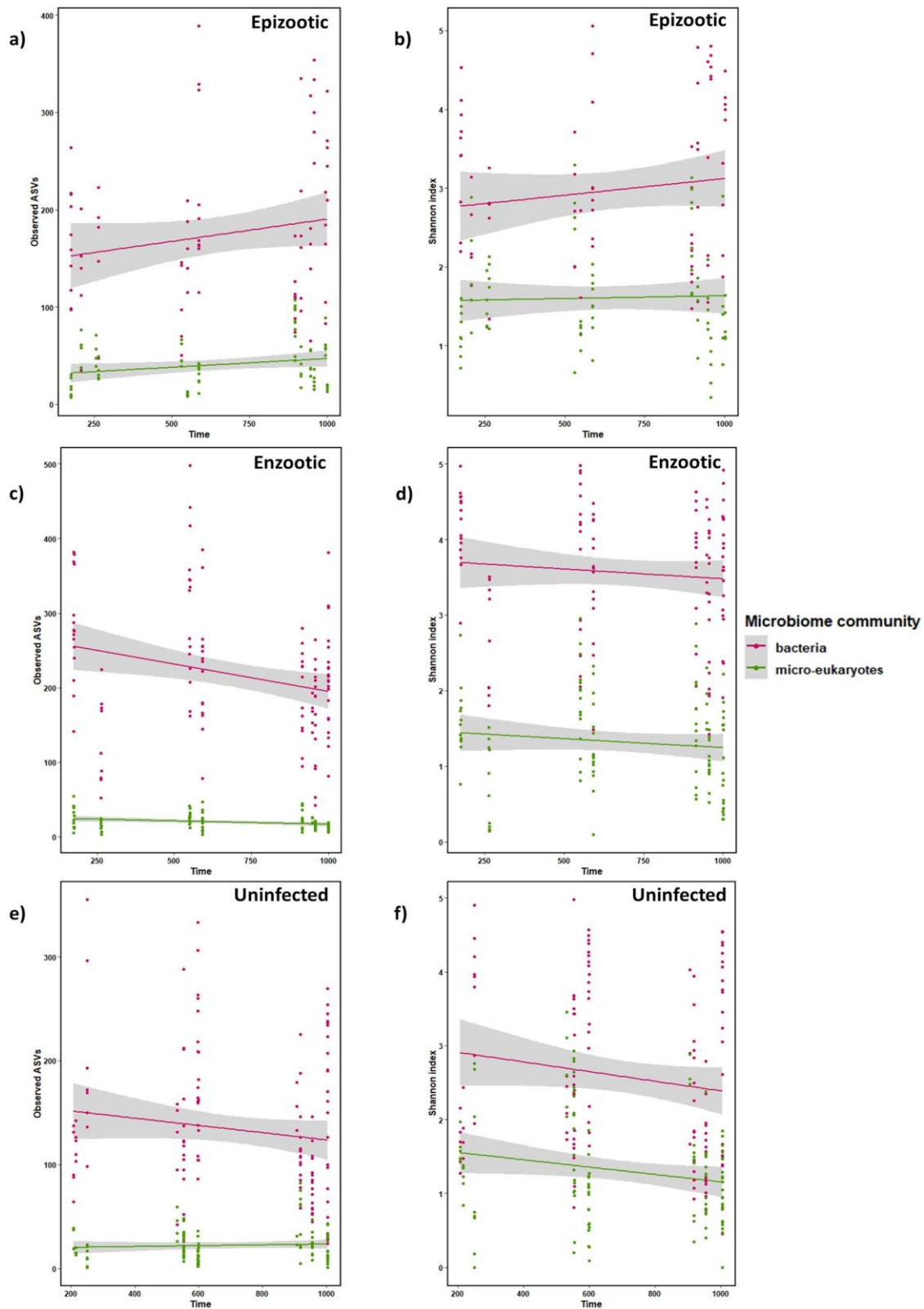


Figure 22: Alpha diversity indices of bacteria and micro eukaryotes (number of observed ASVs and Shannon index) over time, considering the enzootic, epizootic and uninfected populations. The lines were created based on the linear model $y \sim x$ and the shadow area represents a 95% confidence interval.

Generally, we observed the highest microbial diversity on tadpole skin from enzootic populations, followed by tadpoles from epizootic and uninfected populations, both for bacteria and micro-eukaryotes (Figure 23 and Table 10). The highest variance of species richness and abundance (Shannon index) was observed in tadpoles from uninfected populations, while the variance of the number of observed ASVs was highest in tadpoles from the epizootic populations (Suppl. Mat. Table 16).

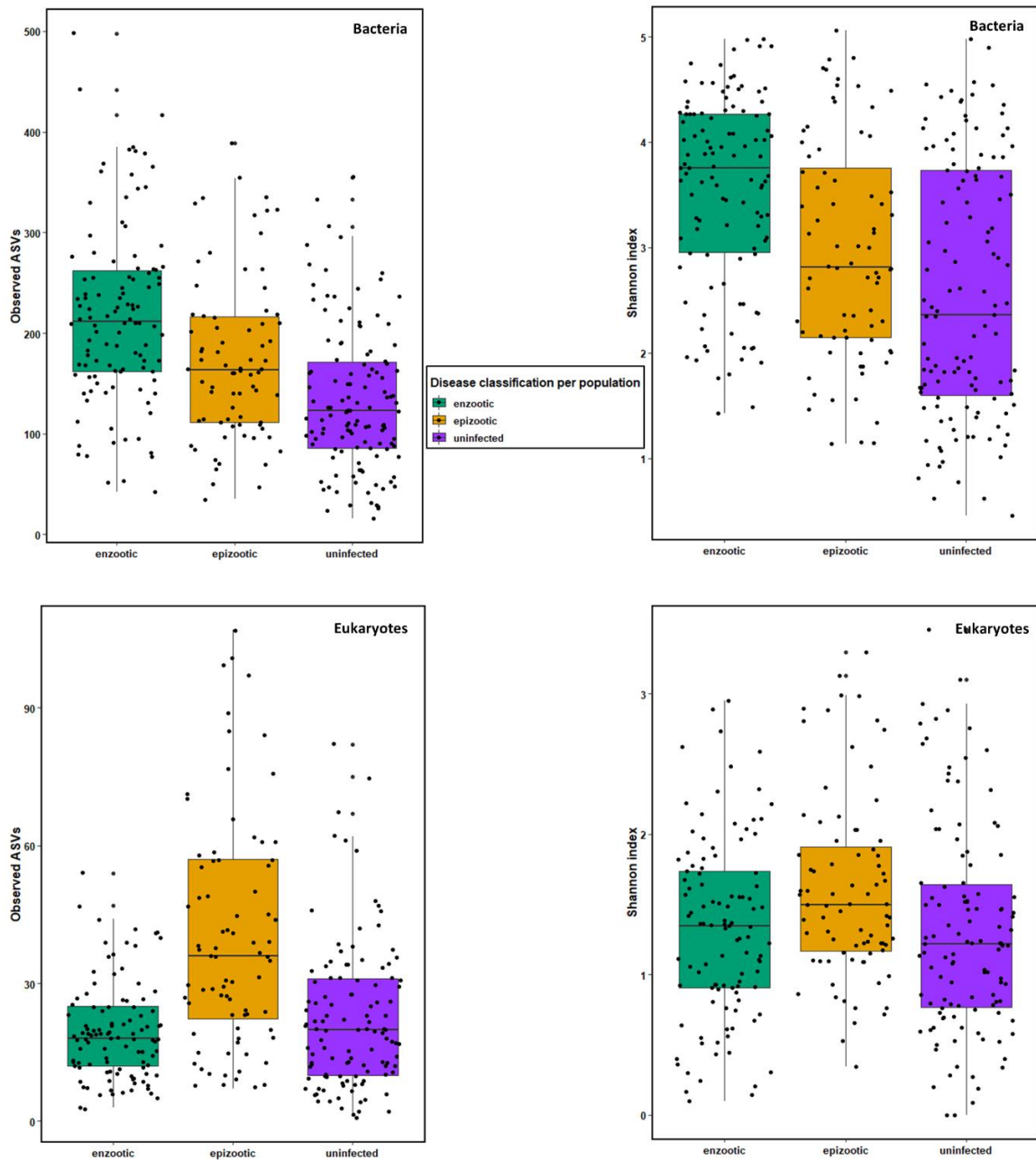


Figure 23: Alpha diversity (number of observed ASVs and Shannon index) of bacteria and micro eukaryotes across epizootic, enzootic and uninfected populations, all samples combined.

Table 10: Results of the pairwise Dunn tests. Comparisons were done between enzootic vs. epizootic populations, enzootic vs. uninfected populations and epizootic vs. uninfected populations. The number of degrees of freedom for all the comparisons is 1. Values in bold are significant ($p < 0.05$). The column “Direction of the relationship” indicates in each group the diversity index tested is higher.

Community	Index	Comparison	Z	p-value	Direction of the relationship
Bacteria	Observed ASVs	Epizootic vs. enzootic	3.27	0.001	enzootic > epizootic
Bacteria	Shannon	Epizootic vs. enzootic	3.48	<0.001	enzootic > epizootic
Bacteria	Observed ASVs	Epizootic vs. uninfected	3.34	0.001	epizootic > uninfected
Bacteria	Shannon	Epizootic vs. uninfected	2.20	0.028	epizootic > uninfected
Bacteria	Observed ASVs	Enzootic vs. uninfected	7.43	<0.001	enzootic > uninfected
Bacteria	Shannon	Enzootic vs. uninfected	6.40	<0.001	enzootic > uninfected
Micro eukaryotes	Observed ASVs	Epizootic vs. enzootic	-6.24	<0.001	enzootic < epizootic
Micro eukaryotes	Shannon	Epizootic vs. enzootic	-2.56	0.016	enzootic < epizootic
Micro eukaryotes	Observed ASVs	Epizootic vs uninfected	5.62	<0.001	epizootic > uninfected
Micro eukaryotes	Shannon	Epizootic vs uninfected	3.22	0.004	epizootic > uninfected
Micro eukaryotes	Observed ASVs	Enzootic vs uninfected	-0.79	0.425	
Micro eukaryotes	Shannon	Enzootic vs uninfected	0.66	0.512	

The skin microbiome in infected and uninfected individuals

We observed significant differences in the microbiome, between infected and uninfected individuals (permanova bacteria: $F=3.30$, $R^2=0.02$, $p=0.001$; betadisper: $F=0.48$, $p=0.48$; permanova eukaryotes: $F=2.09$, $R^2=0.013$, $p=0.04$; betadisper: $F=2.22$, $p=0.13$) (Figure 21c and d). These differences were driven by the relative abundances of a total of 10 bacterial ASVs and 8 micro-eukaryotic ASVs (Suppl. Mat. Figure 30).

We observed generally higher bacterial richness (higher number of observed ASVs) in uninfected individuals (GLMM, Bd infection $F_{1,131}=14.27$, $p<0.001$), while for the eukaryotic community, none of the diversity indices were linked to Bd infection or Bd load class (Figure 24).

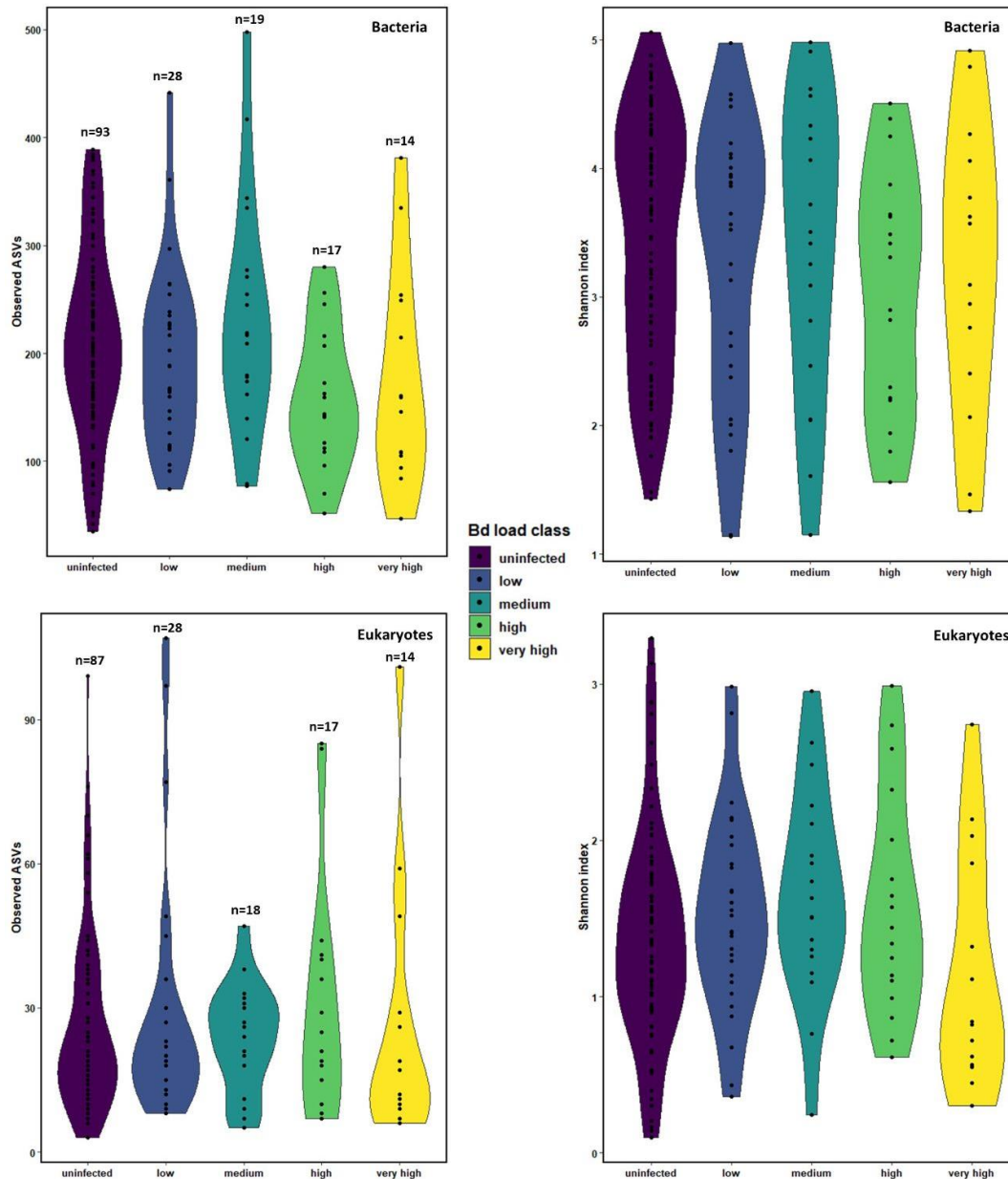


Figure 24: Alpha diversity (number of observed ASVs and Shannon index) indices of bacteria and micro eukaryotes across the different Bd load classes. Bacterial dataset comprised 171 samples and micro eukaryotes 164 samples. The number of samples for each load class is indicated on the plot and the same number of samples were used for both observed ASVs and Shannon index models.

Discussion

Using Bd population data and microbiome sequencing, we compared (1) Bd-positive vs. Bd-negative populations, (2) enzootic vs. epizootic populations over time and (3) infected vs. uninfected individuals from Bd-positive populations, all of them

considering the bacterial and micro eukaryotic skin communities of *A. obstetricans* tadpoles. Our results show that the presence of Bd in the population (Bd positive-negative populations) and the disease dynamic (enzootic-epizootic populations) are stronger drivers than individual Bd loads carried by the tadpoles. We also show that microbiome diversity over time is significantly different in enzootic, epizootic and uninfected populations, with epizootic populations increasing their diversity while enzootic and uninfected ones show a decreasing pattern. This can suggest a selection process on enzootic populations, which allows the individuals to persist with Bd.

We detected a significant link between population infection status (Bd-positive and negative populations) and the microbiome structure. Even though the overdispersion detected in both microbiome communities could have an impact on the output of the community composition comparison, the high number of ASVs which are differentially abundant between the two groups (identified by the LefSe analysis) confirm that the Bd presence in the population is detrimental for the abundance of specific ASVs on the skin microbiome, both for bacteria and micro eukaryotes. The LefSe analysis on Bd-positive and Bd-negative populations indicated that some genera, such as *Pseudomonas* and *Limnohabitans* and some families, like *Burkholderiaceae* may be significantly abundant in both populations (Bd-positive and negative), since different ASVs belonging to these taxa are present in both groups. The same result has been found before (Woodhams et al., 2015), where ASVs from *Pseudomonas* genus could either enhance or inhibit Bd activity on the skin in different amphibian species or life stages. The differential abundance of *Pseudomonas* was clearer on the skin of infected and uninfected tadpoles (from Bd positive lakes). Three out of the five ASVs that were significantly more abundant in infected individuals belonged to this genus, while the other two belonged to the genus *Flavobacterium*, which was already reported to have ASVs with Bd-inhibitory capacities (Lam et al., 2010; Shaw et al., 2014). Many bacterial species or ASVs might, however, fall into a gradient of host-microbiome associations, ranging from mutualism with the host to pathogenicity, depending on the intrinsic host regulation or on a combination of habitat-specific variables (Lam et al., 2010) changing their role in the host microbiome dynamic according to factors which are external to Bd severity. Additionally, four out of the 10 ASVs (bacteria and micro eukaryotic communities combined) characterising infected tadpoles were shared with Bd-positive

populations, indicating that individuals carrying Bd could play a role in defining the signature microbiome of their living site, in case of infection.

Environmental variables, such as presence of livestock and tourism pressure, were significantly linked to the microbiome diversity, while we detected an isolation by distance regarding the bacterial community composition. This suggests that variation of environmental conditions along a spatial gradient can alter both microbiome diversity and composition (differentially for bacteria and micro eukaryotes) in the face of a pathogen, potentially affecting the microbiome capacity to protect the host against Bd. A similar combination of spatial and environmental variation could affect Bd spread, as well, considering the observed clusters of Bd-positive and negative populations (Figure 1) along our sampling sites.

In general, the bacterial community was more sensitive than the micro eukaryotes to disease classification per population and individual infection. Permanova analysis showed a higher explanatory power (R^2) on the bacterial community (in comparison to the micro eukaryotes), when comparing enzootic and epizootic populations and infected and uninfected individuals. Both bacterial alpha diversity indices (observed ASVs and Shannon index) were significantly different across enzootic, epizootic and uninfected populations and were differently affected over time. Micro eukaryotes, however, were less affected by Bd variables. None of the diversity indices was significantly different between enzootic and uninfected populations, only in their comparison with the epizootic one. Shannon index also did not differ over time between enzootic, epizootic and uninfected populations, while the number of observed ASVs was significantly affected by it, but with a less clear tendency, in comparison to the bacterial community. Other studies with micro-eukaryotes (fungi more specifically) found diverging results regarding their relationship with Bd. Medina and colleagues identified a correlation between Bd presence and the abundance of specific OTUs, however no relationship between the community diversity and structure and Bd infection was identified (Medina et al., 2019). In another study, fungi abundance contributed more significantly to Bd defense than bacteria, in an experiment with adults of the amphibian species *Dendrobates sp* (Kearns et al., 2017), while other micro eukaryotic groups have not been indicated as inhibitors of Bd infection (Kueneman et al., 2016, 2017). We observed that the micro eukaryotic community was more influenced by environmental variables, including climate, while the associations with Bd-related variables were weak.

Our results on alpha diversity variation in enzootic, epizootic and uninfected populations and their differences in tendency over time support our second hypothesis. Epizootic populations showed a significant increasing tendency in bacterial alpha diversity, with both the number of ASVs and Shannon index being distinct to enzootic populations. Here, individuals with lower skin bacterial diversity would suffer a stronger selection pressure due to their potential lower capacity to protect the tadpoles against Bd. Hence, individuals with higher skin bacterial diversity would have higher chances of survival. In this context, diversity would increase over time because only individuals with diverse microbiomes would be able to remain in the lake. In enzootic populations, diversity would start higher, in comparison to epizootic ones, due to the first selection process undergone by the surviving individuals. Our results suggest that, in this case, both diversity and composition are continuously regulated by Bd presence in the population and once the taxa which are more capable of protecting the host against Bd undergo a selection process and are retained on the skin, the regulation mainly occurs in the community composition, so there is no more increase in the overall richness of the community, even though it is generally higher in enzootic than in epizootic lakes. In a short-term survey of 2 weeks in a US national park, OTUs that were negatively correlated with Bd tended to decrease over time in an epizootic population, while OTUs that were positively correlated to Bd tended to increase over time (Jani & Briggs, 2014), suggesting a natural re-organization of the microbiome in regard to Bd. A previous study (Bates et al., 2018) with metamorphs conducted in the same region of our work also identified differences in community composition between epizootic and enzootic populations, with a reduction in the Shannon index in epizootic populations, in comparison to enzootic ones. We found a slightly lower Shannon index in epizootic populations compared to enzootics), however it was not significant. Lastly, we observed that uninfected populations showed the same decreasing pattern of alpha diversity as enzootic ones over time. Our results showed that uninfected populations also presented a general difference in the diversity indices, which are lower than enzootic and epizootic populations. Here, however, there is no Bd regulation of any kind and all the variation seen is due to environmental and host-specific factors. The variance comparison reveals that the Shannon index varies more in uninfected populations, while the number of observed ASVs does not vary differently in comparison to the enzootic and epizootic populations. The higher variance could be due to the different selection processes driving the

community in different directions, which is expected in any area under different environmental conditions. The decreasing tendency over time suggests that, even though general diversity is under different pressures, the community composition could be more relevant than the richness itself, causing the decline of the number of observed ASVs.

Finally, the presence and the infection load of Bd on the tadpoles skin was not an important factor defining skin diversity of bacteria and micro eukaryotes. For this analysis, infected and uninfected tadpoles that shared the same habitat were compared, and, as observed in the Mantel test, there was a strong geographic effect on the skin microbiome composition of *A. obstetricans*. This impact could be more relevant for the microbiome than the individual Bd load, making individuals that share the same habitat more similar to each other. Additionally, tadpoles may be less susceptible to Bd than post-metamorphs (Blaustein et al., 2005; Langhammer et al., 2014), probably due to the restricted keratinized mouthparts, so possible microbiome changes due to Bd presence would be less detectable on an individual level. Even though the community composition was significantly affected by the Bd presence on the skin, its influence was lower than the disease classification per population and the population infection status.

Our results show a consistent influence of Bd-related factors on the skin microbiome of amphibians. The population infection status (Bd-positive or negative) showed to be more important than individual loads of Bd in regard to association to the community composition and diversity, for both bacteria and eukaryotes. The diversity variation observed in enzootic, epizootic and uninfected populations over time indicates medium- to long-term changes on the skin communities, caused by Bd oriented selection mainly on bacterial taxa. Different kinds of environmental changes, however, can modify the response of the host to Bd impact (Bernardo-Cravo et al., 2020), and therefore, their survival over time and under different Bd pressures.

Supplementary Material for Chapter 4

Table 11: Number of tadpole samples sequenced for 16S rRNA gene and 18S rRNA gene, across all the lakes over time. The dataset consists of 309 and 300 samples for bacteria and micro eukaryotes, respectively. The ratio represents the number of confirmed Bd-positive samples/ number of total samples sequenced. The positive samples for Bd are the same for both 16S and 18S.

Lake	Altitude (m)	Coordinates	Infection classification	2016								2017						2018									
				june		july		aug		sept		june		july		august		june		july		august		sept		oct	
				16S	18S	16S	18S	16S	18S	16S	18S	16S	18S	16S	18S	16S	18S	16S	18S	16S	18S	16S	18S	16S	18S	16S	18S
Acherito	1858	42.878; -0.709	enzoo	0/5	0/5									5/5	5/5	5/5	5/5			3/10	3/10	0/5	0/5	2/5	1/4		
Ansabere	1876	42.888; -0.709	epizoo	4/5	4/4					3/5	3/5					0/5	0/5					0/5	0/5	1/5	1/5		
Arlet	1974	42.84; -0.615	epizoo	5/5	5/5								5/5	5/5					6/6	6/6							
Ayes	1681	42.844; 1.065	epizoo			0/5	0/5			0/5		0/5	0/5			0/5	0/5	8/10	8/10			0/5	0/5	0/5	0/5		
Bellonguere	1864	42.84; 1.063	uninf			0/5	0/5					0/5	0/5					0/3	0/3								
Embarrat	2180	42.84; -0.19	uninf											0/5	0/5	0/5	0/5			0/5	0/4	0/5	0/4			0/5	0/5
Fache Espagne	2522	42.814; -0.246	uninf							0/4	0/4			0/5	0/5	0/10	0/10									0/10	0/10

Grand Fache	2430	42.813; -0.223	uninf							0/4	0/4			0/5	0/4	0/5	0/5					0/5	0/5				
Lhurs	1691	42.922; -0.702	enzoo	5/5	5/5					4/4	4/5			0/5	0/5	2/5	2/5					2/5	2/4	1/8	1/5		
Puits	1891	42.864; -0.633	enzootic	0/5	0/4					1/5	1/5			5/5	5/5	4/5	4/5			3/3	3/3	0/1 0	0/1 0	4/1 0	4/1 0		
Vallon Amont	2215	42.838; -0.187	uninfected					0/5	0/5					0/5	0/5					0/1 0	0/5	0/7	0/7			0/1 0	0/1 0

Table 12: Prevalence of Bd infected samples considering samples collected for Bd detection in each population (maximum of 30). Epizootic, enzootic and uninfected populations are indicated as enzo, epizo and uninf, respectively, in brackets.

	2016			2017			2018		
	june/ july	august	september	june/ july	august	september	june/ july	august	september/ october
	Bd+	Bd+	Bd+	Bd+	Bd+	Bd+	Bd+	Bd+	Bd+
Acherito (enzo)	0/10			25/25			21/29		3/10
Ansabere (epizo)	4/5		8/10		0/10			0/20	2/10
Arlet (epizo)	5/5			29/29			6/6		
Ayes (epizo)	0/10			0/30	0/5		21/30		
Bellonguere (uninf)	0/30			0/9			0/3		
Embarrat (uninf)				0/30	0/10		0/30	0/10	0/5
Fache Espagne (uninf)			0/10	0/30	0/10			0/30	
Grand Fache (uninf)			0/30	0/25	0/10			0/30	

Lhurs (enzo)	13/13		4/4	0/24	9/30			5/30	2/10
Puits (enzo)	0/5		2/10	25/26	6/10		3/3	0/30	4/10
Vallon Amount (uninf)		0/10		0/30			0/30	0/7	0/10

Table 13: Description of the climatic data used for the analysis, considering some of the Climdex indices.

Index	Description
Length of the growing season	Annual count between the first span of at least 6 days with daily mean temperature >5°C and the first span after July 1 st of 6 days with T<5°C
Number of frost days	Annual number of days when the daily minimum temperature is < 0°C
Consecutive dry days	Maximum number of consecutive days with precipitation level < 1mm
Simple precipitation index	daily precipitation amount on rainy days divided by the number of rainy days

Table 14: Correlation values (R^2) of the annual climatic data from lakes classified either as infected or uninfected (n=32). Values in bold are different from zero and p-values are between brackets.

	Growing season length	Number of frost days	Simple precipitation index	Number of consecutive dry days
Growing season length	1			
Number of frost days	-0.41 (0.02)	1		
Simple precipitation index	-0.13 (0.47)	0.03 (0.87)	1	
Number of consecutive dry days	-0.14 (0.44)	0.12 (0.50)	-0.35 (0.05)	1

Table 15: Correlation values (R^2) of the unique variables, considering the complete dataset of populations classified either as infected or uninfected (n=11). None of the correlation values were significantly different (p-values are between brackets).

	Tourism	Fish	Livestock	pH
Tourism	1			
Fish	0.44 (0.17)	1		
Livestock	0.09 (0.8)	-0.13 (0.70)	1	
pH	0.32 (0.34)	-0.23 (0.50)	0.19 (0.58)	1

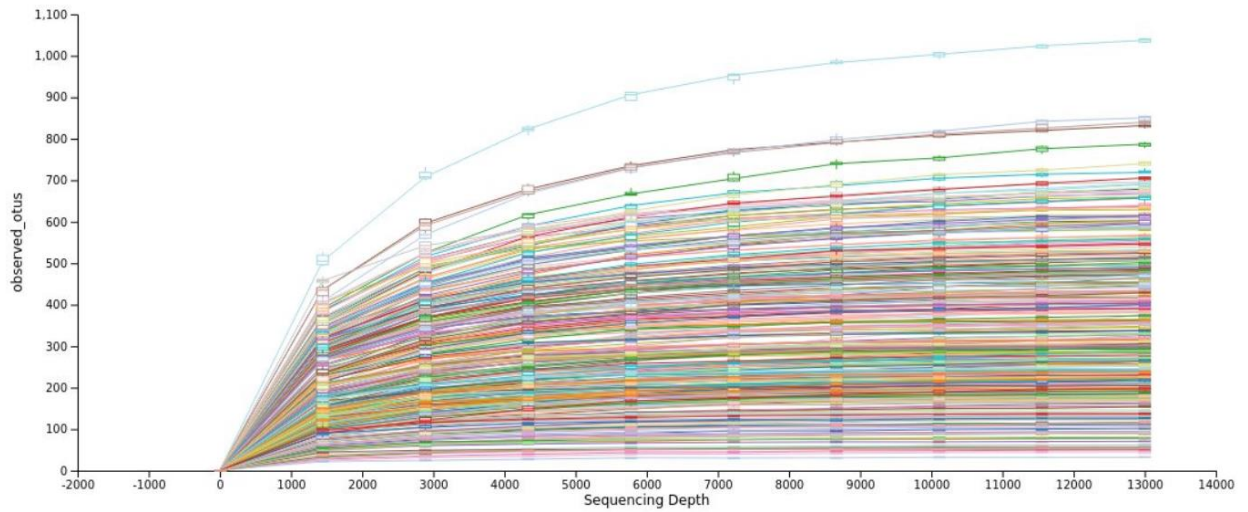
Table 16: Results of the Fligner-Killeen test of homogeneity of variances. Pairwise comparisons were done between enzootic vs. epizootic populations, enzootic vs. uninfected populations and epizootic vs. uninfected populations. The number of degrees of freedom for all the comparisons is 1. Values in bold are significant (p<0.05). The column “Direction of the relationship” indicates in each group the variance is higher.

Community	Index	Comparison	X^2	p-value	Direction of the relationship
Bacteria	Observed ASVs	Epizootic vs. enzootic	0.259	0.611	
Bacteria	Shannon	Epizootic vs. enzootic	2.167	0.141	
Bacteria	Observed ASVs	Epizootic vs. uninfected	0.897	0.343	
Bacteria	Shannon	Epizootic vs. uninfected	4.116	0.042	epizootic<uninfected
Bacteria	Observed ASVs	Enzootic vs. uninfected	2.918	0.088	
Bacteria	Shannon	Enzootic vs. uninfected	15.135	<0.001	enzootic<uninfected
Micro eukaryotes	Observed ASVs	Epizootic vs. enzootic	36.87	<0.001	enzootic<epizootic
Micro eukaryotes	Shannon	Epizootic vs. enzootic	0.149	0.700	
Micro eukaryotes	Observed ASVs	Epizootic vs. uninfected	16.962	<0.001	epizootic>uninfected

Micro eukaryotes	Shannon	Epizootic vs. uninfected	1.536	0.215	
Micro eukaryotes	Observed ASVs	Enzootic vs. uninfected	9.612	0.002	enzootic<uninfected
Micro eukaryotes	Shannon	Enzootic vs. uninfected	0.805	0.367	

Figure 25: Rarefaction curve of all the samples included in both (a) bacterial and (b) micro eukaryotic datasets, considering the minimum number of sequences as 13000 for bacteria and 6440 for micro eukaryotes. Three samples from the bacterial and five from the micro eukaryotic datasets were discarded for having less sequences. Maximum number of sequences/sample was 211.135 and 132.646 for bacteria and micro eukaryotes, respectively.

(a) Rarefaction curve for bacteria (16S rRNA gene)



(b) Rarefaction curve for micro eukaryotes (18S rRNA gene)

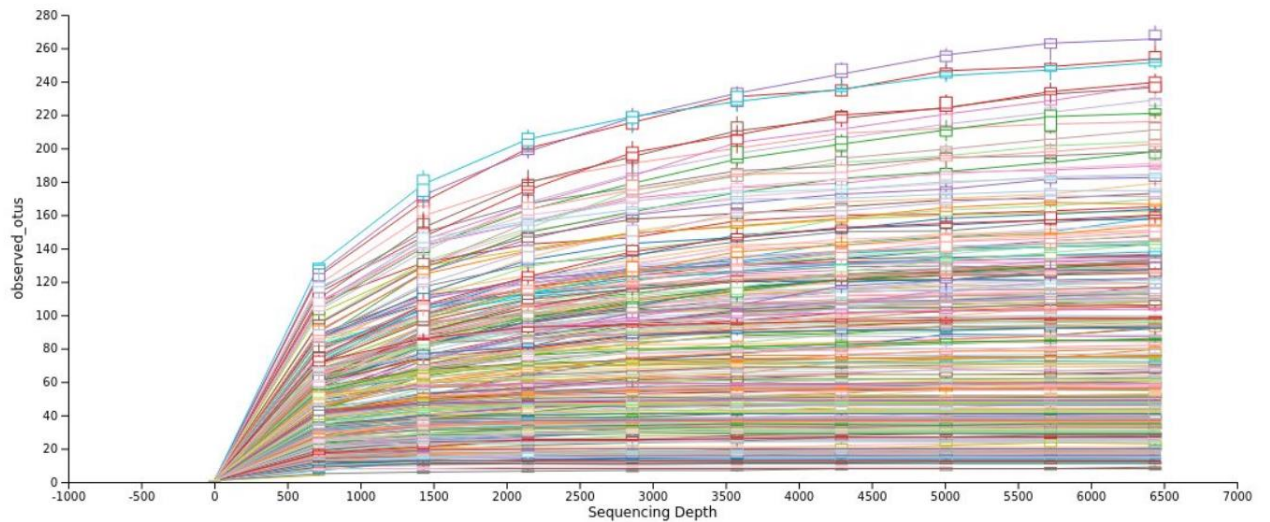


Figure 26: Differently abundant bacterial ASVs of infected and uninfected lakes (LefSe, LDA score >2 and $p \leq 0.01$). Red bars identify ASVs significantly more abundant in Bd-positive populations and green bars identify ASVs which are significantly more present in Bd-negative populations.

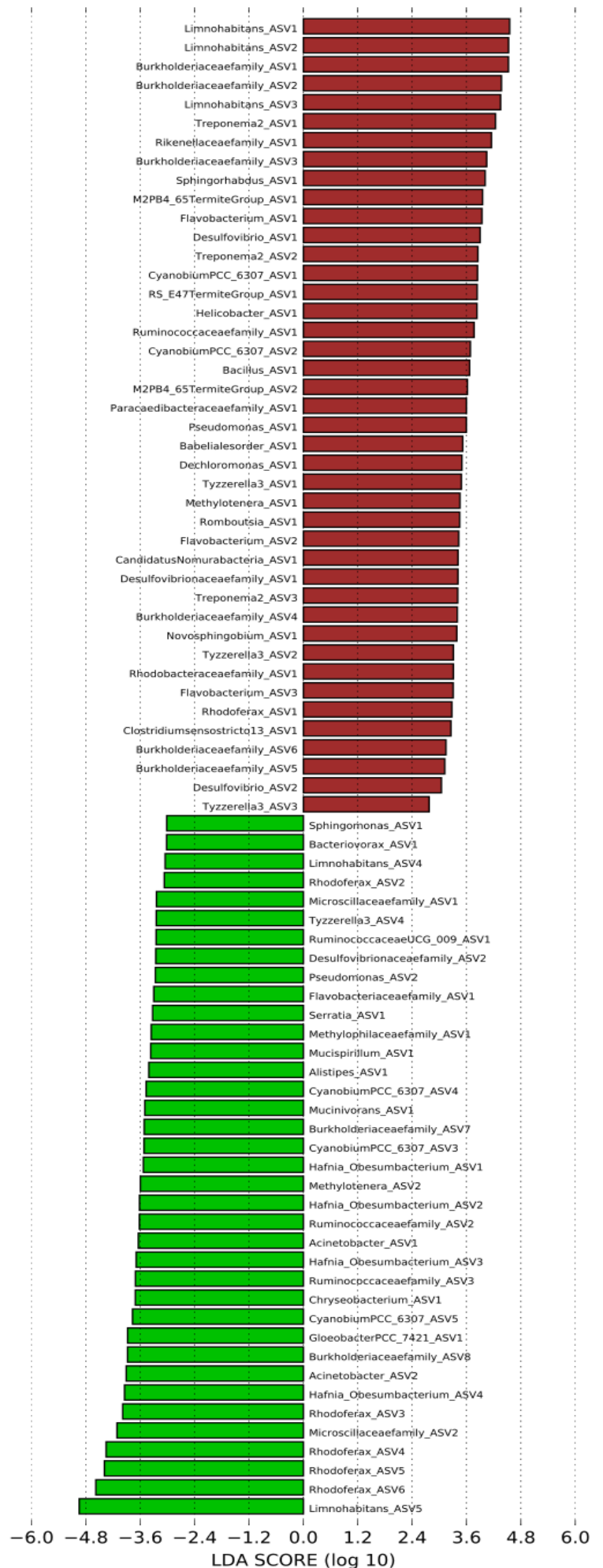


Figure 27: Differently abundant micro eukaryotic ASVs of Bd-positive and negative populations (LefSe, LDA score >2 and $p \leq 0.01$). Red bars identify ASVs significantly more abundant in Bd-positive populations and green bars identify ASVs which are significantly more present in Bd-negative populations.

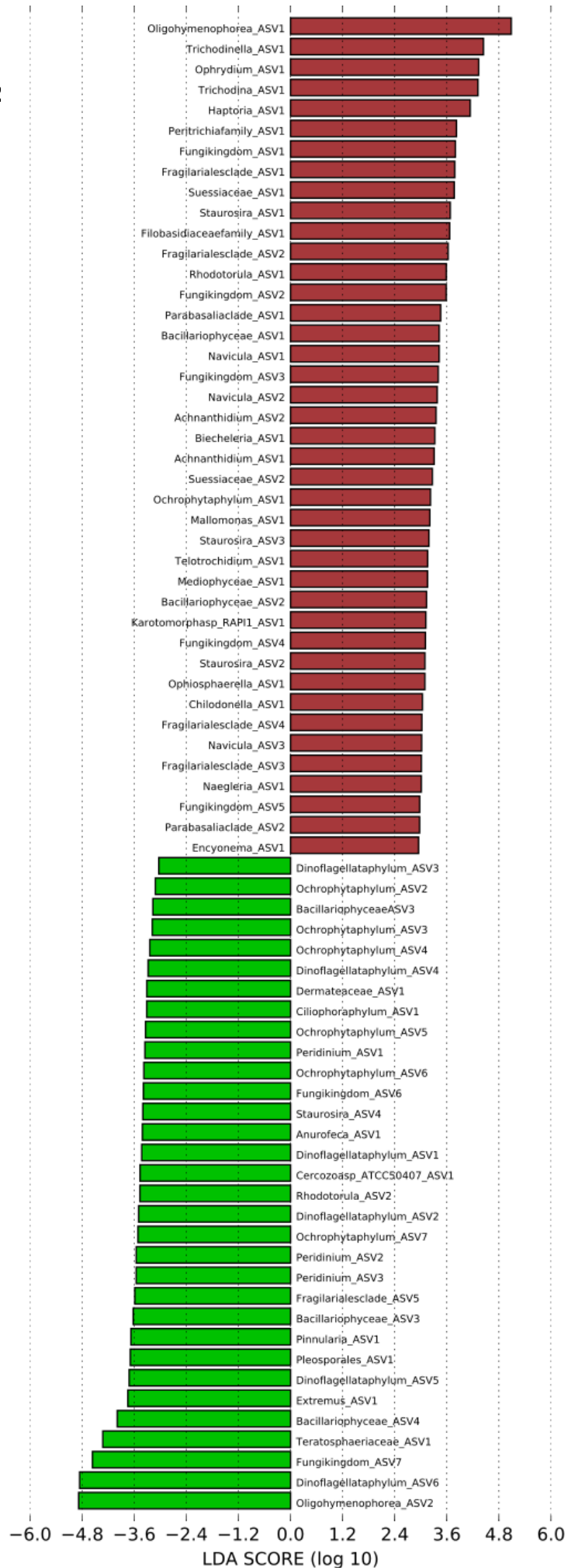


Figure 28: Differently abundant bacterial ASVs of epizootic and enzootic populations (LefSe, LDA score >2 and $p \leq 0.01$). Red bars identify ASVs significantly more abundant in epizootic populations and green bars identify ASVs which are significantly more present in enzootic populations.

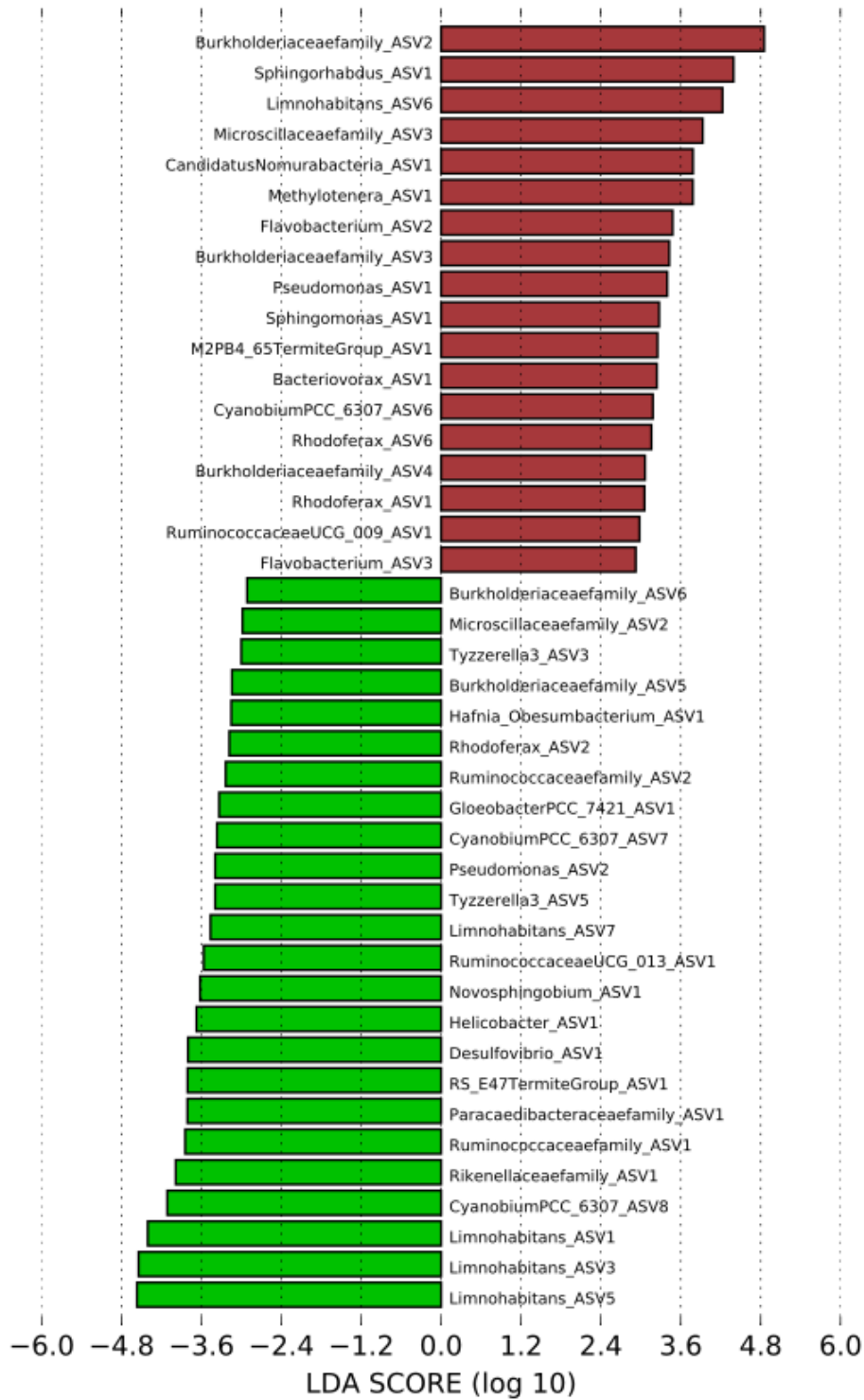


Figure 29: Differently abundant micro eukaryotic ASVs of epizootic and enzootic populations (LefSe, LDA score >2 and $p \leq 0.01$). Red bars identify ASVs significantly more abundant in epizootic populations and green bars identify ASVs which are significantly more present in enzootic populations.

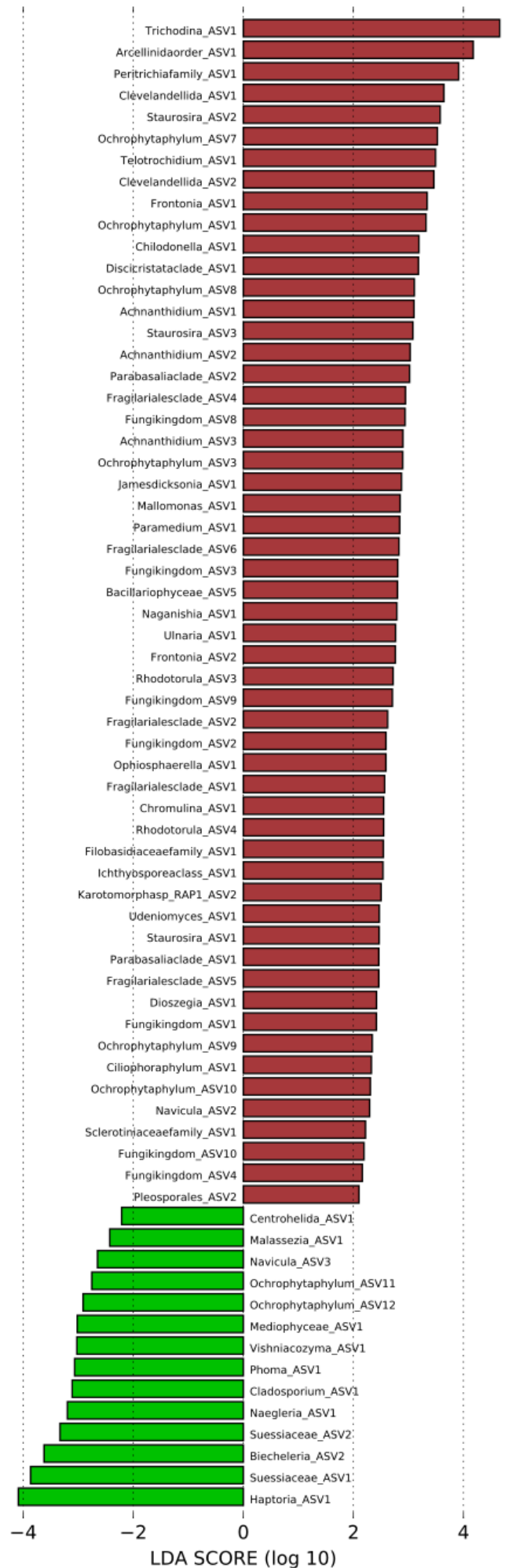
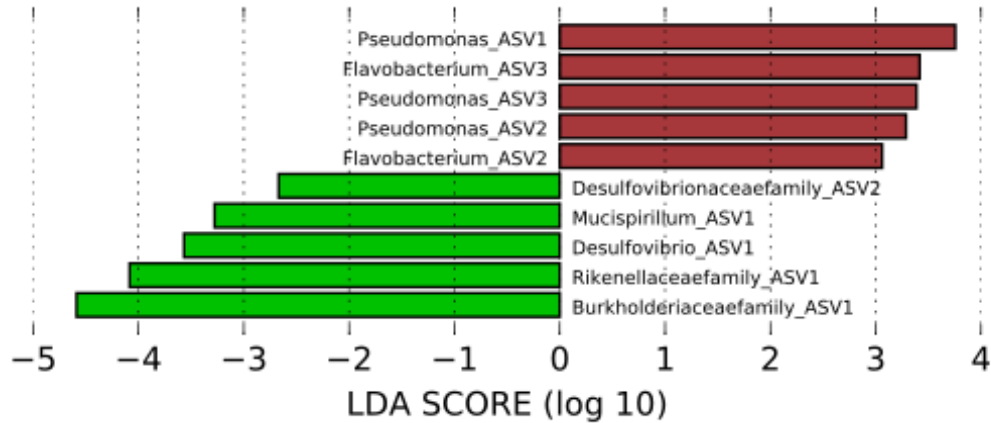
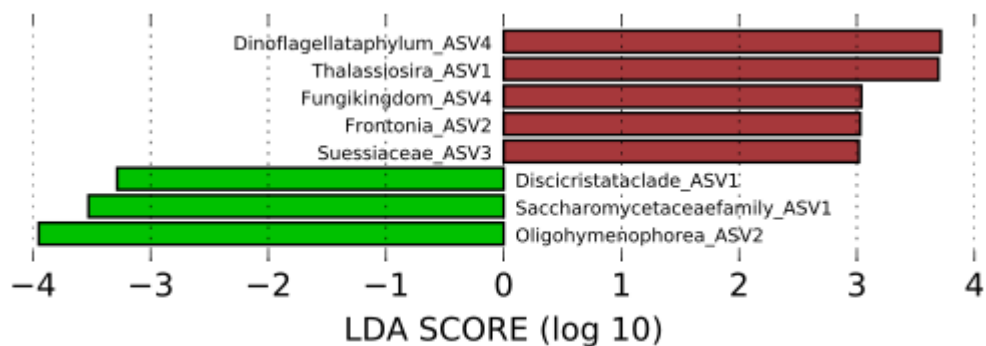


Figure 30: Differently abundant (a) bacterial and (b) micro eukaryotic ASVs of infected (1) and uninfected (0) individuals (LefSe, LDA score >2 and $p \leq 0.01$) from Bd-positive populations. Red bars identify ASVs significantly more abundant in infected individuals and green bars identify ASVs which are significantly more present in uninfected individuals.

a)



b)



Résumé du chapitre 4

Dans le chapitre 4 – « Interactions entre *Batrachochytrium dendrobatidis* et le microbiome cutané des amphibiens chez les crapauds accoucheurs de montagne » – je couvre le dernier facteur de la pyramide des maladies : l'agent pathogène. Dans le chapitre précédent, j'ai détecté l'influence de l'environnement et de l'espèce hôte sur le microbiome cutané des têtards. Dans ce chapitre 3, je me suis donc concentrée sur les relations microbiome-hôte-pathogène des têtards d'*Alytes obstetricans* uniquement, puisqu'il est considéré comme une espèce indicatrice de Bd dans les Pyrénées. Dans ce travail, j'ai analysé non seulement la communauté bactérienne mais aussi la communauté micro eucaryote, fournissant ainsi une vue plus complète du microbiome de la peau. Nous avons divisé les populations d'*A. obstetricans* en considérant les différentes variables de Bd : (1) populations Bd+ et Bd- ; (2) au sein des populations Bd+ : épizootie et enzootie ; et (3) au sein des populations Bd+ : individus infectés et non infectés.

J'ai détecté une altération de l'ensemble de la communauté du microbiome (à la fois pour les bactéries et les micro-eucaryotes) associée à toutes les variables de Bd (vérifiées une par une), démontrant une relation significative entre Bd et le microbiome de la peau des amphibiens, même lorsque l'agent pathogène n'infecte pas directement les individus, mais est présent dans l'environnement. La richesse et la diversité du microbiome des têtards ont également été affectées par Bd, les deux indices étant plus élevés dans les populations Bd+. Afin de voir si les populations épizootiques et enzootiques diffèrent dans leur richesse et leur diversité au cours du temps, j'ai analysé la variation de ces indices sur les années 2016, 2017 et 2018. Le lien entre la dynamique de la maladie au fil des années et la diversité du microbiome n'était significatif que pour les bactéries. J'ai observé une augmentation de la richesse et de la diversité dans les populations épizootiques, alors que dans les populations enzootiques et non infectées, le pattern était opposé. Ceci suggère un processus de sélection favorisant les individus ayant une plus grande richesse et diversité du microbiome, les rendant plus aptes à survivre à Bd. Après cette première étape de sélection, les survivants, dont la composition du microbiome est la plus efficace contre Bd, resteraient dans la population. Par conséquent, dans les populations enzootiques, la régulation de Bd se produirait principalement au niveau de la composition et de l'abondance de ces taxons bactériens déjà sélectionnés, avec un modèle décroissant dans la richesse mais un modèle plus stable dans la diversité. Ce

résultat est cohérent avec d'autres études qui ont trouvé une plus faible diversité du microbiome dans les populations épizootiques, par rapport aux populations enzootiques.

Chapter 5: General conclusions

My thesis addresses the issue of how environmental variables can affect the skin microbiome of tadpoles in the context of the disease pyramid, and how the amphibian skin microbiome interacts with a pathogen (Bd). Such an approach is important to understand possible relationships between Bd (or other pathogens) and the amphibian skin microbiome of populations under different environmental conditions.

In a nutshell, my work was conducted in a model system composed of amphibian hosts (*A. obstetricans*, *R. temporaria* and *B. bufo*), their skin microbiome, and the pathogen Bd, in a montane ecosystem. My results give important insights into how environmental factors, especially anthropogenic and climatic, can affect the skin microbiome composition of different amphibian species. This impact can, therefore, alter the interactions between the host, the host microbiome and the pathogens, influencing the outcome of a disease in a specific population. I also showed differences in the skin microbiome between Bd-positive and Bd-negative amphibian populations, and that the presence of Bd in the population is linked to differences in both microbiome composition and diversity in the bacterial and micro eukaryotic skin communities. Not only does Bd presence is correlated with the structure of the skin microbiome, but also enzootic and epizootic populations show distinct skin microbiome compositions over time, especially the bacterial community, indicating a Bd-driven selection process on the microbiome.

In chapter 2, “Environmental Factors and Host Microbiomes Shape Host – Pathogen Dynamics”, I covered the variables linked to disease dynamic and proposed a new concept for disease ecology, through the transition of the disease triangle (Stevens, 1960) to the disease pyramid (Bernardo-Cravo et al., 2020), by adding the microbiome factor into the host-environment-pathogen dynamics. The new concept is, in fact, a theoretical fusion of many other studies which have considered the amphibian skin microbiome under distinct conditions and associated with different species. We show how interactions between host, host microbiome, pathogen, and the environment may all affect disease outcome. My review incorporates environmental factors and microbiome function into disease theory and may be used for infection disease in general, not only for amphibians. Such interactions have already been discussed for human health as well, with the climatic changes being a key factor for the emergency of infection disease (Lindahl and Grace, 2015) and the human microbiome being essential for colonization

resistance against infectious pathogens (Libertucci and Young, 2019). Therefore, such an approach may be used for both wildlife and human diseases, providing a better view on the complexity of the disease systems.

Chapter 3, “Climate and fish impact tadpole skin microbiome in mountain lakes”, explores three out of the four edges of the disease pyramid: the host, the environment and the host bacterial microbiome. With my results, I could show the host influence on the microbiome composition, through both the network analysis of the most abundant bacterial taxa and the influence of the host species on the presence and/or relative abundance of the selected abundant genera. Such influence had been already confirmed with amphibian adults by other studies (Kueneman et al., 2014; Muletz Wolz et al., 2018; Medina et al., 2019), and was now verified with tadpoles from our mountain lakes. From the anthropogenic proxies analyzed (fish introduction, tourism impact and livestock presence), the introduction of fish was the most important one. Fish was already known to affect tadpoles by predation (Vredenburg, 2004; Knapp et al., 2016), but its impact on the tadpole skin microbiome was still unknown. The top 10 bacterial taxa responsible for the differences observed on the skin of tadpoles from lakes with and without fish were all more abundant in lakes with fish, indicating a higher dominance of abundant taxa. A difference in abundance like this kind on a few taxa could alter the amphibian capacity in dealing with external threats, such as pathogens, for example. No impact of livestock presence was found, even though it has been pointed out as a potential disturbance to the skin microbiome in livestock-fish pond systems (Preuss et al., 2020). The dilution of livestock influence, due to low livestock numbers and the large living area for the cattle was suggested to be the reason for this result.

Lastly, I found an important climatic impact on the amphibian skin microbiome. This impact was mainly due to the length of the growing season and simple precipitation level on both the presence and abundance of the selected top bacterial taxa. Temperature and humidity variables are known to have a strong effect on amphibian hosts due to their ectothermic physiology (Paaijmans et al., 2013; Greenberg and Palen, 2021) and the impact of climatic variables on their skin microbiome just reinforces its influence on amphibians. In the Pyrenees, longer growing seasons are translated into earlier ice-melt, which has been found to increase infection probability by Bd (Clare et al. 2016). Such event could become more frequent and stronger due to climate changes, increasing not only Bd infection probability, but also Bd (and other pathogens) spread and colonization

of new amphibian populations. My results suggest that the (potential) increased chances of infection combined with an altered skin microbiome under longer growing seasons can act together in disturbing the amphibian populations in the mountains and augment external pressures on these populations over time, especially if enhanced by climate change and the spread of other pathogens. The results of this chapter disentangle some of the connections between the host, the host microbiome and the environment in tadpoles from a mountain range, adding a substrate to analyze possible further interactions between the amphibian skin microbiome and pathogens.

In chapter 4, “Interactions between *Batrachochytrium dendrobatidis* and amphibian skin microbiome in montane common midwife toads”, I moved from the environmental and species impact on the bacterial microbiome (chapter 3) to the relationship between the pathogen Bd and both bacterial and micro eukaryotic skin communities of *A. obstetricans*, known to be linked to Bd presence, with population declines already documented. In all comparisons done (Bd-positive vs. Bd-negative populations, enzootic vs. epizootic populations, infected vs. uninfected individuals), I identified specific ASVs which were differently abundant between the compared groups, for both bacterial and micro eukaryotic communities. In comparison to the micro eukaryotic community, the bacterial community composition was, in general, more sensitive to the disease classification per population (enzootic or epizootic) and individual infection status (infected or uninfected). The population infection status (Bd-positive or Bd-negative) was a consistent explanatory variable of the alpha diversity indices (number of observed ASVs and Shannon index) for both bacteria and micro eukaryotes, and all the diversity indices were higher in Bd-positive populations. Livestock presence was also a consistent explanatory variable for both bacterial and micro eukaryotic diversity, while climatic variables were largely important for the micro eukaryotic, but not for bacterial diversity in *A. obstetricans*. This shows again the importance of taking environmental factors into account when analyzing the interaction between skin microbiome of distinct amphibian species and pathogen presence.

Regarding enzootic and epizootic populations, both bacterial and micro eukaryotic communities were significantly different, and a general difference was also present in the alpha diversity indices between enzootic, epizootic and uninfected populations. We observed that in the medium- to long-term, epizootic populations showed an increase in microbiome diversity, while enzootic and uninfected populations

saw their diversity indices go down over the years. We considered this temporal pattern as a result of a selection process acting towards taxa which are more efficient in protecting the amphibians against Bd. We hypothesized that individuals from epizootic populations with lower skin bacterial diversity would be less efficient to protect the host against Bd and, therefore, would suffer negative impacts from Bd infection (death), leaving mainly individuals with high skin microbiome diversity. In the enzootic populations, the microbiome would be regulated on the community diversity (alpha diversity) and composition (beta diversity) as well. Therefore, the diversity indices would not increase continuously, but rather decrease, since the most effective anti-Bd taxa would be maintained, and their abundance would remain stable over time. In this case, bacterial taxa which are not efficient against Bd would not be selected over time.

With the combination of my results, I could cover the climatic, anthropogenic, host-specific and pathogenic relationships with the amphibian skin microbiome of tadpoles. We showed that climatic, anthropogenic (mainly fish introduction) variables and host species can alter the microbiome composition by affecting the presence, relative abundance and the correlation network of the top skin bacterial taxa (chapter 3). Such outcome suggests that future climatic changes, combined with habitat perturbation, can have a significant impact on the amphibian skin microbiome. These alterations could impact the capacity of amphibians to fight diseases, such as chytridiomycosis and ranavirosis, since their initial skin microbiome can be a relevant factor in defining their susceptibility to diseases (Harris et al., 2009; Walke et al., 2015). Once the pathogen is in a population, or an individual is infected, it is difficult to predict how the relationship between the pathogen and the microbiome evolves over time, i.e. if the pathogen alters microbiome composition, or if the initial microbiome composition affects host susceptibility. In my work, I could not define this causal-consequence relation, but I found frequent links between Bd presence (in a population and in infected individuals) and skin microbiome structure. I could also observe a tendency in skin microbiome diversity (mainly in the bacterial community) according to the disease classification per population (enzootic, epizootic and also uninfected), indicating Bd epizootic-enzootic events as causes for changes in skin bacterial diversity over time (chapter 4).

With many interactions occurring at the same time and affecting amphibian species in distinct ways, it is difficult to trace a unique amphibian conservation strategy to be applied on a broad scale. It is, however, possible to work on the strongest local

environmental stressors on amphibian populations. Hundreds of populations of the endangered species *Rana sierrae* that have undergone multiple stressors simultaneously (fish introduction, presence of pesticides in the environment and Bd invasion) were able to show a significant increase of 11% in their populational number per year, over 20 years (Knapp et al., 2016). The main reasons for such a recovery were reduced frog susceptibility to Bd (probably to evolutionary and immunological changes in response to Bd in enzootic populations) and removal of introduced fish from their habitat (creating new habitats for amphibian recolonization) (Knapp et al., 2016). Such kind of recuperation indicates that the best way to preserve wild populations is still through habitat preservation, especially breeding sites (Scheele et al., 2014), but once stressors are present in the environment, it is still possible to reverse population declines through active removal of invasive species, for example.

Other forms of chytridiomycosis mitigation have been studied, but in general they have to follow three steps: identification of disease suppression, modelling disease-population dynamics, and the exploration of adaptive management in the field, which is the most challenging (Woodhams et al., 2011). Recently, mitigation through probiotic treatment have been tested with bacterial taxa potentially effective against Bd. Inoculations of the bacterium *Janthinobacterium lividum*, for example, have increased the survival of amphibians by 40% under controlled conditions (Kueneman et al., 2016), after a significant decrease in skin microbiome diversity due to reduced environmental input. The effectiveness of bacteria, however, may vary considering the amphibian species, the Bd-lineage and the surrounding environment and thus, may not be suitable for a broad range of species (Walke and Belden, 2016). Additionally, amphibians are under different threats in the environment (climatic alteration, pollutants, habitat fragmentation) and the pressure of these factors, combined or not with Bd, can change the results of conservation efforts focused mainly on chytridiomycosis (Garner et al., 2016). An essential step is, therefore, to understand and cover the possible reasons for local population declines, how they occur in a specific habitat/ population over time and which are the consequences for the population. Only then, local priorities can be discussed considering the amphibian species ecology and the possibility of mitigating chytridiomycosis with probiotic treatment in nature.

Conclusions générales

Ma thèse aborde la question de savoir comment les variables environnementales peuvent affecter le microbiome cutané des têtards dans le contexte de la pyramide des maladies, et comment le microbiome cutané des amphibiens interagit avec un pathogène (Bd). Une telle approche est importante pour comprendre les relations possibles entre Bd (ou d'autres agents pathogènes) et le microbiome cutané des amphibiens dans des populations soumises à différentes conditions environnementales.

En bref, mes travaux ont été menés dans un système modèle composé d'hôtes amphibiens (*A. obstetricans*, *R. temporaria* et *B. bufo*), de leur microbiome cutané et de l'agent pathogène Bd, dans un écosystème de montagne. Mes résultats fournissent un aperçu important de la manière dont les facteurs environnementaux, notamment anthropiques et climatiques, peuvent affecter la composition du microbiome cutané de différentes espèces d'amphibiens. Cet impact peut modifier les interactions entre l'hôte, le microbiome de l'hôte et les agents pathogènes, influençant ainsi l'issue d'une maladie dans une population spécifique. J'ai également montré qu'il existe des différences dans le microbiome de la peau entre les populations d'amphibiens Bd-positives et Bd-négatives, et que la présence de Bd dans la population est liée à des différences à la fois dans la composition du microbiome et dans la diversité des communautés bactériennes et micro-eucaryotes de la peau. Non seulement la présence de Bd est corrélée à la structure du microbiome cutané, mais les populations enzootiques et épizootiques présentent également des évolutions distinctes de la composition du microbiome cutané au fil du temps, en particulier la communauté bactérienne, ce qui indique un processus de sélection du microbiome lié à Bd.

Dans le chapitre 2, "Les facteurs environnementaux et les microbiomes de l'hôte façonnent la dynamique hôte-pathogène", j'ai couvert les variables liées à la dynamique des maladies et j'ai proposé un nouveau concept pour l'écologie des maladies, par la transition du triangle des maladies (Stevens, 1960) à la pyramide des maladies (Bernardo-Cravo et al., 2020), en ajoutant le facteur microbiome dans la dynamique hôte-environnement-pathogène. Ce nouveau concept est, en fait, une fusion théorique de nombreuses autres études qui ont considéré le microbiome cutané des amphibiens dans des conditions distinctes et associées à différentes espèces. Nous montrons comment les interactions entre l'hôte, le microbiome de l'hôte, l'agent pathogène et l'environnement

peuvent toutes affecter l'issue de la maladie. Mon examen intègre les facteurs environnementaux et la fonction du microbiome dans la théorie des maladies et peut être utilisé pour les maladies infectieuses en général, et pas seulement pour les hôtes amphibiens. De telles interactions ont également déjà été discutées pour la santé humaine, les changements climatiques étant un facteur clé pour l'émergence des maladies infectieuses (Lindahl et Grace, 2015) et le microbiome humain étant essentiel pour la résistance à la colonisation contre les agents pathogènes infectieux (Libertucci et Young, 2019). Par conséquent, une telle approche peut être utilisée à la fois pour les maladies de la faune sauvage et de l'homme, offrant ainsi une meilleure vision de la complexité des systèmes pathologiques.

Le chapitre 3, « Le climate et les poissons impactent le microbiome cutané des têtards dans les lacs de montagne », explore trois des quatre arêtes de la pyramide des maladies : l'hôte, l'environnement et le microbiome bactérien de l'hôte. Avec mes résultats, j'ai pu montrer l'influence de l'hôte sur la composition du microbiome, à la fois par l'analyse du réseau des taxons bactériens les plus abondants et par l'influence de l'espèce hôte sur la présence et/ou l'abondance relative des genres abondants sélectionnés. Cette influence avait déjà été confirmée avec les adultes d'amphibiens par d'autres études (Kueneman et al., 2014 ; Muletz Wolz et al., 2018 ; Medina et al., 2019), et a maintenant été vérifiée avec les têtards de nos lacs de montagne. Parmi les proxys anthropiques analysés (l'introduction de poissons, l'impact touristique et la présence de bétail), l'introduction de poissons était la plus importante. On savait déjà que les poissons affectent les têtards par prédation (Vredenburg, 2004 ; Knapp et al., 2016), mais leur impact sur le microbiome cutané des têtards était encore inconnu. Les 10 principaux taxons bactériens responsables des différences observées sur la peau des têtards des lacs avec et sans poissons étaient tous plus abondants dans les lacs avec poissons, ce qui indique une plus grande dominance des taxons abondants. Une telle différence d'abondance sur quelques taxons pourrait altérer la capacité des amphibiens à faire face à des menaces extérieures, telles que des agents pathogènes, par exemple. Aucun impact de la présence du bétail n'a été trouvé, bien qu'il ait été signalé comme une perturbation potentielle du microbiome de la peau dans les systèmes d'étangs bétail-poisson (Preuss et al., 2020). La dilution de l'influence du bétail, due au faible nombre d'animaux et à la grande surface de vie du bétail, a été suggérée pour expliquer ce résultat.

Enfin, j'ai constaté un impact climatique important sur le microbiome cutané des amphibiens. Cet impact était principalement lié à la durée de la saison de croissance et à l'indice simple de précipitation sur la présence et l'abondance des principaux taxons bactériens sélectionnés. Les variables de température et d'humidité sont connues pour avoir un effet important sur les hôtes amphibiens en raison de leur physiologie ectothermique (Paaijmans et al., 2013; Greenberg et Palen, 2021) et l'impact des variables climatiques sur leur microbiome cutané ne fait que renforcer son influence sur les amphibiens. Dans les Pyrénées, l'allongement des saisons de croissance se traduit par une fonte des glaces plus précoce, qui s'est avérée augmenter la probabilité d'infection par Bd (Clare et al. 2016). Un tel événement pourrait devenir plus fréquent et plus fort en raison des changements climatiques, augmentant non seulement la probabilité d'infection par Bd, mais aussi la propagation de Bd (et d'autres pathogènes), ainsi que la colonisation de nouvelles populations d'amphibiens. Mes résultats suggèrent que l'augmentation (potentielle) des risques d'infection, combinée à une modification du microbiome cutané lors de saisons de croissance plus longues, peut agir conjointement pour perturber les populations d'amphibiens dans les montagnes et augmenter les pressions externes sur ces populations au fil du temps, en particulier si elles sont renforcées par le changement climatique et la propagation d'autres agents pathogènes. Les résultats de ce chapitre démêlent certaines des connexions entre l'hôte, le microbiome de l'hôte et l'environnement chez les têtards d'une chaîne de montagnes, ajoutant un substrat pour analyser d'autres interactions possibles entre le microbiome cutané des amphibiens et les agents pathogènes.

Dans le chapitre 4, « Interactions entre *Batrachochytrium dendrobatidis* et le microbiome cutané des amphibiens chez les crapauds accoucheurs de montagne », je suis passée de l'impact de l'environnement et des espèces sur le microbiome bactérien (chapitre 3) à la relation entre l'agent pathogène Bd et les communautés cutanées bactériennes et micro eucaryotes d'*A. obstetricans*, connues pour être liées à la présence de Bd, avec des déclin de population déjà documentés. Dans toutes les comparaisons effectuées (populations Bd-positives vs. Bd-négatives, populations enzootiques vs. épizootiques, individus infectés vs. non infectés), j'ai identifié des ASV spécifiques qui étaient différemment abondants entre les groupes comparés, tant pour les communautés bactériennes que micro-eucaryotes. En comparaison avec la communauté micro-eucaryote, la composition de la communauté bactérienne était, en général, plus sensible

à la classification de la maladie par population (enzootique ou épizootique) et au statut d'infection individuel (infecté ou non infecté). Le statut d'infection de la population (Bd-positif ou Bd-négatif) était une variable explicative cohérente des indices de diversité alpha (nombre d'ASVs observés et indice de Shannon) pour les bactéries et les micro-eucaryotes, et tous les indices de diversité étaient plus élevés dans les populations Bd-positives. La présence de bétail était également une variable explicative cohérente pour la diversité bactérienne et micro-eucaryote, tandis que les variables climatiques étaient largement importantes pour la diversité micro-eucaryote, mais pas pour la diversité bactérienne chez *A. obstetricans*. Cela montre à nouveau l'importance de prendre en compte les facteurs environnementaux lors de l'analyse de l'interaction entre le microbiome cutané d'espèces distinctes d'amphibiens et la présence de pathogènes.

Concernant les populations enzootiques et épizootiques, les communautés bactériennes et micro-eucaryotes étaient significativement différentes, et une différence générale était également présente dans les indices de diversité alpha entre les populations enzootiques, épizootiques et non infectées. Nous avons observé qu'à moyen et long termes, les populations épizootiques ont montré une augmentation de la diversité du microbiome, tandis que les populations enzootiques et non infectées ont vu leurs indices de diversité diminuer au fil des ans. Nous avons considéré ce schéma temporel comme le résultat d'un processus de sélection agissant en faveur des taxons les plus efficaces pour protéger les amphibiens contre Bd. Nous avons émis l'hypothèse que les individus des populations épizootiques ayant une diversité bactérienne cutanée plus faible seraient moins efficaces pour protéger l'hôte contre Bd et, par conséquent, subiraient des impacts négatifs de l'infection par le pathogène (disparition), laissant principalement des individus avec une diversité élevée du microbiome cutané. Dans les populations enzootiques, le microbiome serait également régulé au niveau de la diversité de la communauté (diversité alpha) et de sa composition (diversité bêta). Par conséquent, les indices de diversité n'augmenteraient pas continuellement, mais diminueraient plutôt, puisque les taxons anti-Bd les plus efficaces seraient maintenus, et leur abondance resterait stable dans le temps. Dans ce cas, les taxons bactériens qui ne sont pas efficaces contre Bd ne seraient pas sélectionnés au cours du temps.

Avec la combinaison de mes résultats, j'ai pu couvrir les relations climatiques, anthropogéniques, spécifiques à l'hôte et aux pathogènes avec le microbiome cutané des têtards d'amphibiens. Nous avons montré que des variables climatiques, anthropiques

(principalement l'introduction de poissons) et l'espèce de l'hôte peuvent modifier la composition du microbiome en affectant la présence, l'abondance relative et le réseau de corrélation des principaux taxons bactériens de la peau (chapitre 3). Ces résultats suggèrent que les changements climatiques futurs, combinés à la perturbation de l'habitat, peuvent avoir un impact significatif sur le microbiome cutané des amphibiens. Ces altérations pourraient avoir un impact sur la capacité des amphibiens à lutter contre des maladies telles que la chytridiomycose et la ranavirose, puisque leur microbiome cutané initial peut être un facteur pertinent pour définir leur susceptibilité aux maladies (Harris et al., 2009; Walke et al., 2015). Une fois que l'agent pathogène est dans une population, ou qu'un individu est infecté, il est difficile de prévoir comment la relation entre l'agent pathogène et le microbiome évoluera dans le temps, c'est-à-dire si l'agent pathogène modifiera la composition du microbiome, ou si la composition initiale du microbiome affectera la susceptibilité de l'hôte. Dans mon travail, je n'ai pas pu définir cette relation causalité-conséquence, mais j'ai trouvé des liens fréquents entre la présence de Bd (dans une population et chez les individus infectés) et la structure du microbiome cutané. J'ai également pu observer une tendance dans la diversité du microbiome cutané (principalement dans la communauté bactérienne) en fonction de la classification de la maladie par population (enzootique, épizootique et également non infectée), indiquant que les événements épizootiques-enzootiques liés à Bd sont les causes des changements dans la diversité bactérienne cutanée au cours du temps (chapitre 4).

De nombreuses interactions se produisant en même temps et affectant les espèces d'amphibiens de manière distincte, il est difficile de tracer une stratégie unique de conservation des amphibiens à appliquer à grande échelle. Il est toutefois possible de travailler sur les facteurs de stress environnementaux locaux les plus forts sur les populations d'amphibiens. Des centaines de populations de l'espèce menacée *Rana sierrae* ayant subi plusieurs facteurs de stress simultanément (introduction de poissons, présence de pesticides dans l'environnement et invasion par Bd) ont pu montrer une augmentation significative de 11% de leur nombre de population par an, sur 20 ans (Knapp et al., 2016). Les principales raisons d'une telle récupération étaient la réduction de la sensibilité des grenouilles à Bd (probablement en raison de changements évolutifs et immunologiques en réponse à Bd dans les populations enzootiques) et le retrait des poissons de leur habitat (créant de nouveaux habitats pour la recolonisation des

amphibiens) (Knapp et al., 2016). Ce type de récupération indique que la meilleure façon de préserver les populations sauvages reste la préservation de l'habitat, en particulier des sites de reproduction (Scheele et al., 2014), mais une fois que les facteurs de stress sont présents dans l'environnement, il est toujours possible d'inverser le déclin des populations en éliminant activement les espèces envahissantes, par exemple.

D'autres formes d'atténuation de la chytridiomycose ont été étudiées, mais en général elles doivent suivre trois étapes : (1) l'identification de la suppression de la maladie, (2) la modélisation de la dynamique de la maladie dans la population, et enfin (3) l'exploration de la gestion adaptative sur le terrain, ce qui est le plus difficile (Woodhams et al., 2011). Récemment, l'atténuation par traitement probiotique a été testée avec des taxons bactériens potentiellement efficaces contre Bd. Les inoculations de la bactérie *Janthinobacterium lividum*, par exemple, ont augmenté la survie des amphibiens de 40% dans des conditions contrôlées (Kueneman et al., 2016), après une diminution significative de la diversité du microbiome cutané due à la réduction des apports environnementaux. Cependant, l'efficacité des bactéries peut varier en fonction de l'espèce d'amphibien, de la lignée de Bd et du milieu environnant, et peut donc ne pas convenir à un large éventail d'espèces (Walke et Belden, 2016). De plus, les amphibiens sont soumis à différentes menaces dans l'environnement (altération climatique, polluants, fragmentation de l'habitat) et la pression de ces facteurs, combinée ou non à Bd, peut modifier les résultats des efforts de conservation axés principalement sur la chytridiomycose (Garner et al., 2016). Une étape essentielle est donc de couvrir les raisons possibles du déclin des populations locales, de comprendre comment elles se produisent dans un habitat/une population spécifique au fil du temps et quelles sont les conséquences pour la population. Ce n'est qu'ensuite que les priorités locales peuvent être discutées en tenant compte de l'écologie des espèces d'amphibiens et de la possibilité d'atténuer la chytridiomycose par un traitement probiotique dans la nature.

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