UAB
Universitat Autònoma de Barcelona

Amphibian communities facing pathogen threats: understanding the epidemiology of chytridiomycosis in Catalonia

Maria Puig Ribas

Amphibian communities facing pathogen threats:

understanding the epidemiology of chytridiomycosis in Catalonia

Maria Puig Ribas

PhD Thesis 2025

UAB
Universitat Autònoma
de Barcelona

Amphibian communities facing pathogen threats: understanding the epidemiology of chytridiomycosis in Catalonia

Maria Puig Ribas

Supervisors

Oscar Cabezón Ponsoda

Albert Martínez Silvestre

Tutor

Ignasi Marco Sánchez

PhD Thesis

Departament Medicina i Cirurgia Animals

Facultat de Veterinària

Universitat Autònoma de Barcelona

2025

Wildlife Conservation Medicine
Research group

IRTA
CReSA
Centre de Recerca
en Sanitat Animal

CRARC
Centre de Recuperació
d'Amfibis i Rèptils
de Catalunya

Els doctors **Oscar Cabezón Ponsoda**, investigador associat al Wildlife Conservation Medicine Research Group i al Centre de Recerca en Sanitat Animal (IRTA-CReSA), i **Albert Martínez Silvestre**, director científic del Centre de Recuperació d’Amfibis i Rèptils de Catalunya,

Informen:

Que la memòria titulada “**Amphibian communities facing pathogen threats: understanding the epidemiology of chytridiomycosis in Catalonia**”, presentada per **Maria Puig Ribas** per a la obtenció del títol de Doctora per la Universitat Autònoma de Barcelona, s’ha realitzat sota la nostra direcció i, un cop considerada satisfactòriament finalitzada, autoritzem la seva presentació per tal que sigui avaluada per la comissió corresponent.

I perquè així consti als efectes que siguin oportuns, firmem el present informe a Bellaterra, 17 de Maig de 2025.

Óscar Cabezón Ponsoda **Albert Martínez Silvestre**

Table of contents

Abstract

> Tremoleu, miserables! Que el turment més terrible us espera, per tota l’eternitat!
>
> — Josep M. Folch i Torres, Els Pastorets

Amphibians are the most threatened vertebrates on Earth, with emerging infectious diseases playing a critical role in their global declines. In Europe, chytridiomycosis, caused by the fungal pathogens *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal), has contributed to severe population losses. Despite its ecological significance, Catalonia (NE Spain), home to 15 native amphibian species, remains largely understudied in terms of amphibian health and pathogen surveillance. This thesis addresses key knowledge gaps in the epidemiology of chytridiomycosis in Catalonia by combining methodological validation, pathogen surveillance, and ecological analyses across multiple amphibian hosts. The overarching goal was to assess chytridiomycosis prevalence, identify environmental and anthropogenic drivers of infection, and evaluate host-pathogen-environment interactions through a series of field studies conducted between 2018 and 2024. The thesis comprises four empirical chapters.

In **Chapter 1**, we evaluated the use of passive integrated transponder (PIT) tags for marking urodeles. We found that tag retention and wound healing varied significantly by species, emphasizing the need for species-specific validations. PIT tagging proved reliable in *Salamandra Salamandra* and *Pleurodeles waltl*, but not in *Calotriton asper*, reinforcing the importance of ethical and evidence-based tagging protocols in wildlife research.

Chapter 2 presents the first region-wide Bd and Bsal survey in Catalonia, encompassing over 3,200 amphibians from 14 species and 41 sites. Bd was detected in 141 individuals from eight species, indicating widespread endemicity, while Bsal was not detected. Bd occurrence was significantly influenced by summer temperatures and human disturbance. Notably, *Alytes obstetricans* larvae and *Hyla meridionalis* showed high infection prevalence and loads without overt disease signs, suggesting they may act as tolerant reservoir hosts. Other species showed lower prevalence, with variable susceptibility.

In **Chapter 3**, we investigated the potential of the amphibian skin microbiome as a health indicator. Using 16S rRNA long-read sequencing, we analyzed the microbiomes of *H. meridionalis* and *Pelophylax perezi*, two species with contrasting Bd responses. Our results showed species-specific microbial profiles, but no consistent association with Bd infection status. This suggests that the skin microbiome may contribute to interspecific differences in susceptibility, warranting further investigation into its role in resistance and tolerance. These findings support incorporating microbiome analyses into long-term monitoring programs.

Chapter 4 documents one of the first cases of Bd-Bsal coinfection in wild European amphibians and the first in Iberian urodeles. Coinfection was observed in two urodele species – one endemic, one introduced – during a 2018 chytridiomycosis outbreak. Infection loads were high, and histopathology revealed severe lesions and inflammation, suggesting synergistic interactions between the pathogens and raising concerns about compounded impacts on host immunity and population viability.

Collectively, this thesis reveals the complexity of chytridiomycosis epidemiology in a biodiverse but under-monitored region. We demonstrate that Bd is widespread in Catalonia, with variable responses among amphibian species that span a continuum of resistance and tolerance. Subclinically infected species may act as pathogen reservoirs, while more susceptible taxa risk population declines. Importantly, we show that anthropogenic disturbance and climate are key drivers of infection dynamics, highlighting the need for integrative conservation approaches that consider both environmental and biological factors.

This thesis provides a foundational framework for chytrid disease surveillance and amphibian health assessment in Catalonia. It emphasizes the urgency of ethical monitoring methodologies, microbiome-informed strategies, and multispecies disease management. As amphibians continue to face escalating threats from habitat degradation, climate change, and infectious diseases, such comprehensive and context-specific research is essential for effective conservation action – both in Catalonia and globally.

Resum

Els amfibis són els vertebrats més amenaçats del planeta, amb les malalties infeccioses emergents com a causa clau del seu declivi global. A Europa, la quitridiomicosi, causada pels fongs *Batrachochytrium dendrobatidis* (Bd) i *B. salamandrivorans* (Bsal), ha provocat declivis poblacional severs. Tot i la seva importància ecològica, Catalunya (NE d'Espanya), amb 15 espècies d'amfibis autòctones, ha estat poc estudiada pel que fa a la salut dels amfibis i la vigilància de patògens. Aquesta tesi aborda buits clau sobre l'epidemiologia de la quitridiomicosi a Catalunya mitjançant la combinació de validació metodològica, estudis de vigilància i anàlisis ecològiques en múltiples hostes amfibis. L'objectiu principal ha estat avaluar la prevalença de la quitridiomicosi, identificar factors ambientals i antropogènics que influeixen en la infecció, i analitzar les interaccions hoste-patogens-ambient a través d'una sèrie d'estudis de camp entre 2018 i 2024. La tesi s'estructura en quatre capítols empírics.

Al **Capítol 1**, hem avaluat l'ús de microxips per al marcatge d'urodels. Els nostres resultats constaten que la retenció dels xips i la cicatrització de ferides varia segons l'espècie, destacant la necessitat de validar el seu ús en cada espècie. Mentre que el marcatge amb xips va ser fiable en *Salamandra salamandra* i *Pleurodeles waltl*, no ho va ser en *Calotriton asper*, reforçant la importància d'aplicar protocols ètics i basats en l'evidència en la recerca de fauna salvatge.

El **Capítol 2** presenta la primera avaluació a escala regional de Bd i Bsal a Catalunya, amb més de 3.200 amfibis de 14 espècies i 41 localitats mostrejades. Bd es va detectar en 141 individus de vuit espècies, indicant una endemicitat generalitzada, mentre que Bsal no es va detectar. La presència de Bd es va veure significativament influenciada per la temperatura estival i per l'impacte antropogènic. Les larves d'*Alytes obstetricans* i *Hyla meridionalis* van mostrar altes prevalences i càrregues infeccioses sense signes clínics, suggerint un possible paper com a hostes reservori. Altres espècies van mostrar prevalences menors, amb una susceptibilitat variable.

Al **Capítol 3**, es va explorar el potencial del microbioma cutani com a indicador de salut en amfibis. Mitjançant la seqüenciació del gen 16S rRNA

de lectura llarga, es van analitzar els microbiomes de *H. meridionalis* i *Pelophylax perezi*, dues espècies amb respostes contrastades davant Bd. Es van detectar perfils microbians específics per espècie, però sense cap associació consistent amb l'estat d'infecció per Bd. Això suggereix que el microbioma cutani podria contribuir a les diferències de susceptibilitat, i posen de manifest la necessitat d'aprofundir en el seu paper en la resistència i la tolerància. Aquest capítol posa en valor la integració de l'anàlisi del microbioma en la vigilància de la salut d'amfibis a llarg termini.

El **Capítol 4** documenta un dels primers casos de coinfecció Bd-Bsal en amfibis salvatges europeus, i el primer en urodels ibèrics. Aquesta coinfecció es va observar en dues espècies de urodels – una endèmica i una introduïda – durant un brot de quitridiomicosi al 2018. Es van detectar càrregues infeccioses elevades i lesions greus i inflamació, suggerint interaccions sinèrgiques entre patògens. Aquestes troballes generen preocupació sobre els impactes acumulatius de la coinfecció en la immunitat dels hostes i la viabilitat poblacional.

En conjunt, aquesta tesi revela la complexitat de l'epidemiologia de la quitridiomicosi en una regió biodiversa però escassament monitoritzada. Demostrem que Bd està àmpliament distribuït a Catalunya, amb respostes variables entre espècies d'amfibis que abasten un espectre de resistència i tolerància. Les espècies que mantenen infeccions subclíniques poden actuar com a reservoris del patogen, mentre que les més susceptibles poden patir declivis poblacionals. A més, identifiquem que l'alteració humana i el clima són factors clau en la dinàmica de la infecció, reforçant la necessitat d'estratègies de conservació integradores que considerin tant factors ambientals com biològics.

Aquest treball proporciona un marc fonamental per a la vigilància de la quitridiomicosi i la salut dels amfibis a Catalunya. Subratlla la urgència d'utilitzar metodologies ètiques de mostreig, estratègies basades en el microbioma, i enfocs de gestió multispecífics. Davant l'escalada de les amenaces que enfronten els amfibis, com la degradació de l'hàbitat, el canvi climàtic i les malalties infeccioses, aquesta recerca representa una contribució essencial per a guiar accions de conservació efectives, tant a escala local com global.

Introduction

Pare, digueu-me què li han fet al riu que ja no canta.
Rellisca com un barb
mort sota un pam d'escuma blanca.
Pare, que el riu ja no és el riu.
Pare, abans que torni l'estiu,
amagui tot el que és viu.

— Joan Manuel Serrat, Pare

Biodiversity in crisis

Earth's biodiversity is currently suffering dramatic and unprecedented declines. Biodiversity encompasses all species of animals, plants, fungi and microorganisms, the genes they contain, and the ecosystems and ecological processes they are part of. Simply put, biodiversity is an umbrella term for nature's variety and function. The existential threats biodiversity faces are now evident across all species and ecosystems, reaching the level of a global crisis.

We are witnessing species extinctions at rates 100 - 1,000 times higher than the background extinction rates observed in the fossil record, with projections suggesting these rates could increase by an additional 10 - 100 times over the next 50 years (Pimm et al., 2014). The International Union for Conservation of Nature (IUCN) estimates that more than 45,300 species are threatened with extinction and extinction risk is increasing for all assessed taxa (IUCN, 2024). Among vertebrates, the rates of population loss are striking, with 32% of species decreasing in population size and range and 40% of mammals having lost over 80% of their populations (Ceballos et al., 2017). The most comprehensive analysis of the global state of nature, the Living Planet Index (LPI), shows an average 69% decline in animal populations across the globe since 1970 (WWF, 2022). At an ecosystem level, initial assessments are also concerning. In Europe alone, 36% of terrestrial and 19% of marine ecosystems are endangered (IUCN-CEM, 2022).

Overall, it is now clear that we are in the midst of the Earth's sixth mass extinction and studies indicate that this will be the most alarming in history, both in terms of magnitude and root causes (Ceballos et al., 2017; Cowie et al., 2022; McCallum, 2015; Pimm et al., 2014; Wake & Vredenburg, 2008).

Drivers of biodiversity loss

In contrast to the five previous mass extinction events, the current biodiversity losses are predominantly caused directly and indirectly by a single actor, the *Homo sapiens*. The impact of humans on biodiversity has escalated rapidly since the 1970s, due to the demands of an unceasing population growth and affluence. Today, Earth's ecosystems are largely and increasingly dominated by humans and no ecosystem is free of human influence (Vitousek et al., 1997). Human activities have and continue to transform land surfaces, alter biogeochemical cycles and modify species assemblages and genetics. These disturbances further alter environmental processes by driving global climate and biodiversity losses, perpetuating a vicious cycle of ecosystem degradation and dysfunctionality. According to the IPBES report, the ultimate (direct) drivers of biodiversity loss are land/sea-use change, overexploitation, invasive species, pollution and climate change (IPBES, 2019; Millennium Ecosystem Assessment, 2005).

Land/sea-use change is the most pervasive driver of species extinctions and ecosystem collapse. Natural areas have been converted to anthropic cover types including agricultural lands, pastures and cities (IPBES, 2019). Agricultural expansion is the primary form of land use change, representing over one third of the terrestrial surface. Moreover, technological advancements continually enhance land conversion and extraction capacity, increasing the magnitude and extent of impacts. Species native to these transformed lands suffer from habitat loss and the remaining natural areas are often too fragmented to allow biological processes such as migration or reproduction.

Overexploitation refers to the extraction of living beings and non-living materials from nature. A striking example of this is seen in fisheries. About three-quarters of major marine fish stocks are fully exploited, overexploited or depleted (IPBES, 2019). Most fisheries focus on larger species or top predators, whose disappearance has cascading effects that threaten the stability of marine food webs and ecosystems (Myers et al., 2007). Furthermore, the incidental capture of non-target species (bycatch) such as marine mammals, turtles or seabirds, is common. Besides fisheries, humans exploit natural resources like freshwater or timber and deplete highly valued species for status goods, traditional medicine and pet trade.

Humanity has rearranged biological systems by moving and mixing floras and faunas that had evolved separately, known as biological invasions. Invasive species are present around the globe and are particularly harmful on islands. Most invasions are effectively irreversible, compromise the survival of native species and can disrupt the entire ecosystem. Pollution and human-induced climate change are the most recent anthropogenic impacts on biodiversity. Many chemicals used in industry or agriculture are toxic to living beings, including humans, and persist in the environment for decades. Even non-toxic compounds can cause environmental problems, for example, volatile chlorofluorocarbons lead to the breakdown of stratospheric ozone. Also, the emission of greenhouse gases contributes directly to climate change. Human-induced climate change is having far-reaching impacts on geophysical and biological processes. Climate change includes global warming, increasing extreme weather events, changes in precipitation, sea level rise, etc. As a consequence, species are shifting their distribution toward higher latitudes or altitudes (Root et al., 2003) or succumbing to extreme events; primary productivity is being altered, with effects propagating upward through the food web, forcing entire ecosystems to either adapt or collapse. Moreover, climate change tends to amplify other drivers and interactions among different drivers result in synergistic impacts on biodiversity.

The impact of disease

Emerging infectious diseases (EID) are increasingly recognized as key threats to biodiversity conservation (Daszak et al., 2000). The increasing frequency of EID is also the result of human activities, which alter the ecology of hosts, pathogens or both. As mentioned above, globalization and human transport of animals, plants and their products have resulted in biological invasions. Similarly, these activities can introduce non-native infectious agents into naïve host populations, known as pathogen pollution. Naïve populations, lacking defenses against novel infectious agents, may subsequently experience overt disease, mass mortalities, population declines, or even extinctions. Human-induced climate change is also expanding the native range of parasites, by enhancing the survival of free-living agents or increasing the range of arthropod vectors (Gray et al., 2009). However, not only novel pathogens have the potential to drive biodiversity losses. Environmental stress caused by human encroachment into wildlife habitats, habitat disturbance and loss, pollution and climate change can impair host immune defenses. This can increase host susceptibility to endemic agents that were previously innocuous and trigger disease outbreaks (Acevedo-Whitehouse & Duffus, 2009). Disease also interacts with other threats to biodiversity and populations already depleted have a higher risk of extinction due to unpredictable events such as disease (De Castro & Bolker, 2005). Moreover, small populations generally present low genetic variability associated with reduced immune response effectivity and increased susceptibility to disease. On the other hand, disease can also drive host populations to such low densities that extinction risk rises due to demographic fluctuations or stochasticity.

An intertwined future

Biodiversity loss is acknowledged as one of humanity's most significant challenges in the coming decades (UNEP, 2023). Biodiversity is tightly linked to human well-being by providing food, water and energy security, protection against natural disasters, physical and mental health, social relations and cultural values. The emergence of human and domestic animal diseases shares common causal themes with emerging wildlife diseases (Fig. 1) (Daszak et al., 2000). Moreover, mounting evidence indicates that biodiversity loss frequently increases the transmission of both well-established and novel diseases (Keesing et al., 2006; Keesing & Ostfeld, 2012). The COVID-19 pandemic perfectly exemplifies how anthropogenic activities drive zoonotic disease emergence and stresses the importance of preserving intact ecosystems and their endemic biodiversity in securing human health (Lawler et al., 2021).

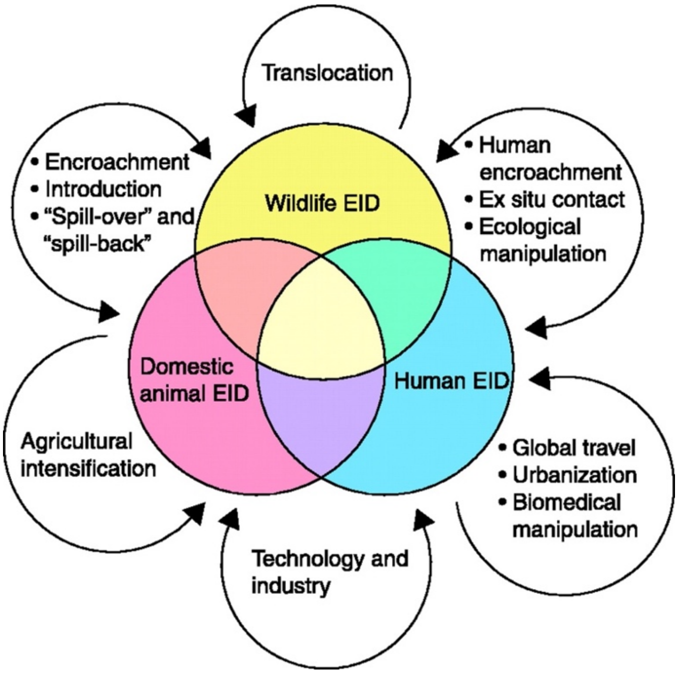


Figure 1. The host-pathogen continuum between wildlife, domestic animals and humans. Arrows present the key factors driving disease emergence, which share common themes among all three groups and are the consequence of human activities. From Daszak et al. (2000).

The benefits humanity obtains from well-functioning natural communities are collectively known as ecosystem services and they can be divided into supporting, regulating, provisioning and cultural services (Millennium Ecosystem Assessment, 2005). The global monetary value of ecosystem services is estimated to range from \$125 to \$145 trillion annually, contributing more than twice as much to human well-being as the global domestic product (Costanza et al., 2014). The loss of wild populations and degradation of ecosystems, thus, compromises the functioning of our life-support system, the sustainment of economic activity, and domestic animal and human health. Despite 30 years of policy interventions aimed at halting biodiversity loss, the problem is still far from being addressed. Therefore, we now have the inescapable responsibility and necessity of monitoring, protecting and restoring our planet's natural heritage to ensure the welfare of current and future generations.

Amphibians: the creatures that live a double life

Amphibians were the first vertebrates ever to conquer the land. Amphibians evolved in the Devonian period, around 370 million years ago, from a lobe-finned fish ancestor with functional lungs. They flourished during the Carboniferous and Permian periods, even becoming dominant species in ancient ecological communities. However, the rise of reptiles, birds and mammals later challenged the hegemony of amphibians in terrestrial environments. Despite this, amphibian's ecological adaptations such as morphological diversity, niche specialization, reproductive strategies and skin physiology, cannot be overshadowed by any other taxa. Modern amphibians continue to be a diverse and ecologically significant group, highlighting the remarkable adaptability and resilience of life on Earth.

Amphibians are ectothermic, anamniotic, tetrapod vertebrates characterized by their unique ability to live both in water and on land. This dual lifestyle is reflected in their name, meaning "double life" in Greek, and refers to the generalized life history trait of amphibians undergoing metamorphosis. Metamorphosis typically implies a complete transformation from aquatic larval forms into terrestrial adults. However, numerous variations and exceptions exist to this classical process, allowing amphibians to exploit a wide range of ecological niches. Amphibians thrive in aquatic, semi-aquatic and terrestrial environments, including freshwater, wetland, forest, grassland, desert, alpine, subterranean, arboreal and human-modified ecosystems, and are native to all continents except for Antarctica.

Taxonomic features

Today, the Amphibian class comprises 8,838 species divided into three Orders (Lissamphibia): Anura (7,783 species), Urodela/Caudata (830 species) and Gymnophiona (225 species) (Frost, 2024). Anurans and Urodeles are phylogenetically more closely related to each other than either is to Gymnophiona (Fig. 2). The three orders are morphologically unmistakable and distinctly distributed around the world (Fig. 3).

The order Anura, meaning "without tail" in Greek, includes all species of frogs and toads, characterized by the lack of a tail in their adult form and long hindlimbs compared to shorter forelimbs. These singular long hind limbs enable them to make prodigious leaps, exceeding 2 meters in some species. The common nomenclature of "frog" and "toad" has no taxonomic justification. Species with mainly aquatic lifestyles and smooth, moist skins are usually referred to as frogs, while terrestrial species with warty, dry skins are typically considered toads. Anurans can range in size from the 7.7-millimeters Amau frog (*Paedophryne amanuensis*), the smallest vertebrate on Earth, to over 30 cm in the Goliath frog (*Conraua goliath*). They are the amphibian order with the greatest number of described species and widest distribution, although they are especially abundant in the tropics.

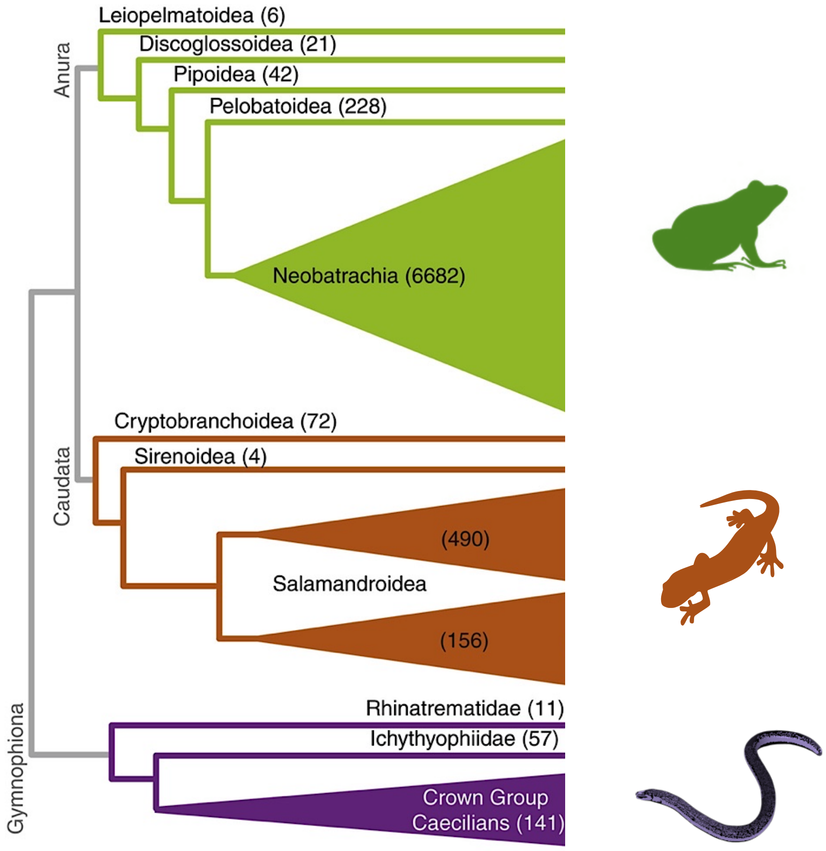


Figure 2. The amphibian tree of life. Phylogenetic tree representing the three amphibian orders with the number of species for major suborders and/or clades in parentheses. Figure adapted from Wake & Koo (2018).

The order Urodela, “tailed ones” in Greek, consists of salamanders and newts, which present a tail both in their larval and adult stages. Salamander bodies are slender, with short limbs projecting at right angles and an overall lizard-like appearance. As with frogs and toads, the common names “salamander” and “newt” are not taxonomically straightforward. Salamander usually refers to species with a terrestrial lifestyle while newts are viewed as mostly aquatic species. However, some classifications consider that salamander is an inclusive group, and newts are salamanders within the subfamily Pleurodelinae. Urodele size can also vary greatly, reaching up to 1.8 m in the Chinese Giant Salamander (*Andrias davidianus*), the largest amphibian in the world. Urodeles are mainly distributed in northern regions and are absent from most large continental and all oceanic islands.

The order Gymnophiona, “naked serpents” in Greek, represents the caecilians, long, cylindrical, limbless, worm-like amphibians. Caecilians remain poorly known to science due to their cryptic, mostly fossorial, lifestyle. This way of living determines that most caecilians have rudimentary, sometimes nonexistent eyes. They instead possess a pair of unique sensory organs, the tentacles, located between the eyes and nostrils that are thought to provide additional olfactory capabilities. Caecilians are also unusual among amphibians due to their bizarre parental care strategies, including dermatophagy and milk provisioning to their young. Their distribution is limited to the tropics, the most restricted among amphibian groups.

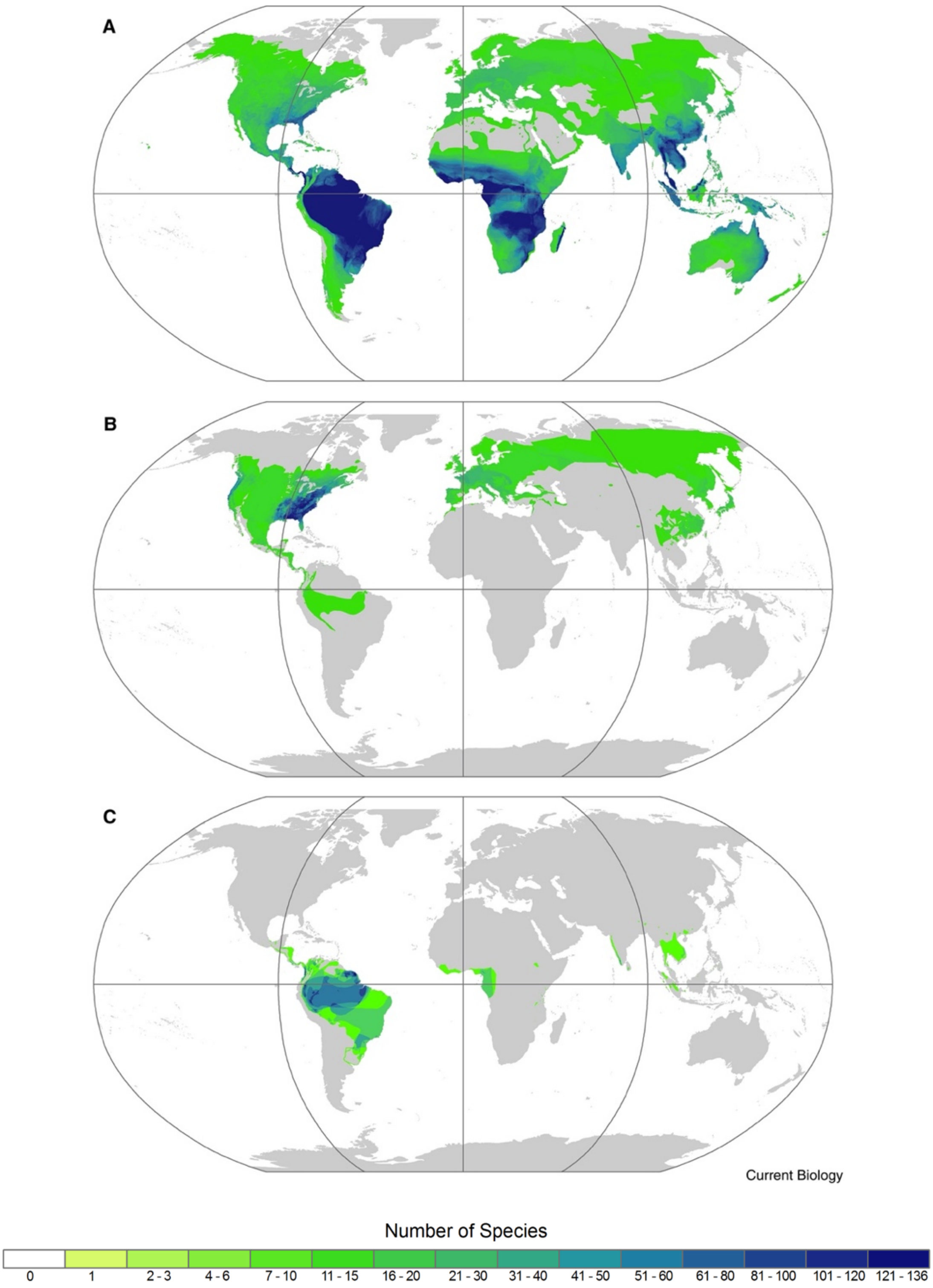


Figure 3. Global amphibian species distribution and richness. The three amphibian orders are distributed differently: Anurans (A) are widely distributed but more abundant in the southern hemisphere, Urodeles (B) are mostly found in the northern hemisphere, and Gymnophiona (C) are restricted to the tropics. Figure adapted from Wake & Koo (2018) and based on data for 7,011 species from AmphibiaWeb and IUCN 2018.

Life cycle and reproductive strategies

The general life cycle of amphibians involves three stages or phases: egg, larva or tadpole and post-metamorphic. Eggs are usually laid in water or moist environments to prevent desiccation. Additionally, they are commonly encased in a gelatinous substance that provides protection and helps retain humidity. The egg stage can last from a few days to several weeks, depending on the species and environmental conditions. Upon hatching, larvae, called tadpoles in Anurans, are primarily aquatic and possess features suited for life in water such as gills or a tail. Urodele larvae typically resemble miniature adults with external gills, whereas tadpoles differ greatly from post-metamorphic forms. Initially, larvae grow in size until they undergo a series of changes, known as metamorphosis, in transition to adult life (Fig. 4). Larval period is very variable, lasting for years in some species and environmental conditions.

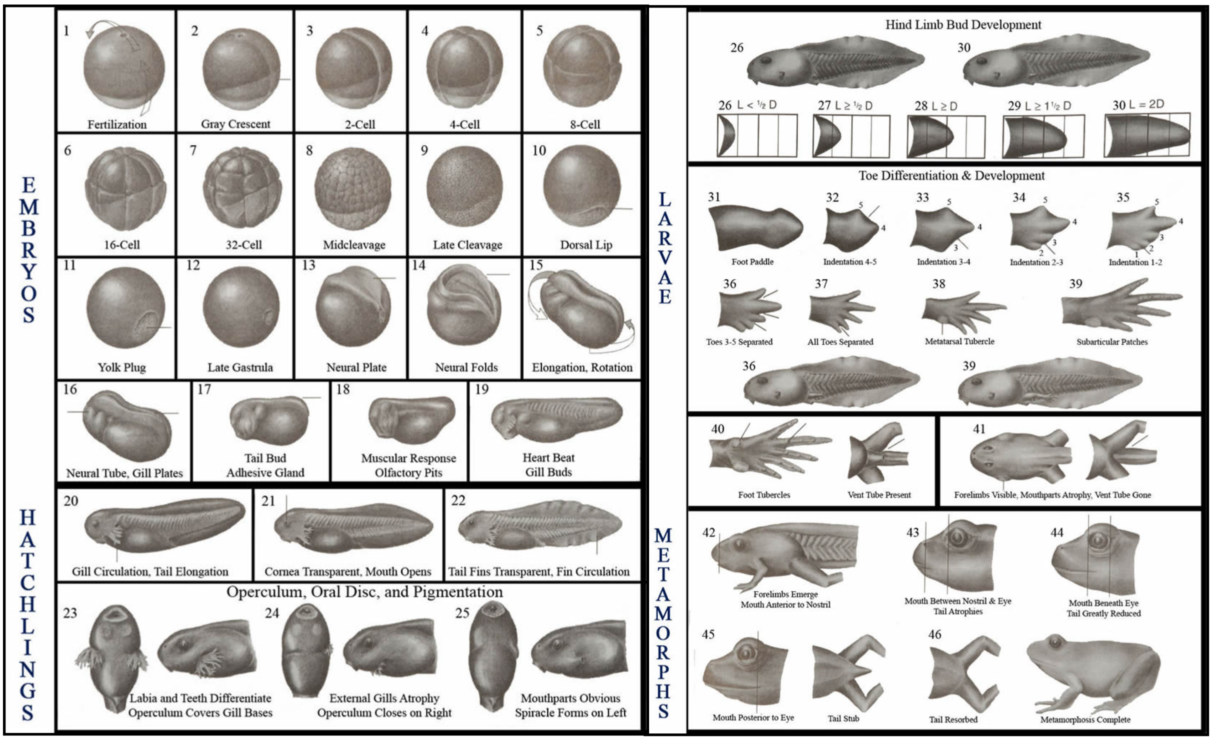


Figure 4. Developmental stages of Anurans according to Gosner (1960). Stages are divided between embryonic, hatchling, larval and metamorph development. The hatchling stage is a transitional period from immobile embryos to active larvae and is sometimes included in the larval period. The larval period is the longest stage, marked by size and limb development growth. Metamorphosis climax occurs between stages 42 and 46, although some body transformations begin earlier.

Metamorphosis is a remarkable biological process that involves a major and usually abrupt post-embryonic transformation of a larva into a juvenile (Laudet, 2011). This transformation invariably includes extensive morphological changes, such as the loss of gills, the development of lungs, the growth of limbs or the reabsorption of the tail. Morphological remodeling is always paired with physiological, biochemical and histological transformations in several organs and tissues. Metamorphosis also implies an ecological transformation, in which different life stages do not share or compete for the same resources. For example, in many frog species, tadpoles are aquatic and herbivorous while adults are terrestrial and carnivorous. Finally, metamorphosis is strictly connected to environmental conditions, with changes triggering the shift from one life stage to another.

Physiologically, these transformations are regulated by the production of thyroid hormones that induce the transcription of target genes. Thyroid hormone levels reach a maximum at the climax of metamorphosis, when the greatest morphological changes occur. Upon completion of metamorphosis, the animal emerges as a juvenile and is considered an adult once it reaches sexual maturity. Generally speaking, post-metamorphic amphibians possess lungs, although these may not be the primary means for respiration and are absent in the Plethodontidae family of salamanders; limbs, which are not present in Gymnophiona; and a skin adapted to prevent desiccation that contains glands for mucus and sometimes toxin production.

Despite metamorphosis being a highly successful life history strategy for amphibians, numerous variations to this process exist, reflecting species' adaptations to different environmental conditions and ecological niches. Two main variations in metamorphosis are the contrasting strategies of direct development and pedomorphosis (Elinson & del Pino, 2012; Laudet, 2011). Direct development involves the deletion of the larval stage and it has been described in all three amphibian orders. The eggs hatch directly into miniature adults, passing through the larval stage within the egg. This strategy allows species to survive in the absence of waterbodies by mating and depositing the eggs directly on land and avoiding predation of vulnerable larvae. On the other hand, pedomorphosis is the retention of larval morphology in sexually mature adults and is only present in Urodela.

Pedomorphosis can be obligate, where individuals never undergo metamorphosis and live in aquatic habitats throughout their lives, or facultative depending on the species. Facultative pedomorphosis provides phenotypic plasticity and adaptation to changing and heterogeneous environments.

Reproductive strategies in amphibians are equally varied, perhaps the most diverse among vertebrates (Kühnel et al., 2010). Both internal and external fertilization occur, with offspring produced via oviparity, ovoviviparity or viviparity. Internal fertilization is a condition for viviparity, which is widespread in Gymnophiona but is seen across orders. External fertilization, in contrast, is the predominant strategy in Anurans. Courtship, mating behavior and cloacal morphology are extremely complex and adapted to each species' reproductive strategy (Fig. 5). Many species display sexual dimorphism, with females often being larger than males or sexes differing in coloration. Despite only about 10% of species showing parental care, these are incredibly varied, with all biparental, female and male uniparental care exhibited (Roland & O'Connell, 2015). Parental care may involve bizarre forms of egg defense, egg incubation, larval transport and/or offspring feeding.

Figure 5. Reproductive strategies of three European amphibians. Left: *Epidalea calamita* on amplexus, a mating position where the male firmly grasps the female from behind with his forearms. Centre: *Alytes obstetricans* after courtship; the male has wrapped the string of eggs around his back legs and will look after them for over a month, before placing them in the water to hatch. Right: *Calotriton asper* on amplexus, where the male grasps the female's trunk with his tail underwater, an adaptation to fast-moving alpine streams. All three species are oviparous but both toads exhibit external fertilization while the newt uses internal fertilization. Both toads also use calls to locate each other during the reproductive season, with the natterjack toad producing extremely loud calls and the common midwife toad emitting high-pitched whistles. Photo credits: Ignasi Marco.

Respiratory and skin physiology

Amphibians utilize different organs for respiration including gills, lungs and skin (Gargaglioni & Milsom, 2007). In most species, at least two mechanisms are functional at any given species and life stage, which can be independently perfused in different proportions. Additionally, two different respiratory media can be used: water and air. Aquatic larvae and paedomorphic species primarily rely on gills for gas exchange. Lungs are used for air-breathing and usually develop during metamorphosis, although they may be already present in larvae. Amphibian lungs are primitive, saclike structures with few internal septa and large alveoli. Therefore, the oxygen diffusion rate into blood is slower than in other vertebrates. Gill and lung ventilation is achieved by buccal or pharyngeal pumping of water or air, respectively.

Finally, all species and life stages can exchange respiratory gases from water or air through the skin (Tattersall, 2007). Amphibian skin is highly permeable and has a rich vasculature that facilitates oxygen uptake and elimination of carbon dioxide. To allow gas exchange, the epidermis needs to be relatively thin, consequently causing high water loss rates in amphibians and explaining their close link with water. Cutaneous respiration represents a different proportion of all respiration depending on species, life stage, season and even local skin areas. In some species, it may be the only means of respiration, such as in the terrestrial lungless salamanders or aquatic species without gills. Regulation of this respiratory mechanism probably involves neural and hormonal control of the cutaneous vasculature.

In addition to gas exchange, skin is responsible for osmoregulation, hydration status, thermoregulation, defense and communication functions in amphibians. Essential ions, such as Na^+ or Cl^-, are actively transported and absorbed through the skin (Larsen, 2021). Water intake is also controlled by the skin, through aquaporin channels, rather than by oral drinking. The ventral pelvic region, known as the drink patch, is the body region of highest water absorption in most species since it has increased vascularization and surface area. Hence, amphibian skin governs and ensures whole-body homeostasis.

Two types of glands are present on amphibian skin and are essential for physical and chemical defense against predators and pathogens (Toledo & Jared, 1995). Mucous glands produce a cutaneous surface fluid that prevents desiccation, aids respiration and skin shedding, and makes the animal slippery, facilitating escape from predators. On the other hand, granular or poison glands produce noxious or toxic secretions that are discharged when adrenergic receptors are stimulated after alarm or injury. Some bioactive molecules secreted by granular glands are categorized as antimicrobial peptides (AMPs)(Rollins-Smith et al., 2005). They have potent activity against bacteria, fungi, protozoa and viruses, representing a first line of defense against pathogens. AMPs, hence, play a role in the innate immune response of the skin and contribute to immune modulation, chemotaxis and healing among others. As in other vertebrates, amphibian immune defenses consist of both innate and adaptive components. However, the immune mechanisms in amphibian skin are complex and remain poorly understood.

Global amphibian declines

During the First World Congress of Herpetology in 1989, scientists began to be concerned about potential worldwide amphibian population declines and extinctions. Some declines started as early as the 1970s, were occurring simultaneously across vast distances and were even happening in protected and pristine areas (Collins & Storfer, 2003). The lack of scientific consensus and comprehensive knowledge triggered the IUCN to establish an investigation task force, the Global Amphibian Assessment (GAA) in 2004 (Stuart et al., 2004). This assessment aimed to gather data on the distribution, abundance, population trends, habitat and threats of all described amphibian species. The results of the GAA and later assessments are clear: amphibians are the most endangered vertebrates on Earth.

Currently, over 41% of all amphibian species are globally threatened (i.e. listed as Vulnerable, Endangered or Critically Endangered in the IUCN Red List), which is far above the levels of threat for mammals (26%) or birds (12%) (IUCN, 2024). At least 3,603 species are experiencing population declines whereas only 27 species show positive trends (IUCN, 2024).

Although the exact number of recent extinctions is difficult to prove, the GAA estimated that between 9 and 122 amphibian species might have gone extinct since 1980 (Stuart et al., 2004). Moreover, these numbers are probably underestimated since 911 species are still too poorly studied to be assessed, and classified as Data Deficient in the Red List (IUCN, 2024).

The drivers of these declines were largely unknown, and scientists explored the potential contribution of habitat loss, overexploitation (human trade and consumption), introduced predators, ultraviolet radiation, pollution, climate change, diseases and interactions among these (Alford & Richards, 1999; Collins & Storfer, 2003; Pounds et al., 2006). The GAA classified declining species based on the causes of their decline into "overexploited", "reduced-habitat" and "enigmatic decline" (Stuart et al., 2004). This last group included species disappearing even when suitable habitat remained and for reasons not fully understood. Also, the number of "enigmatic-decline" species increased with increasing levels of threat, suggesting that the causal factors were rapid and powerful extinction drivers. Rapidly declining species were frequent in the Neotropics, but while reduced-habitat species occurred widely in different continents, overexploited species were concentrated in East and Southeast Asia and enigmatic-decline species were restricted to South and Meso America and Australia (Fig. 6).

Despite the GAA confirming that amphibian population declines were rapid and could not be explained by the typical causes of biodiversity loss, the ultimate driver of these enigmatic declines was still unknown. During the early 2000s, accumulating evidence indicated that infectious diseases were the most plausible explanation for these declines and extinctions. In particular, the emergence of amphibian chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis,* provided the clearest link between disease and declines (Berger et al., 1998; Daszak et al., 2003; Lips et al., 2006). Nowadays, there is no doubt that chytridiomycosis has decimated amphibian populations worldwide and has even been considered "the worst vertebrate disease in recorded history" (Gascon, 2007).

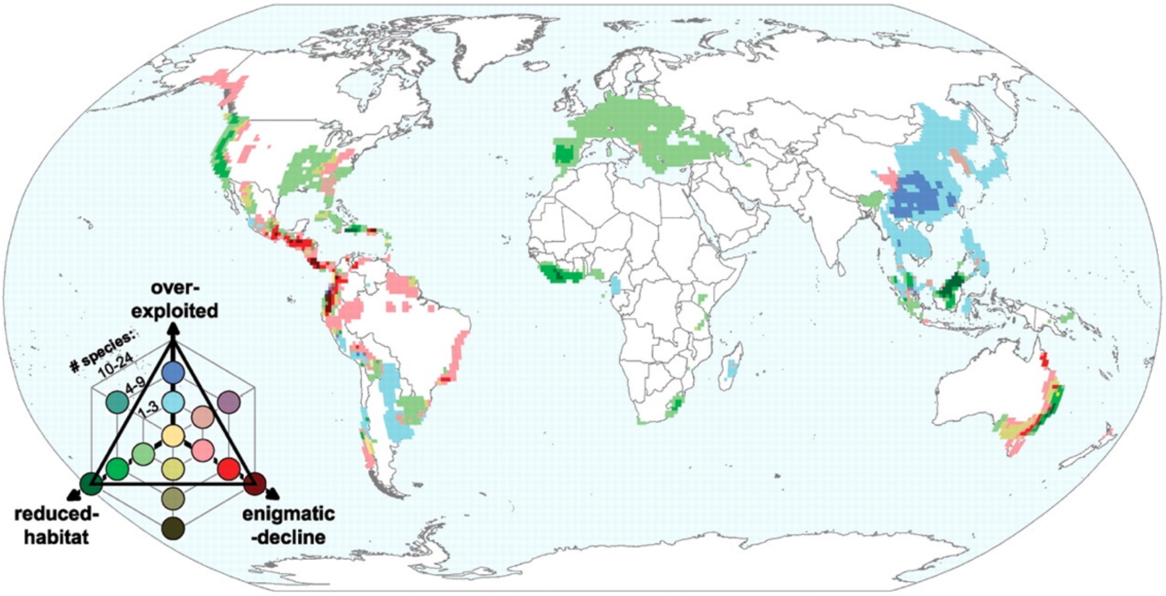


Figure 6. Distribution of rapidly declining amphibian species according to the dominant causal factors. Blue = overexploited; Green = reduced-habitat; Red = enigmatic declines. Darker colors indicate larger numbers of declining species. From Stuart et al. (2004).

The value of amphibians

Modern amphibians may go unnoticed in human daily life and have traditionally been neglected species for conservation science. However, amphibians are vital in maintaining healthy and resilient ecosystems and provide ecosystem services to human societies. Amphibians are virtually ubiquitous and provide services in all four categories of ecosystem services: supporting, regulating, provisioning and cultural (Millennium Ecosystem Assessment, 2005).

Supporting services include contributions to the ecosystem's physical structure and functioning. Amphibians are important contributors to regional diversity and aquatic and terrestrial food webs (Hocking & Babbitt, 2014). Herbivorous tadpoles regulate primary production, maintaining water quality and preventing eutrophication (Alonso et al., 2024). Adults and carnivorous larvae feed primarily on invertebrates and exert top-down control over prey populations. On the other hand, amphibians are essential prey for numerous larger predators such as reptiles, birds or fish. The decrease in amphibian populations has even been linked to the collapse of predator populations in some areas (Zipkin et al., 2020). Overall, amphibians contribute to ecosystem functions by facilitating nutrient cycling and energy flow between terrestrial and aquatic ecosystems. They also directly affect ecosystem structure by physical habitat modification through soil burrowing and bioturbation.

Regulating services provided by amphibians include the control of pest outbreaks and disease transmission (Hocking & Babbitt, 2014). Predation of mosquitoes by amphibians has been demonstrated to be significant, particularly in ephemeral wetlands that cannot support fish. The ability to reduce mosquito larvae abundance is well exemplified by certain species, such as the mole salamander (*Ambystoma talpoideum*), with individuals capable of consuming up to 900 mosquito larvae per day (DuRant & Hopkins, 2008). This mosquito reduction can, in turn, decrease the transmission of mosquito-borne diseases. In Central America, amphibian collapses have been associated with increased human malaria incidence (Springborn et al., 2022). This suggests that amphibians may have an underappreciated impact on public health and human welfare.

Amphibians also contribute to provisioning services by producing useable products such as food or medicine (Hocking & Babbitt, 2014). Frog legs are consumed worldwide and amphibian meat is a significant source of protein in regions like Southeast Asia. Numerous medical advantages have also become possible thanks to amphibians, from traditional medicine and pregnancy tests in the 20th century to modern medical research. The diverse array of chemicals and antimicrobial peptides produced by their skin offer starting points for developing new drugs. Moreover, amphibians are excellent research models for studying tissue regeneration due to their remarkable ability to regrow limbs and even organs.

Cultural services of amphibians to human societies extend globally and across centuries. They feature prominently in mythology, such as in China and Japan, biblical plagues, and medieval Europe, where frogs and toads were associated with magic and witchcraft. Amphibians also appear frequently in the arts, including music, literature, decorations, and jewelry (Fig. 7). In modern times, amphibians are depicted in children's books, movies, and toys. Additionally, their aesthetic appeal and relative ease of maintenance make them popular pets.

Figure 7. Examples of art depicting amphibians. Left: mural fragment from the 7th to 10th century from Cacaxtla, Mexico. Center: print of a frog by the Japanese artist Matsumoto Hoji, from 1814. Right: Kermit the Frog, arguably Jim Henson's most famous Muppet character, created in 1955.

Amphibians are highly sensitive to environmental changes due to their permeable skin and dual life cycle, which exposes them to both aquatic and terrestrial threats. Therefore, declines in amphibian populations can signal broader environmental issues and serve as indicator species for ecosystem health. By conserving amphibian populations, we not only protect these species but also sustain the intricate web of ecological interactions and services that support our environment and well-being. Promoting amphibian conservation can thus have far-reaching socio-economic benefits, highlighting the importance of these often-overlooked creatures in maintaining a healthy and resilient planet.

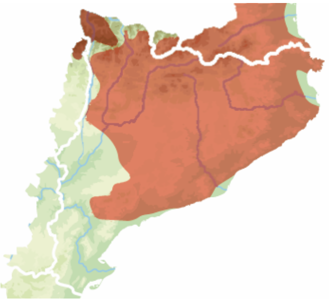
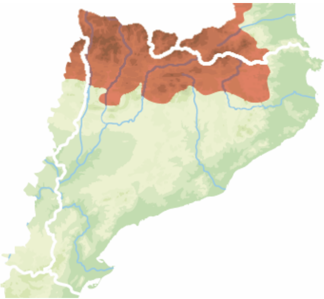
Amphibians in Catalonia

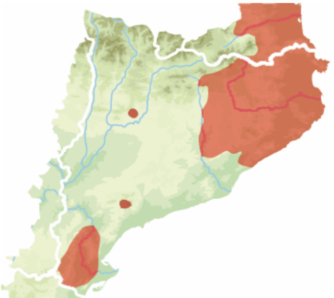
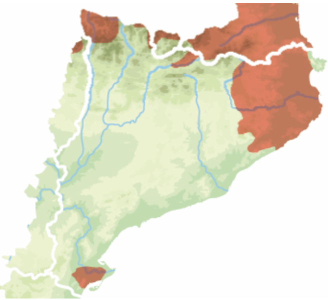
Catalonia, in the northeastern corner of Spain, is home to 15 native amphibian species. It is considered an area of high amphibian richness, as species from different bioregions, habitats and altitudes are present (Pleguezuelos et al., 2002). Two endemic species inhabit Catalonia, the Pyrenean brook newt (*Calotriton asper*), endemic to the Pyrenees, and the Montseny brook newt (*C. arnoldi*). The Montseny brook newt is uniquely endemic to Catalonia and is classified as critically endangered by the IUCN red list due to its low population numbers and extremely limited distribution, a single mountain range. One species, the Graf's hybrid frog (*Pelophylax kl. grafi*) is a hybridogenic species and is grossly indistinguishable from the Perez's frog (*Pelophylax perezi*). For the purpose of this thesis, this species has not been treated separately or included in species counts and descriptions.

The main characteristics, nomenclature and distribution of Catalonian amphibians are summarized below in a series of tables, organized by amphibian Order and Family.

Urodela

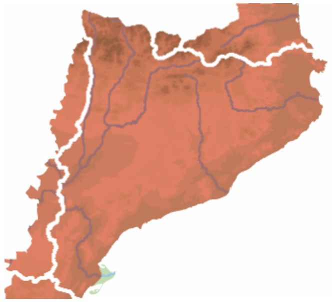
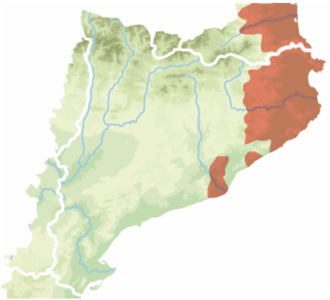
Salamandridae

Picture	Species	Common name	Distribution	IUCN status	Comments
	*Salamandra salamandra*	Fire salamander		VU	Mostly terrestrial and nocturnal habits. Females approach water to give birth to live larvae. Prefers deciduous forests where it is usually seen on rainy nights, but found on diverse habitats and altitudes.
	*Pleurodeles waltl*	Iberian ribbed newt		LC	Largest European newt. As a defense mechanism it can protrude the ribs through the dorsolateral tubercles in the skin. Mostly aquatic and limited to areas with Mediterranean climate.
	*Calotriton asper*	Pyrenean brook newt		LC	Endemic to the Pyrenees and pre-Pyrenean areas. Mostly aquatic, lives in cold and well oxygenated lakes and streams. Mating occurs underwater, where the male wraps the tail around the female's body.

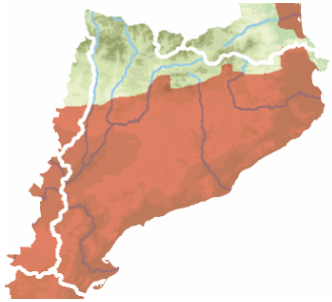
	*Calotriton arnoldi*	Montseny brook newt		CR	Very similar to the Pyrenean brook newt. More aquatic, smaller and with smoother skin. Distribution limited to a few streams in a single mountain range, the Montseny.
	*Triturus marmoratus*	Marbled newt		VU	Medium to large colorful newt. Habitat opportunist. Generally only aquatic during reproduction, when males display a large wavy crest from the neck to the tip of the tail.
	*Lissotriton helveticus*	Palmate newt		LC	Small smooth-skinned newt. Found in a great variety of habitats and altitudes. Only aquatic during reproduction, when males develop a caudal filament, a dorsal crest and webbed hind feet.

Anura

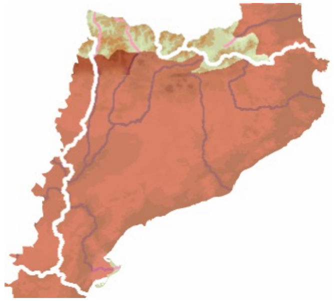
Alytidae

	*Alytes obstetricans*	Common midwife toad		LC	Small, robust and cryptic toad. Terrestrial and nocturnal habits. Found in a wide variety of habitats and altitudes, from montane to urbanized. Dwells close to water bodies, where males deposit the eggs to hatch.
	*Discoglossus pictus*	Painted frog		LC	Medium-sized frog that was introduced into Catalonia from Algeria in the XIX century. Opportunistic in habitat and lifestyle but preference for Mediterranean areas. Two morphotypes exist, spotted and stripped.

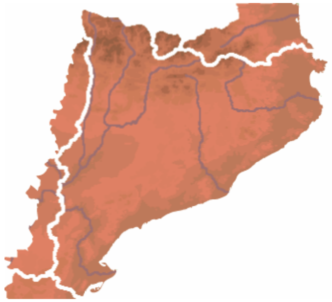
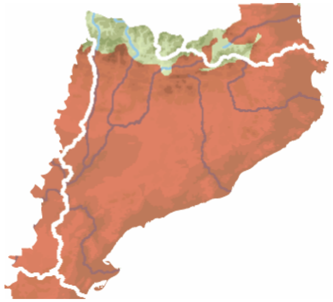
Pelobatidae

	*Pelobates cultripes*	Western spadefoot		VU	Big smooth-skinned toad, has a black spade on hindfeet that helps it dig burrows where it hides during the day. Terrestrial, except for reproduction, and adapted to sandy and agricultural open habitats.

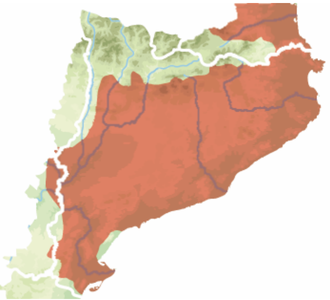
Pelodytidae

	*Pelodytes punctatus*	Common parsley frog		LC	Small and slender frog with warty skin. Nocturnal and fairly cryptic. Breeds in sunny open, shallow or temporary waters and even in brackish waters or cultivated areas. Is a skillful climber.

Bufonidae

	*Bufo spinosus*	Spiny toad		LC	Classical toad, bulky and with warty skin. Terrestrial and nocturn. Found in a great variety of habitats from forests to wetlands, open fields, urban, montane areas and often away from water bodies.
	*Epidalea calamita*	Natterjack toad		LC	Medium-sized toad, moves with short runs instead of hopping. Adapted to sandy and heathland areas and even urban/agricultural areas. Opportunistic breeder throughout the year in temporary shallow ponds.

Hylidae

	*Hyla meridionalis*	Stripeless tree frog		LC	Small smooth frog with long hind legs. Fingertips have adhesive discs adapted to climb. Found in diverse lowland habitats mostly in vegetation near waterbodies. Males have large vocal sacs to produce very loud calls.

Ranidae

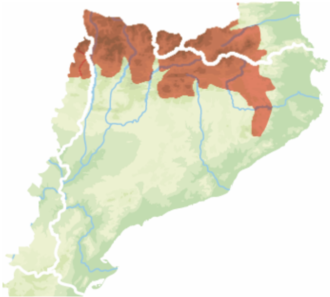
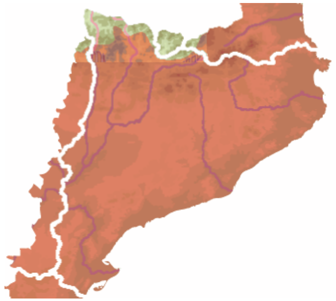
	*Rana temporaria*	European common frog		LC	Medium-to-large frog. Confined to montane cold areas in southern Europe, Pyrenees and pre-Pyrenees in Catalonia. Semi-aquatic, breeds explosively in lakes and fens after the snowmelt.
	*Pelophylax perezi*	Iberian green frog		LC	Large frog with brown to green coloration. Widespread and common, usually diurnal and conspicuous due to its loud calls. Mostly aquatic, found in all kinds of water bodies but absent in alpine ecosystems.

Photo credits: Ignasi Marco, Xavier Fernández Aguilar, Johan Espunyes, Life Tritó Montseny (Fèlix Amat). Distribution maps edited from García-salmerón et al. (2022). IUCN status: LC = Least Concern, VU = Vulnerable, CR = Critically Endangered (IUCN, 2024).

Chytridiomycosis: the worst disease ever recorded

Amphibian chytridiomycosis is a disease caused by two chytrid fungi: *Batrachochytrium dendrobatidis* and *B. salamandrivorans* (hereafter Bd and Bsal, respectively). Chytridiomycosis was first reported as a cause of mortality in wild and captive frogs from Australia and the Americas (Berger et al., 1998). Simultaneously, Bd was discovered as a new species of chytridomycete fungi and as the causal agent of these mortality outbreaks (Longcore et al., 1999). However, Bd probably emerged during the 1970s, when amphibian declines became noticeable around the globe (Lips, 2016). Since its discovery, Bd has been demonstrated as the sole cause of catastrophic declines and extinctions in numerous amphibian populations. In 2010, a second chytrid pathogen was discovered causing lethal disease and steep declines in salamander populations in northwestern Europe (Martel et al., 2013). The extent of the amphibian crisis and the demonstration of chytridiomycosis as a cause of some declines stimulated an extensive research effort. Although most studies focus on Bd, we currently know many aspects of chytrid fungi phylogeny, origin, distribution, ecology and impact. Nevertheless, disease mitigation remains a major challenge and chytridiomycosis continues to be a significant threat to amphibian conservation.

Life cycle and pathogenesis

Both Bd and Bsal have a non-mycelial morphology, characterized by two main life stages (Fig. 8): a motile flagellated zoospore and a reproductive thallus that produces zoosporangium with asexual zoospores (Berger et al., 2005; Longcore et al., 1999). Zoospores are free-living and act as the dispersal stage, being able to survive in the environment for a variable period. Zoospores invade the basal layers of the amphibian epidermis where they germinate, the flagellum is reabsorbed and a cell wall is formed. Immature sporangia are transported from the deeper layers to the skin surface, following epidermal cell maturation and suggesting a long history of evolution of these pathogens with their amphibian hosts. Large numbers of infectious zoospores are then generated inside the maturing sporangia and released into the environment through a discharge tube that opens on the cell surface. These new zoospores are immediately infectious and therefore self-reinfection is possible.

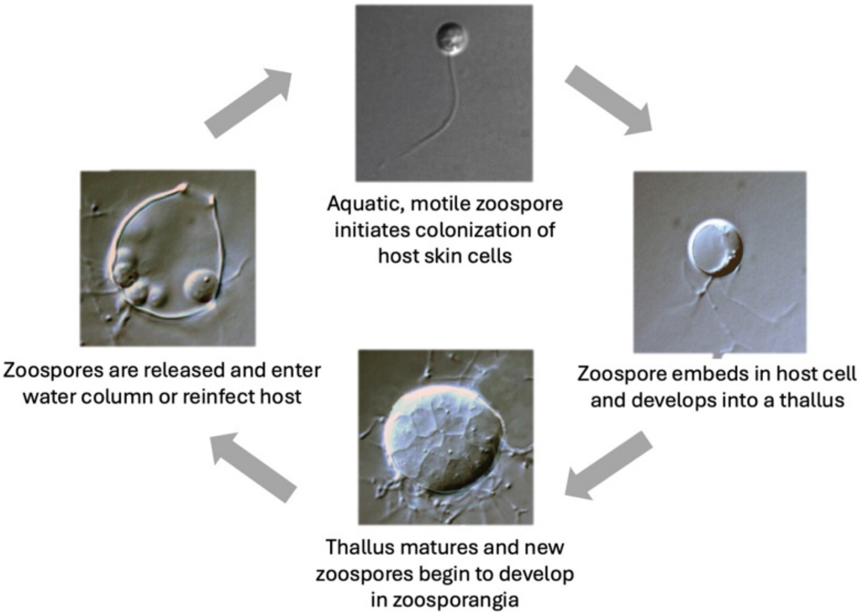


Figure 8. Life cycle of the pathogenic chytrid fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*. Adapted from Rosenblum et al. (2010).

The life cycle of chytrid fungi is highly temperature-dependent with optimal growth occurring between 17-25ºC and 10-15ºC for Bd and Bsal, respectively (Longcore et al., 1999; Martel et al., 2013). Growth can still take place outside these ranges at slower rates and both pathogens appear able to survive temperatures below 5ºC, whereas death may occur at >28ºC for Bd and >25ºC for Bsal (Van Rooij et al., 2015). Under laboratory conditions at 22ºC, the life cycle of Bd takes 4 to 5 days to complete and is expected to be similar in amphibian hosts (Berger et al., 2005). Environmentally resistant non-motile spores have been identified only in Bsal (Stegen et al., 2017).

Chytrid fungi only colonize amphibian keratinizing stratified epidermis. Therefore, the distribution of sporangia follows the distribution of keratin in adults and larvae. In tadpoles, colonization is restricted to their keratinized mouthparts and during metamorphosis infection progresses following the keratinization of epithelia which begins on the hindlimbs (Marantelli et al., 2004). By the end of metamorphosis, the infection has spread throughout the skin of the amphibian host, covering the whole body, head, limbs and tail. The skin is an essential organ for amphibians and chytrid colonization impairs many of the skin's vital functions. The presence of sporangia disrupts the physical structure of the epidermis, interfering with osmoregulation. This results in reduced plasma sodium, potassium and chloride ion concentrations, leading to abnormal cardiac electrical activity and cardiac arrest (Voyles et al., 2009).

Clinical signs in post-metamorphic amphibians may be undetectable and sudden death without obvious disease is common. When present, signs include excessive skin shedding, erythema or skin discoloration, lethargy, anorexia, abnormal posture, aberrant swimming and loss of righting reflex (Van Rooij et al., 2015). The pelvic patch, feet and toes are predilection sites of infection. In tadpoles, clinical signs are limited to depigmentation of the mouthparts, although sub-lethal effects may be possible (Hanlon et al., 2015). Conversely, Bsal chytridiomycosis is characterized by multifocal superficial erosions and ulcerations throughout the body. Microscopically, numerous sporangia can be seen in the keratinized layers of the skin, associated with severe hyperkeratosis, hyperplasia, erosions and ulcerations (Fig. 9). Inflammatory response is typically absent; however mild inflammation may be seen associated with secondary bacterial infections (Grogan et al., 2018). Dissemination to deeper skin layers or internal organs does not occur.

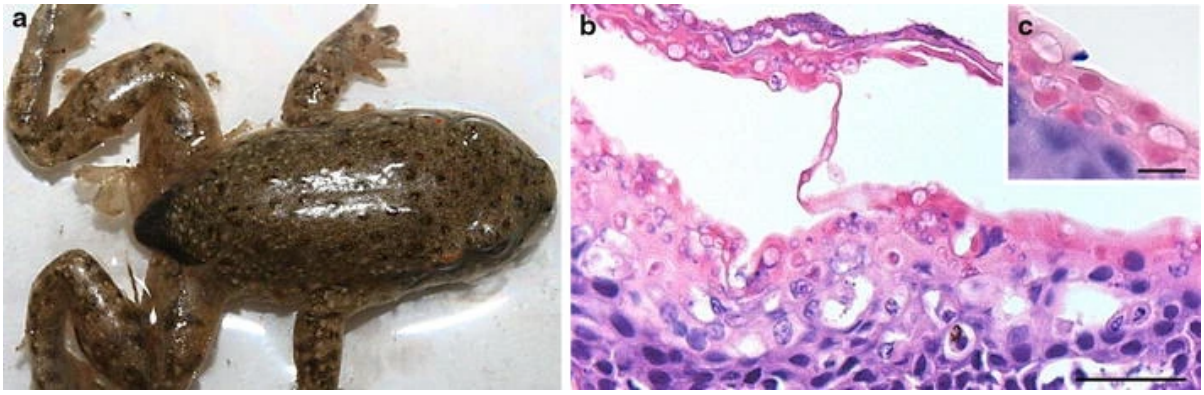


Figure 9. Clinical signs and pathology of chytridiomycosis caused by *Batrachochytrium dendrobatidis*. (a) Naturally infected *Alytes obstetricans* showing excessive skin shedding. (b) Microscopy of a skin section from the same animal revealing diffuse hyperkeratosis, hyperplasia and numerous intracellular zoosporangia; HE, scale bar 50 μm. (c) Intracellular septate zoosporangia; HE; scale bar 10 μm. From Van Rooij et al. (2015).

Emergence and impact

Until recently, there has been much debate about the origins Bd and Bsal, particularly about whether they were recently introduced pathogens (Novel Pathogen Hypothesis), or whether they were endemic pathogens and amphibian mortalities were due to shifts in host susceptibility, pathogen virulence or environmental changes (Endemic Pathogen Hypothesis). These were crucial questions since they had implications for understanding the drivers of emergence and for successful disease mitigation and conservation actions.

The documentation of Bd arrival and spread coinciding with wave-like amphibian declines provided some support for the novel pathogen hypothesis (Lips et al., 2006; Skerratt et al., 2007). However, it wasn't until the application of whole-genome sequencing that the spatiotemporal origins of chytrid fungi were solved (Hanlon et al., 2018). Hanlon et al., (2018) demonstrated that Bd had different genetic lineages, with one, *Bd*ASIA-1, exhibiting the greatest genetic diversity and hallmarks of an ancestral population. Therefore, East Asia is now believed to be the origin of Bd and the source of other Bd lineages. Bd is currently distributed worldwide, but the amphibian pandemic is attributed to the emergence of a single lineage, *Bd*GPL (Global Pandemic Lineage), which shows a recent and near-simultaneous expansion (Hanlon et al., 2018). This expansion took place in the 20th century, coinciding with the global expansion of amphibian trade for exotic pets, medical and food purposes. In fact, the commercial trade of amphibians has been identified as the main driver of the chytridiomycosis pandemic. Live traded amphibians are commonly infected with Bd and intercontinental transmission is ongoing (Hanlon et al., 2018; Schloegel et al., 2009). Similarly, Bsal likely originated in Asia and has recently been introduced through amphibian trade into European naïve populations (Martel et al., 2014). To date, Bsal has only been detected outside Asia in the Netherlands, Belgium, Germany and Spain (Martel et al., 2014, 2020; Sabino-Pinto et al., 2015). Owing to the importance of trade in chytrid fungi emergence, the infection by both pathogens has been included in the World Organization for Animal Health (WOAH, previously OIE) list of reportable diseases (WOAH, 2024a).

Amphibian chytridiomycosis pandemic has been referred to as the worst disease ever recorded due to its impacts on biodiversity. Recent assessments, demonstrate the role of chytridiomycosis in the decline of at least 501 amphibian species in all three amphibian orders, including 90 presumed total extinctions (Scheele et al., 2019). All the declines were attributed to Bd, except for the decline of *Salamandra salamandra* in Europe, which was caused by Bsal. The greatest impacts have been noted on large-bodied and range-restricted anurans from wet climates in the Americas and Australia, probably due to reduced reproductive potential of hosts and optimal environmental conditions for Bd (Fig. 10). No declines have been reported in Asia, where amphibians are considered resistant due to their long interactions with the pathogens. Of all declined species, only 12% are showing signs of population recovery, while population trends are unknown for 49% and 39% are suffering ongoing declines. After initial invasion, some susceptible host populations can survive the epidemic phase and persist with endemic infections. However, endemic disease can have cryptic effects by causing slow declines, suppressing population densities or increasing the vulnerability to other threats and inducing population extirpation even at low pathogen prevalence (Valenzuela-Sánchez et al., 2017).

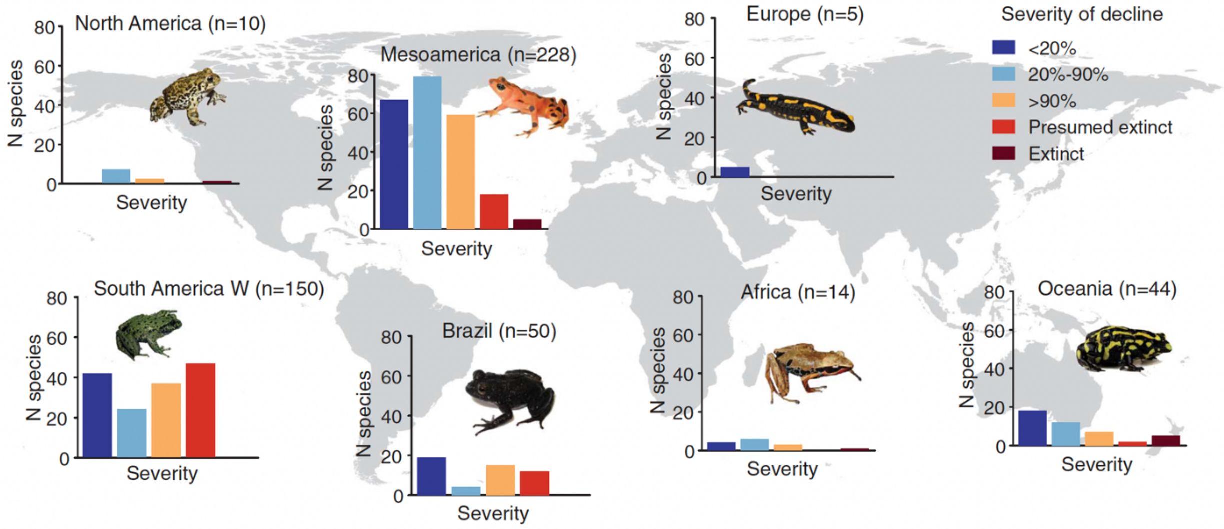


Figure 10. Worldwide distribution and severity of amphibian declines caused by chytridiomycosis. Bars indicate the number (N) of species that suffered declines grouped by geographic region and severity. From Scheele et al. (2019).

The ability of chytrid fungi to cause population declines and extinctions is attributable to its broad host range (Olson et al., 2021), high transmissibility within and between amphibian species, persistence in reservoir hosts and the environment (De Castro & Bolker, 2005) and maintenance of high virulence (Voyles et al., 2018). Chytrid fungi have an exceptionally broad host range, with Bd capable of infecting over 1,375 amphibian species (Olson et al., 2021). Conversely, Bsal exhibits greater host specificity, primarily parasitizing urodele species whereas most anurans and caecilians appear refractory or resistant to infection (Martel et al., 2014). Nevertheless, there is a substantial variation in responses to exposure and infection between host species, populations and life stages (Scheele et al., 2017; Searle, Gervasi, et al., 2011; Tobler & Schmidt, 2010). The range of outcomes to chytrid fungi infection progresses from no infection, infection without disease, disease with limited mortality, mass mortalities with subsequent recovery, population declines and extinctions. Laboratory and field studies have demonstrated that several independent but interacting factors are responsible for these variations in disease dynamics and outcomes including pathogen, host and environmental determinants.

The pathogen

Spatial or temporal variations in chytrid fungi virulence can influence amphibian host survival and population impacts. As virulence factors are genetically determined, genetic differences between pathogen lineages and isolates can translate into variations in virulence. For Bd, phylogenetic studies show four deeply diverged lineages: *Bd*ASIA-1, *Bd*ASIA-2*Bd*BRAZIL, *Bd*CAPE and *Bd*GPL. The *Bd*GPL has rapidly spread worldwide and is considered a hypervirulent lineage, based on its infectivity and lethality in experimental exposures and its common association with chytridiomycosis outbreaks (Hanlon et al., 2018). However, depending on the local context, other lineages can also be responsible for lethal disease and population declines, as seen with the hypovirulent *Bd*CAPE and *Alytes muletensis* declines in Mallorca (Doddington et al., 2013). The discovery of hybrid genotypes shows that Bd can exchange haplotypes among lineages, continuously generating new genomic diversity with potential virulence shifts (Hanlon et al., 2018).

Phenotypic differences, including virulence, have been demonstrated *in vitro* between Bd isolates, even over small geographical scales and within strains (Brannelly et al., 2021). Within the same pathogen isolates, virulence can also be maximized at optimal growth temperatures. Bd seems susceptible to attenuation, which can be achieved *in vitro* after successive passages on culture media. However, there is scant evidence that Bd attenuation is occurring in nature and contributing to the persistence or recovery of host populations. Conversely, the best evidence indicates that Bd virulence has remained stable over time or even increased in endemic populations (Brannelly et al., 2021). Finally, pathogen infection dose determines the development of disease and mortality in individuals and may explain different population-level outcomes (Blaustein et al., 2018; Briggs et al., 2010). For Bsal, many of these questions remain to be elucidated since most experimental work has been performed using only one pathogen isolate and a small range of infectious doses.

Moreover, interactions between coinfecting pathogens can affect infection dynamics and outcomes. Coinfection with Bd and Ranaviruses has been studied in several systems with contrasting results, ranging from no interaction (Thumsová, Alarcos, et al., 2024; Warne et al., 2016) to positive associations among pathogens (Whitfield et al., 2013). Different Bd lineages may also co-occur within the same host, generating hybrids (Schloegel et al., 2012) and potentially modulating pathogen impacts (Rodriguez et al., 2014). The study of coinfections with Bd and Bsal has only recently started to gain attention (Lötters et al., 2018), and initial results show probable synergistic effects between both chytrid fungi. As Bsal continues to expand its range, coinfections with Bd will be increasingly common, potentially exacerbating the impacts of chytridiomycosis on amphibian diversity.

The amphibian host

Central to understanding pathogen dynamics and population outcomes are the effects of variations in host responses among different amphibian species, populations and life stages. Animal defenses against infection involve two distinct but complementary mechanisms: resistance and tolerance (Grogan et al., 2023; Råberg et al., 2009). Resistance describes the host's ability to prevent the establishment or limit the intensity of infection (pathogen loads), while tolerance measures the ability to limit detrimental effects on fitness or survival at a given pathogen load (Fig. 11). Therefore, pathogen transmission is reduced with greater host resistance, but amplified with greater host tolerance.

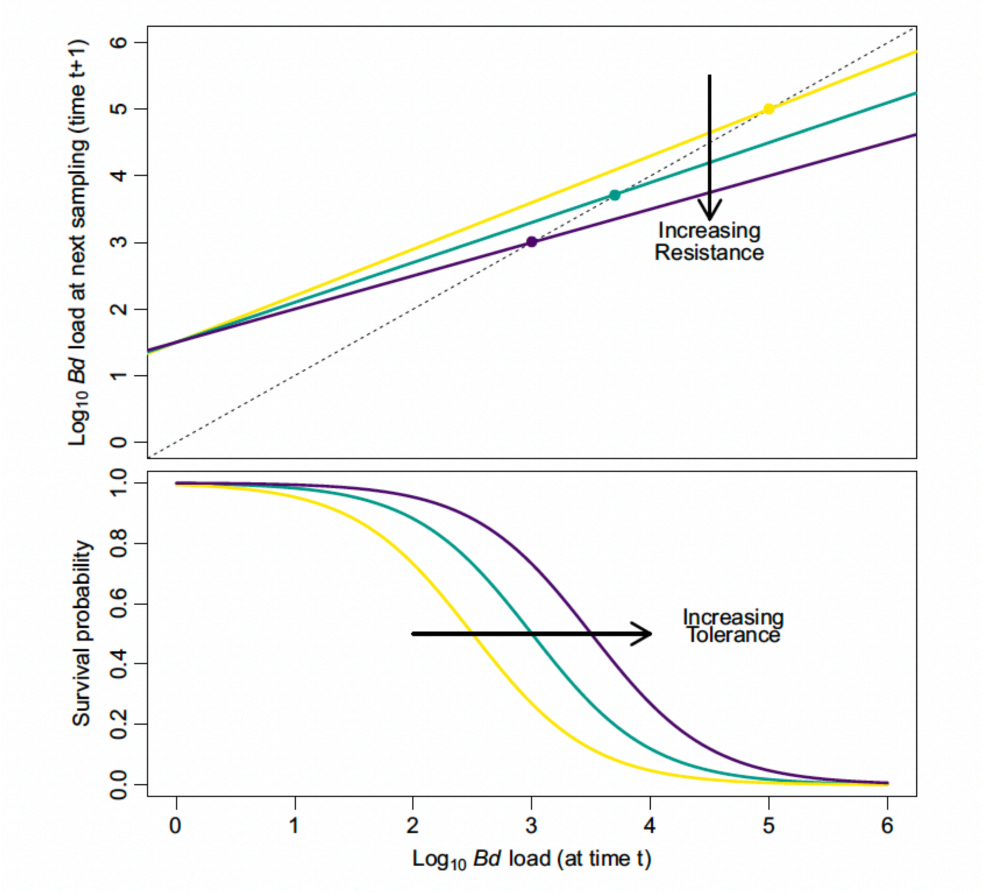


Figure 11. Graphical representation of resistance and tolerance. Top graph: lines show pathogen growth over time, pathogen load at time t +1 (Y-axis) given load at time t (X-axis). Lines with shallower slopes indicate higher resistance and different lines represent different host species, populations, life stages or individuals. The dashed line illustrates where pathogen growth is zero (1:1 line). Bottom graph: curves show survival probability as a function of pathogen load. Curves further to the right indicate higher tolerance and different curves represent different host species, populations, life stages or individuals. From Brannelly et al. (2021).

These traits are especially valuable for describing and comparing relative differences among amphibian hosts since both resistance and tolerance undoubtedly contribute to the diverse impacts observed for chytridiomycosis. Tolerant/resistant hosts within a single amphibian species may be crucial long-term survivors, contributing to population persistence or recovery after disease invasion. Simultaneously, tolerant hosts are typically reservoir hosts in a community, contributing to pathogen maintenance by sustaining high pathogen loads without succumbing to disease. A widespread example are anuran tadpoles, which become infected with high infection prevalence and intensity, yet rarely suffer mortality (Blaustein et al., 2018). Furthermore, tadpoles of some species have very long developmental periods and act as continuous biotic reservoirs, maintaining the infection source year-round. This is in stark opposition with the devastating mortalities seen in recently metamorphosed amphibians. Metamorphosis is generally the least resistant and tolerant stage, likely due to the expansion of keratinized epidermis and the re-modeling of the immune system and vital organs (Grogan et al., 2023). However, the detriment of tolerant tadpoles may be outweighed when they act as a source of new recruits, allowing population persistence even in highly susceptible species (compensatory recruitment is further discussed below).

Variations in resistance and tolerance may arise from differences in hosts' immune function or host behavior. However, environmental conditions can confound measures of intrinsic resistance and tolerance and are often considered as an additional underlying mechanism of variation (Brannelly et al., 2021; Grogan et al., 2023). Although many aspects of the immune system of amphibians remain poorly understood, the basic immune components and mechanisms are fundamentally conserved across all vertebrates. Both innate and acquired immune components play a role in defense against chytrid fungi, and variations in these functions can lead to differences in resistance or tolerance among hosts (Grogan et al., 2018).

Regarding constitutive innate defenses, skin sloughing may physically reduce or clear pathogen burdens, and skin secretions such as lysozyme or antimicrobial peptides (AMP) can inhibit the growth of chytrid fungi.

Similarly, the commensal microbial communities that inhabit amphibian skin can secrete antimicrobial compounds that repel, prevent colonization and/or inhibit fungal growth. Both AMP and Bd-inhibitory bacteria seem reliable predictors of species susceptibility and population impacts and are perhaps the most well-studied components of the amphibian's immune system (Grogan et al., 2018). Conversely, much less is known about other innate immune mechanisms such as pathogen recognition, inflammation and complement activation. Across host species, cellular immune responses are inconsistent and generally diminished, often only present in areas of ulceration and associated with secondary bacterial infections. Given this mostly inadequate innate immune response, the subsequent activation of adaptive immune responses seems also unsurprisingly defective. While there is evidence that some adaptive immune mechanisms are activated during chytrid fungi infections, these appear weak and non-protective to subsequent infection and disease in most species (Brannelly et al., 2021; Grogan et al., 2018; Van Rooij et al., 2015).

Overall, there is an apparent lack of immune response of amphibians to chytrid fungi that could be explained by (1) lack of pathogen recognition due to intracellular location, immune evasion or masking antigens; (2) pathogen-induced immunosuppression by downregulation of genes important for protective responses; or (3) a combination of both mechanisms. The major determinants of host resistance appear to be variations in constitutive innate defenses, including AMP and skin microbiome (Grogan et al., 2023). On the other hand, tolerance is associated with mechanisms related to the pathological outcome of infection, such as direct pathogen damage to host tissues and dysregulation of host defense mechanisms. Highly susceptible hosts have been found to mount massively dysregulated and non-protective immune responses that disrupt homeostatic mechanisms. This immunopathology contributes to morbidity and mortality and, together with mechanisms of tissue regeneration, could explain tolerance variation among hosts (Grogan et al., 2023).

Variations in hosts' behavior could also drive differences in resistance and tolerance by decreasing exposure, growth or further transmission of the pathogen (Brannelly et al., 2021). Increased exposure to warm temperatures or behavioral fever induced by infection in some species or individuals could reduce pathogen load and promote clearance. Behaviors that reduce aggregation of hosts or interactions with reservoir species or environments may also decrease exposure and transmission. Moreover, behavior can be dynamic and vary over time in a population or with infection status. However, there is little empirical evidence of behavior actually shaping individual resistance or tolerance and no studies have explored variations in behavior at the population level.

Besides resistance and tolerance, populations experiencing high adult mortality due to chytrid fungi may persist or recover due to increased recruitment. Compensatory recruitment is effective only if the infection has minimal impact on juveniles or larvae and if young adults successfully breed before the prevalence and intensity of infection rise. The exact mechanisms behind compensatory recruitment are not well understood but may involve earlier maturation in response to low adult abundance or to infection itself. Nevertheless, these populations may never recover to pre-epidemic abundances and are left more vulnerable to stochastic events.

Finally, host community composition also alters disease dynamics and the impact on target (declining or endangered) species. The presence and abundance of tolerant reservoir hosts or life stages in a community are key in keeping the force of infection high enough to drive susceptible host species to extinction. Moreover, chytridiomycosis epidemics have caused substantial shifts in amphibian communities, altering the relative abundance of species with different resistance and tolerance and subsequent pathogen transmission (Brannelly et al., 2021). The force of infection is predicted to increase or decrease with increases in the relative abundance of tolerant and resistant hosts, respectively (Fig. 12). Indisputably, this will impact the density and population outcome of the target species in a particular community.

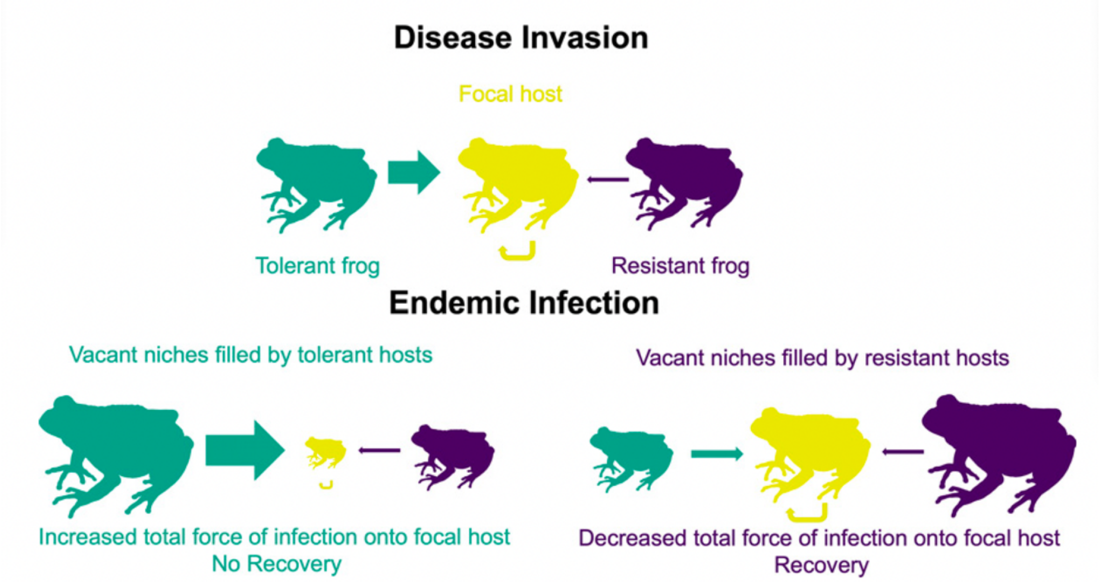


Figure 12. Consequences of changes in the frog community on a target/focal species after disease invasion. The focal species (in yellow) represents susceptible species of particular conservation concern. Resistant species (in purple) maintain low pathogen loads while tolerant species (in green) maintain high pathogen loads without major fitness effects. The size of the frog silhouette indicates relative population sizes. The force of infection that the population of each host exerts on the focal host is represented by the thickness of the arrows. During epidemic disease, the focal host declines leaving an open niche. Once chytridiomycosis becomes endemic, the impact on the persistence and recovery of the focal host depends on whether the niche is filled by resistant or tolerant hosts. If the niche is filled by tolerant hosts, the force of infection on the focal host increases, leading to continued declines. If resistant hosts fill the niche, the force of infection is reduced, allowing the persistence or recovery of the focal host. From Brannelly et al. (2021).

The environment

Environmental factors strongly influence the biology of chytrid fungi and their ectothermic amphibian hosts. Consequently, environmental variations can affect the prevalence or infection loads by regulating pathogen growth and survival or the resistance/tolerance of amphibian hosts (Scheele, Foster, et al., 2019). Areas within a species distribution that are climatically unsuitable or where local conditions are less favorable for chytrid fungi growth, or more favorable for amphibian immunocompetence and reproduction are known as climatic or environmental refuges (Fig. 13). Disease-induced mortality is reduced in these refuges, promoting host population persistence or recovery in a subset of sites while other populations are extirpated. Temperature is arguably the most crucial environmental factor influencing chytrid fungi dynamics (Blaustein et al., 2018), and likely accounts for many variations in infection outcomes. Chytridiomycosis impacts have been more severe in montane amphibian species from tropical and subtropical regions, where temperatures are optimal for Bd. Examples of divergent infection outcomes mediated by temperature can also be seen among populations of the same species. For example, Bd-infected *Alytes muletensis* populations are declining in narrow, cool canyons, while remaining stable in more open, warmer canyons (Doddington et al., 2013). Similarly, despite Bd infection having a wide elevational distribution in *Alytes obstetricans,* disease and mortality are only observed in a restricted elevation, coinciding with optimal temperatures for the pathogen (Walker et al., 2010). Besides temperature, other abiotic environmental factors including humidity, salinity or water chemistry variables can mediate infection dynamics and impacts (Brannelly et al., 2021).

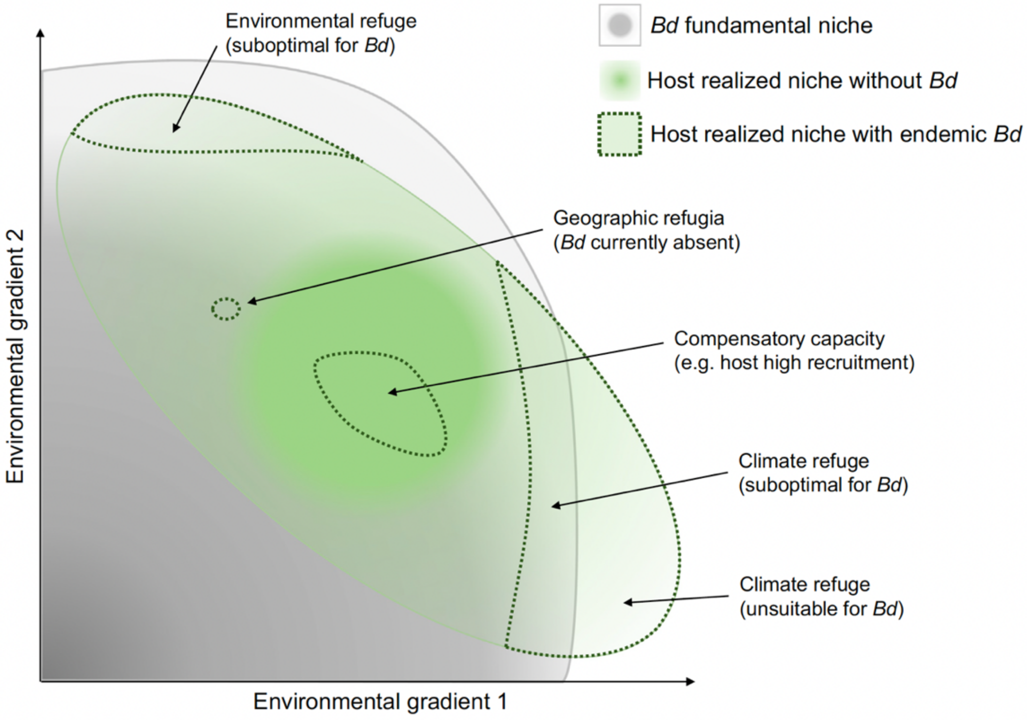


Figure 13. Representation of potential host responses to chytrid fungi infection in a two-dimensional space. The environmental niche of the pathogen (gray) and the amphibian host (green) are depicted, with color fading illustrating reductions in biological fitness in both species. Host responses include contraction to geographical refuges where the pathogen has not been introduced, and contraction to climatic or

environmental refuges with unsuitable/suboptimal conditions for chytrid fungi or where amphibians can reduce or offset mortality. Susceptible amphibian species can show all or none of these responses across their range. Bd = *Batrachochytrium dendrobatidis*. Adapted from Scheele, Foster, et al. (2019).

Environmental refuges may also arise due to variations in biotic processes that influence the presence and abundance of chytrid fungi free-living stages. For example, aquatic microfauna in mountain lakes may predate on free-living zoospores, lowering the environmental abundance of infectious stages (Schmeller et al., 2014). This leads to a decrease in both the prevalence and intensity of infection in susceptible hosts, thereby reducing the overall impact of the disease. The aquatic macroinvertebrate community and algae can also affect chytrid fungi dynamics, either directly by competition or predation, or indirectly by releasing antifungal metabolites (Van Rooij et al., 2015).

Managing chytridiomycosis in nature

Despite global consensus on the risk chytridiomycosis poses for amphibian conservation and over 20 years of intensive research, effective management strategies in free-living populations are scant. Preventing pathogen introduction into new areas by controlling the movement of amphibian reservoirs and other infection sources remains the primary and most effective objective. However, Bd is already widespread and mitigating disease threats or promoting host-pathogen coexistence is the most urgent need (Brannelly et al., 2021; Garner et al., 2016). Many reviews of potential mitigation strategies have been published. However, few studies have tested the efficacy of specific actions empirically and, to date, most management efforts have failed or had little success.

Reintroductions and supplementations of individuals have been frequently attempted since they are highly appealing to the general public and promote the idea that “something is being done” (Garner et al., 2016). The objective of these actions is to add animals into a habitat to re-establish a viable population of the target species. Nevertheless, translocation projects require large and continued funds, careful planning and evaluation and long-term monitoring (IUCN/SSC, 2013). Furthermore, for translocations to be successful, it is essential to address the threatening processes that initially caused the species' decline. Unfortunately, this has been generally ignored and most failures have been attributed to the continued presence of the pathogen in the environment and the re-emergence of chytridiomycosis. Complete elimination of chytrid fungi from the environment is virtually impossible (but see Bosch et al. 2015); thus, translocations are unlikely to be successful when undirected or as a sole strategy.

Several considerations and complementary strategies have been identified for reintroductions and supplementations to be successful. These include the translocation of animals with higher resistance/tolerance to infection or the release into sites less conducive to chytrid fungi. In principle, resistant or tolerant hosts could be sourced from populations naturally coexisting with the pathogen or from selectively bred captive colonies. However, selective breeding implies that the mechanisms behind resistance and tolerance are known and are genotypic traits that can be selected for (Garner et al., 2016). Currently, there are no reports of such artificial selection for resistant/tolerant phenotypes in amphibians. Only a few translocations tested the use of individuals from naturally coexisting populations, with largely unsuccessful results (Brannelly et al., 2016). The host and the habitat could be also modified to reduce chytrid fungi impacts, either as part of translocation programs or as independent strategies.

Host modification attempts have involved vaccination, treatment with antifungals and bioaugmentation of skin microbiome. Vaccines, including dead zoospores, fungal metabolites, infection with hypovirulent strains and infection followed by treatment, have only been tested under laboratory conditions to date with mixed results (Cashins et al., 2013; McMahon et al., 2014; Stice & Briggs, 2010; Waddle et al., 2021). This likely indicates that protective responses cannot be generalized across species, life stages and scenarios. Additionally, questions regarding safety, commercial production and large-scale administration into free-living populations still need to be addressed before vaccination can be considered feasible. Antifungal treatment such as topical itraconazole is highly effective in captive amphibians (Brannelly et al., 2012), but is unsuitable as a long-term strategy *in situ* since they only reduce infection directly after treatment (Geiger et al., 2017; Hudson et al., 2016).

Successful treatment in free-living amphibians has only been reported in an isolated single-host system in which pathogen eradication was achieved in combination with chemical disinfection of the environment (Bosch et al., 2015). Finally, probiotic therapy through bioaugmentation of bacteria with antifungal properties in the skin microbiota of amphibians holds promise (Bletz et al., 2013). Increased survival and decreased infection prevalence and loads have been reported in both laboratory experiments (Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Kueneman et al., 2016) and in a single trial in a natural system (Vredenburg et al., 2011), demonstrating the potential of skin microbiome modification as a mitigation strategy for chytridiomycosis.

Habitat modification is already part of many conservation programs addressing habitat loss and its application for chytridiomycosis management is likely to be readily accepted by conservationists. Interventions would aim to create disease refuges by decreasing the environmental suitability for chytrid fungi, excluding reservoir species, and/or facilitating compensatory mechanisms in target species (e.g. increased recruitment or reduced mortality from other sources) (Garner et al., 2016; Scheele, Foster, et al., 2019). These strategies could involve the manipulation of physical variables, for example, canopy opening may elevate water temperatures or promote desiccation, conditions detrimental for chytrid fungi. Alternatively, chemical variables like salinity could be manipulated, or the aquatic biotic community could be modified by favoring the presence of zoospore predators. Artificial structures could also be added, for example, heat sources could provide refuges for infected individuals to reduce or clear infection (Waddle et al., 2024). Where habitat modification is not feasible, new habitats for target species could be created, even without environmental refugia, to promote habitat connectivity and population persistence (Heard et al., 2018). Chemical disinfection of waterbodies with fungicides has also proven useful in controlling chytrid fungi infections in amphibians, either as a standalone approach (Thumsová, González-Miras, et al., 2024) or in combination with host treatment (see above). While using chemicals in natural environments is generally undesirable, it may serve as a last-resort strategy for threatened species until more environmentally friendly alternatives are developed.

Regardless of the strategy, a comprehensive understanding of host-pathogen dynamics and the ecology of amphibian hosts and chytrid pathogens in each system is crucial for success. Recently, the importance of studying the mechanisms underlying host persistence or recovery after disease emergence has been highlighted (Brannelly et al., 2021). Appropriate management actions differ depending on which mechanism is responsible for coexistence and failure to identify them could lead to ineffective or even detrimental actions. Moreover, it is unlikely that disease mitigation can be accomplished through a single management approach (Garner et al., 2016). By strategically combining multiple methods, it may be possible to offset the limitations of individual strategies and enhance the likelihood of success. Structured decision-making for selecting optimal management options and identifying knowledge gaps, post-management monitoring to assess the effectiveness of actions and adaptive management approaches, should also be considered integral components of any disease management plan.

Hypotheses and Objectives

> Tots els ocells són pardals, tots van per-dalt.
>
> — Josep Puig Subirana (Putxi)

In Iberia, montane amphibians (>1600 masl) suffered the greatest impacts following the introduction and spread of Bd (BdGPL) (Walker et al., 2010). The first European outbreaks of chytridiomycosis occurred in Peñalara (Guadarrama National Park, Spain) and the Pyrenees in 1997 and 2002, respectively (Bosch et al., 2001; Walker et al., 2010). In Peñalara, chytridiomycosis caused the near extirpation of *Alytes obstetricans* populations and secondarily affected other sympatric species such as *Salamandra salamandra* and *Bufo spinosus* (Bosch & Martínez-Solano, 2006). In the western French Pyrenees, recurrent mortalities of the highly-susceptible *A. obstetricans* continue to be observed and Bd is now considered endemic in the area, with prevalence reaching 100% in overwintering tadpoles (Clare, 2014; Clare et al., 2016). In Portugal, studies also indicate that Bd caused severe declines in the populations of *A. obstetricans* in *Serra da Estrela* Natural Park (Rosa et al., 2013). In all these systems, *A. obstetricans* tadpoles are considered the main Bd reservoir and chytridiomycosis dynamics are broadly understood. However, current amphibian monitoring and chytrid fungi studies in the Iberian Peninsula are largely biased towards a single species, A. *obstetricans*, in a handful of sites. This leaves vast areas and the majority of the over 30 native amphibian species unexamined, resulting in a limited understanding of the epidemiology of chytridiomycosis.

Catalonia is an autonomous community of Spain located in the northeast of the Iberian Peninsula, representing a total area of 32,108 km^2 with a remarkable ecological diversity. Catalonia's habitats, climate and species are diverse, from alpine ecosystems reaching 3,143 masl in the Pyrenees to Mediterranean ecosystems across the coastal ranges and lowlands. This diversity is well-represented in the amphibian taxa, as Catalonia supports 15 species adapted to different habitats and altitudes, as well as two endemic species. Despite the relevance of Catalonia, the health status of amphibian populations and the study of chytrid fungi have received little attention. Current chytridiomycosis reports mostly rely on opportunistically collected data and low sample sizes, without systematic approaches to site selection, species representation and sampling methodology (Bargalló et al., 2016; Martínez-Silvestre et al., 2023). Due to the conservation status of *C. arnoldi*, efforts were made to rule out the presence of Bd in its populations between 2007 and 2011 (Obon et al., 2013). However, the study only examined 158 animals, mostly *C. arnoldi,* and no other surveys have been conducted since. Another species that has recently received attention is *C. asper*, with asymptomatic Bd-positive individuals detected in the Catalan Pyrenees (Martínez-Silvestre et al., 2020). Although the survey included 486 animals from 29 sites across the Pyrenees, the Catalan part of the mountain range was only partially represented and no other species of the amphibian community were tested. Perhaps the most surprising omission is the lack of studies on *A. obstetricans* in the Catalan Pyrenees, considering the devastating mortalities and the potential reservoir role of tadpoles in other montane systems.

Moreover, in 2018, an outbreak of Bsal chytridiomycosis was reported in *Montnegre i el Corredor* Natural Park in Catalonia, 1,000 km from its nearest known occurrence in northern Europe (Martel et al., 2020). Nevertheless, the presence of Bsal in adjacent areas and across Catalonia was not explored after the outbreak. As Bsal is clearly expanding its range, it is imperative to know whether Bd-Bsal coinfections are occurring and how this may alter our current knowledge about chytridiomycosis. Overall, long-term and region-wide studies across multi-host communities are lacking, hampering our understanding of the epidemiology of chytridiomycosis in Catalonia. Therefore, there is an urgent need to determine chytrid fungi distribution, potential impacts, and the role of environmental factors and different host species in the infection dynamics.

We hypothesize that Bd is already widespread across Catalonia, infecting amphibian communities regardless of ecosystem or habitat type, while Bsal remains absent or extremely limited in distribution. We also expect that different species will exhibit a range of responses to Bd infection, spanning a spectrum of resistance and tolerance. Species with higher resistance or tolerance are more likely to act as reservoirs, maintaining subclinical infections and facilitating pathogen persistence, while more susceptible species may experience severe disease outcomes and population declines.

The overarching goal of this thesis was to understand the epidemiology of chytridiomycosis in Catalonia by optimizing amphibian monitoring approaches and studying host-pathogen-environment interactions.

The specific objectives were to:

1. Evaluate the use of passive integrated transponder tags for marking urodele species in capture-mark-recapture studies (**Chapter 1**).

2. Determine the distribution and prevalence of chytrid fungi in amphibian communities across Catalonia (**Chapter 2**).

3. Assess the role of environmental determinants in shaping the occurrence of *Batrachochytrium dendrobatidis* across Catalonia (**Chapter 2**).

4. Investigate the resistance and tolerance of different amphibian hosts and their potential roles in *Batrachochytrium dendrobatidis* maintenance in Catalonia (**Chapter 2, 3.2, 4**).

5. Evaluate microbiome as a health indicator and study the interplay between *Batrachochytrium dendrobatidis* infection and amphibians' skin microbiome (**Chapter 3**).

6. Analyze the interaction between *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in coinfected amphibians during an outbreak of chytridiomycosis (**Chapter 4**).

Chapter 1

Evaluation of passive integrated transponder tags for marking urodeles

Maria Puig Ribas
Paula Alonso-Almorox
Johan Espunyes
Albert Martínez-Silvestre
Oscar Cabezón

Ecological Indicators 145 (2022) 109690

The use of passive integrated transponder (PIT) tags in urodeles has become popular for individual marking in population and disease ecology studies. However, mark loss or mark-induced mortality can introduce biases and decrease precision in parameter estimates, leading to ineffective population management strategies. In this study we aimed to 1) analyze the existing literature on the use of PIT tags in urodeles; 2) determine whether species characteristics and PIT tagging methods influenced PIT tag rejection across studies; and 3) experimentally assess the adequacy of a subcutaneous PIT tagging method without anesthesia in three European urodele species. We systematically and quantitatively reviewed a database of literature related to the use of PIT tags in urodeles, classified and examined urodele species details, study design, PIT tagging methods, and outcomes across studies. Among the 51 peer-reviewed papers that fit our criteria, the most striking finding was the lack of reporting and standardization of the PIT tagging procedures. The majority of studies presented incomplete information on factors that could strongly influence the probability of PIT tag rejection as well as impact individual welfare (i.e. PIT tag size, its anatomical placement in the animal, anesthesia use, sterility or skin closure methods). We could not identify significant predictors of PIT tag loss, suggesting that the effectivity of PIT tags may be highly specific to the species and method used. Our PIT tagging method proved reliable in *Salamandra salamandra* and *Pleurodeles waltl*, whereas it did not seem a suitable technique for *Calotriton asper* (PIT tag loss was 0% and 66.6%, respectively, and significantly different among species). Overall, we recommend a greater emphasis on reporting implantation methods, ensuring animal welfare and performing species and protocol specific laboratory trials before using PIT tags in urodeles in the field. Critically analyzing PIT tagging methods as well as testing their use in different species is essential to ensure the validity of future research studies and conservation strategies in urodeles.

Introduction

Amphibians are currently the most threatened vertebrate taxa on Earth, with over 41% of species threatened with extinction and declining more rapidly than either birds or mammals (IUCN, 2021; Stuart et al., 2004). Moreover, the conservation status of amphibians is certainly underestimated since 16.4% of species are too poorly known to assess (classified as “Data Deficient” by the IUCN). Within amphibian orders, urodeles may be facing greater conservation challenges, reaching 400 out of 701 (57%) species under threatened categories. The global decline of amphibian populations can be attributed to diverse interacting processes including habitat loss and degradation, overexploitation, invasive species and infectious diseases such as chytridiomycosis (Beebee & Griffiths, 2005; Scheele, Pasmans, et al., 2019; Stuart et al., 2004). In order to effectively manage and conserve amphibian diversity, our ability to monitor population dynamics and the impact of specific threats is crucial (Grant et al., 2016; Pickett et al., 2014).

Capture-mark-recapture (CMR) studies are key tools for population ecology, providing estimates of survival, recruitment, abundance and movement (Amstrup et al., 2010; Nichols, 1992; Pradel, 1996). Relevant aspects of disease ecology and the impact of disease on free-living populations can also be investigated through CMR data (K. A. Murray et al., 2009; Wobeser, 2007). All CMR models operate under several assumptions, a critical one being that marks are not lost and do not affect individual survival. Mark loss or mark-induced mortality can introduce biases and decrease precision in parameter estimates, leading to ineffective population management strategies (Arnason & Mills, 1981; T. L. McDonald et al., 2003). The choice of a marking technique that has proven reliable for the species under study is clearly important to avoid this issue.

Numerous marking techniques have been used in amphibians including toe clipping, branding and tattooing, subcutaneous injection of fluorescent dyes, Passive Integrated Transponder (PIT) tags and pattern mapping (Ferner, 2010). All of the above-mentioned methods have been used in urodeles, however, each one has apparent advantages and pitfalls. Toe clipping has been a common method for marking urodeles due to its simplicity and low cost. However, mutilation techniques provoke pain and distress and may impact the survival and behavior of marked individuals, raising animal welfare and ethical concerns (McCarthy et al., 2009; J. H. Waddle et al., 2008). The potential of toe regeneration, common in urodeles, is another disadvantage of toe clipping and this technique may be inappropriate for long-term studies. Mark durability and readability can also be an issue with fluorescent dye injection and pattern identification (Bailey, 2004; Dalibard et al., 2021; Heemeyer et al., 2007; Moon et al., 2022). Pattern mapping holds promise as a non-invasive and cheap technique, but it can only be used for species with natural patterns or marks and it can be time-consuming in large populations and long-term studies (Arntzen et al., 2004). Nevertheless, constant computational advances are steadily overcoming these issues, simplifying the use of pattern mapping for wildlife studies (Sannolo et al., 2016). At the same time, the use of PIT tags for marking urodeles has become more popular over recent decades, especially as technological advances allow for smaller tags (Cooke et al., 2013b; Moon et al., 2022).

Passive Integrated Transponder (PIT) tags are glass-encapsulated circuit chips that, once scanned, provide a unique alpha-numeric code which offer permanent and unambiguous identification (Gibbons & Andrews, 2004). These characteristics satisfy many criteria for an ideal marking technique, such as providing individual identification, potentially lasting indefinitely and being easily read (Ferner, 2010). Tags as small as 7 mm long are currently available, which allows for adaptability to different animal sizes, yet, tag size is still a limitation for its use in smaller urodele species. Tagging can be performed via injection with a pre-loaded needle or by surgical implantation either under the skin, into the muscle or the celomic cavity. Despite being a relatively simple procedure, tagging can involve tissue and organ damage, potentially affecting behavior and even survival of marked individuals. Anesthesia may be required (e.g. depending on the site of tag implantation) and personnel expertise as well as validation of tagging protocols are highly recommended to ensure animal welfare and tag retention (Cooke et al., 2013). As a consequence, PIT tags still present challenges for their applicability in urodeles and in a wide range of situations.

The effectiveness of different PIT tagging techniques has been reviewed for other aquatic taxa such as fishes (Cooke et al., 2011; Ebner, 2009;

Musselman et al., 2017) and reptiles (Doody et al., 2009), and there is a large body of literature, dating back to the 1980s, describing its use and implantation in anuran amphibians (L. J. Brown, 1997; Camper & Dixon, 1988; Christy, 1996; Pyke, 2005). Conversely, studies assessing the use of PIT tags in urodeles are limited, detailed protocols are rare and data appear scattered. Summarizing PIT tagging techniques and outcomes as well as testing their use in different species is, therefore, essential to ensure the validity of future research studies and conservation strategies in urodeles. In this study, we aimed to 1) systematically review the existing literature on the use of PIT tags in urodeles in order to evaluate gaps of knowledge and inform future research; 2) determine whether a relationship between species characteristics and PIT tagging methods on tag rejection was apparent across studies; 3) experimentally assess the adequacy of a subcutaneous PIT tagging method without anesthesia in three European urodele species.

Methods

Literature review

We conducted a systematic quantitative literature review, in order to provide a comprehensive and reproducible overview of the current knowledge on the use of PIT tags in urodeles (Pickering & Byrne, 2014). We used *Scopus* (Elsevier) and *Web of Science* (WoS; Thomson Reuters) databases using ‘title, abstract, keyword’ and ‘all fields’ searches respectively. We used the keywords “(urodel* OR caudat* OR salamand* OR newt) AND (PIT-tag* OR transponder* OR microchip*)” (29 articles from *Scopus*, 35 articles from *WoS*). We also conducted a search on Google Scholar and the reference list of extracted articles was screened to retrieve additional relevant literature.

We then assessed the title and abstract of each entry individually for inclusion in our database. Criteria for inclusion were peer-reviewed primary literature and English-language full text; whereas exclusion criteria were study/paper duplicates, non-peer reviewed/grey literature (i.e., books, workshops, theses, governmental reports), studies reporting the use of implantable marks/tracking devices other than PIT tags (e.g. radiotransmitters) and studies with a single time point, where study design precluded information about PIT tag effectivity. The full-text of the remaining articles was assessed in order to further filter the results. For each paper that met our inclusion/exclusion criteria we thoroughly reviewed their content and extracted the following data to construct a database: literature details, urodele species details, study details, PIT tagging methods and outcomes.

Under ‘urodele species details’ we recorded taxonomic family, species identity and IUCN status (IUCN, 2021) and population origin. We also added the preferred habitat and mean total length of the species in our database (Oliveira et al., 2017). For ‘study details’ we extracted study focus, sample size, location (i.e., laboratory, mesocosm or field study) and duration of the study. In study focus, we classified studies according to their main objectives in 1) method validation, when aiming to estimate PIT tag effectivity, health impacts or compare it with other marking methods; 2) population level, when PIT tags were used to estimate population parameters; 3) animal movement, when PIT tags were used to evaluate activity patterns, habitat preferences or dispersal; and 4) others, when PIT tags were used as a reference mark for other studies. ‘PIT tagging methods’ included PIT tag size, tag location, use of anesthesia, skin closure and sterility assurance. Under ‘outcomes’ we recorded or calculated information on survival (proportion of animals alive at the end of the experiment), tag rejection (proportion of animals that expelled PIT tags) and recapture (proportion of animals recaptured at least one time at the end of the experiment).

Data was extracted from all publications included in the systematic database independently by two investigators and then compared for consistency. For our analysis we split studies into experiments if they used different species, methodologies, times or study locations. If retention, survival or recapture were reported at multiple times through an experiment, we used the information from the last time step. Finally, we summarized and analyzed the resulting database to detect patterns.

Data analysis

We used the extracted data to test whether a relationship between PIT tag rejection and species characteristics and tagging methods was apparent. For the meta-analysis, we used the studies that reported PIT tag rejection data and that had complete records on all other variables of interest. The potential predictors of PIT tag rejection were selected based on similar studies in other taxa (Musselman et al., 2017) and on information availability. We constructed a generalized linear model with PIT tag rejection as response variable and taxonomic family, habitat preferences, tagging anatomical location and the ratio of tag length to animal length (tag:urodele length) as predictors. Animal length rather than mass was used since length measurements are more commonly reported in PIT tagging studies.

Experimental PIT tagging

We conducted laboratory experiments on three species of European urodeles to evaluate the validity of a subcutaneous PIT tagging method without anesthesia. The species included in the study were *Salamandra salamandra* (n=18), *Calotriton asper* (n=9) and *Pleurodeles waltl* (n=12). The individuals used in this experiment were confiscated from illegal collectors and were admitted at the Catalonian Reptiles and Amphibians Rescue Center (Barcelona, Spain). They were housed in groups of 5-10 individuals of the same species in 600 x 400 x 300 mm aquaria (room temperature 20ºC, water temperature 14-18ºC, water pH 7, vegetable soil for fire salamanders and water and pebbles for the newts). Water was regularly changed and aerated by aeration stones, and the daylight/dark pattern was set at 14/10h according to the local natural photoperiod. All the individuals included in the study were apparently healthy, chytrid fungi and ranavirus-negative adults that had been acclimated to captive conditions for a minimum of one month.

In 2020, we implanted PIT tags (ID-100A, 8 x 1.4 mm, Trovan Ltd.) subcutaneously in the dorsum of each individual following a previously described protocol for anurans (Christy, 1996; Newell et al. 2013). This method does not require anesthesia and has shown no effects on behavior, appetite, mobility or cause of morbidity/mortality of marked anurans

(Christy, 1996). Transponders were supplied in individually-packed sterilized needles and were applied using a spring-loaded plastic syringe. The needle was inserted subcutaneously in the dorsum of the animal, parallel to the spine, at the level of the caudal thorax and facing cranially. At a distance of 1-2 cm from the entrance wound, the tag was injected by applying pressure to the syringe plunger. This tag placement ensured that internal organs were not damaged since they were protected by the rib cage. The site of injection was sealed with Vetbond® tissue adhesive and the animals were then returned to their aquaria. The animals were followed up at 2, 7, 14, 21 and 28 days after PIT tag placement. At each follow-up, we recorded survival and we inspected each individual for tag loss and stage of wound healing. Wounds were considered in an inflammatory stage when marked inflammation was observed; in a proliferative stage when granulation tissue was present and wound edges began to contract; and in a maturation stage when scar tissue formed (Poll, 2009; Young & McNaught, 2011).

Tagging was performed by a trained veterinary surgeon (M.P.R.) using a pair of new and previously moistened nitrile gloves. Survival (percentage alive at the end of the trial), tag retention (percentage retaining tags at the end of the trial), and time to healing were calculated at the conclusion of the experiment. Kaplan-Meier survival curves were constructed for each species using PIT tag rejection as the response variable, and differences among species were tested using a log-rank test. All statistical analyses were performed with R 4.0.3 (R Core Team, 2023) and using “ggplot2” version 3.3.6 (Wickham, 2016) and “survival” version 3.3-1 (Therneau, 2022) packages.

Results

Literature review

The literature search returned 29 articles identified by *Scopus*, 35 articles identified by *WoS* and 37 articles identified by Google Scholar and paper citations, resulting in 76 research papers after removing duplicates. Seven articles were excluded based on title and abstract and 18 additional articles were excluded after full-text screening. Overall, we obtained 51 original peer-reviewed research papers that met our criteria of PIT tag studies on urodeles (Full list of studies and extracted data can be found online at https://doi.org/10.1016/j.ecolind.2022.109690. Publication dates ranged from 1993 to 2022, with an increase in the number of published papers since 2006 and a peak in publications in 2017 (n=7). Papers were published in 29 different journals and the three journals publishing the highest number of papers were *Herpetological Review* (n=8), *Herpetological Journal* (n=5) and *Journal of Herpetology* (n=5). The majority of studies were conducted in United States (58.86%; 29/51). Six studies were carried out in France; three each in Germany and Austria; one each in Belgium, Canada, Czech Republic, Italy, Japan, Laos, Mongolia, Spain and UK; and one study was carried out in both France and Spain. Regarding the focus of the studies, 19 were centered on method validation, 15 on population level estimations, nine on animal movement, and eight studies used PIT tags as a reference mark for other studies (Fig. 1).

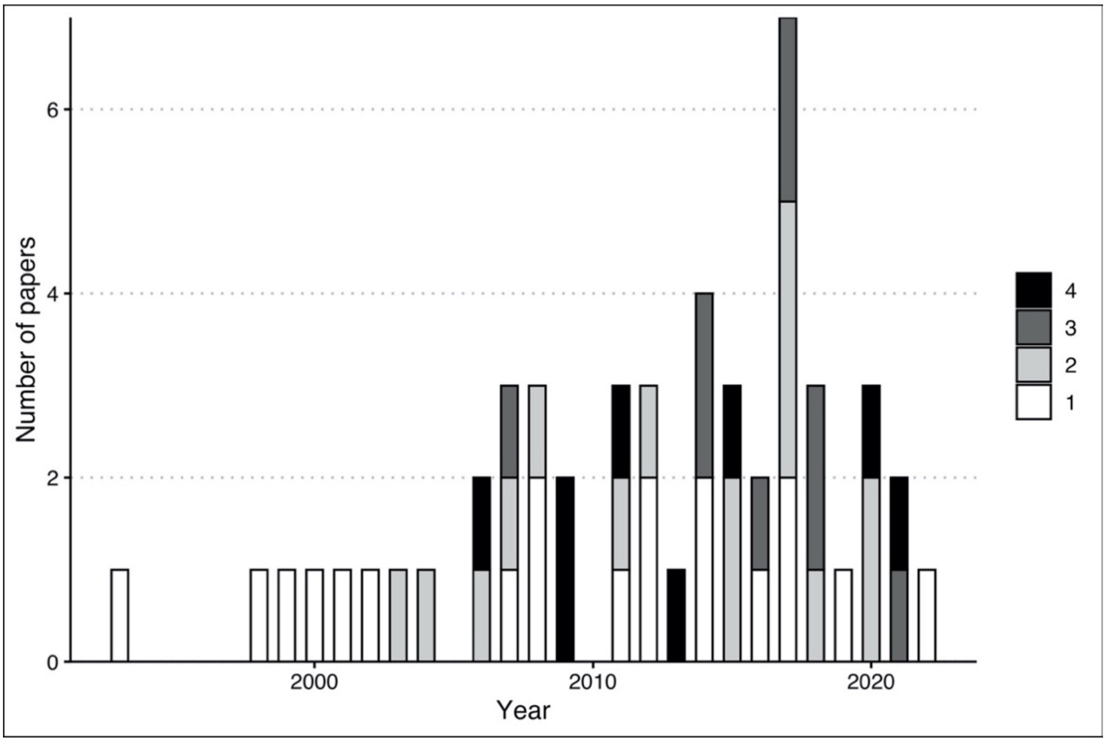


Figure 1. Bar graph showing the number of papers published in each of the study foci by publication year. 1: method validation; 2: population level; 3: animal movement; 4: other study focus.

Within the 51 retrieved studies we could identify 65 individual experiments. These experiments were conducted on 29 species of urodeles, with 12 species used in multiple experiments (Table 1). These species corresponded to seven families: Salamandridae (n = 10), Ambystomatidae (n = 7), Plethodontidae (n = 6), Sirenidae (n = 2), Hynobiidae (n=2), Cryptobranchidae (n = 1), Proteidae (n = 1). Most papers focused on a single urodele species (82.35%; 42/51) and eight studies used two species. A single study contained information on three species (*Ambystoma annulatum, A. maculatum* and *A. texanum*), however, the authors reported study outcomes together for the three species and we considered them as a single experiment (i.e., *Ambystoma spp*. on Table 1). The adult mean length of the studied species ranged from 87.5 mm of *Ichthyosaura alpestris* to 978 mm of *Siren lacertina* (median = 164.25).

Table 1. Taxonomic families and species of urodeles on which the 65 reviewed experiments were conducted.

Family **Species**	**Nº experiments**
Ambystomatidae	**23**
Ambystoma annulatum	5
Ambystoma laterale	4
Ambystoma macrodactylum	1
Ambystoma maculatum	7
Ambystoma opacum	3
Ambystoma tigrinum	2
Ambystoma spp	1
Cryptobranchidae	**5**
Cryptobranchus alleganiensis	5
Hynobiidae	**2**
Hynobius nebulosus	1
Salamandrella keyserlingii	1
Plethodontidae	**8**
Desmognathus monticola	2
Desmognathus quadramaculatus	1
Eurycea rathbuni	1
Plethodon albagula	1
Plethodon metcalfi	1
Plethodon shermani	2
Proteidae	**1**
Necturus maculosus	1
Salamandridae	**24**

Calotriton asper	3
Ichthyosaura alpestris	8
Pleurodeles waltl	1
Salamandra salamandra	4
Taricha torosa	1
Triturus carnifex	1
Triturus cristatus	3
Triturus dobrogicus	1
Triturus marmoratus	1
Tylototriton podichthys	1
Sirenidae	**2**
Siren intermedia	1
Siren lacertina	1
Total	**65**

Experiments were disproportionally conducted on species classified as 'least concern' (52/65), followed by 'near threatened' (10/65), and only three of the 65 experiments were performed on 'vulnerable' species (IUCN, 2021, Fig 2a). No experiments included species under other threatened categories. Species with terrestrial habits were more commonly used in experiments (42/65) than either species with aquatic habits (14/65) or shared terrestrial-aquatic preferences (9/65) (Fig. 2b). Experiments were also more frequently conducted on adult life-stages (55/65), with only eight experiments conducted on juveniles and one each on metamorphs and larvae (Fig. 2c). Experiments also varied in the location where they were conducted, the majority being performed in the field (40/65), but laboratory settings (19/65) and mesocosms (6/65) were also used (Fig. 2d). There were three articles that included both laboratory and field setups and one that combined laboratory and mesocosm. The sample size (number of PIT tagged urodeles) per experiment ranged from 6 to 3,745 individuals (median = 79) and it was higher in field studies (median = 147) than in laboratory and mesocosm settings (median = 18). Experiment duration was highly variable, ranging from 7 days to 20 years (median = 377.5 days) and it was also greater for field (median = 730 days) than for laboratory and mesocosm settings (median = 106.5 days). Sample size and experiment duration were not specified in three experiments each.

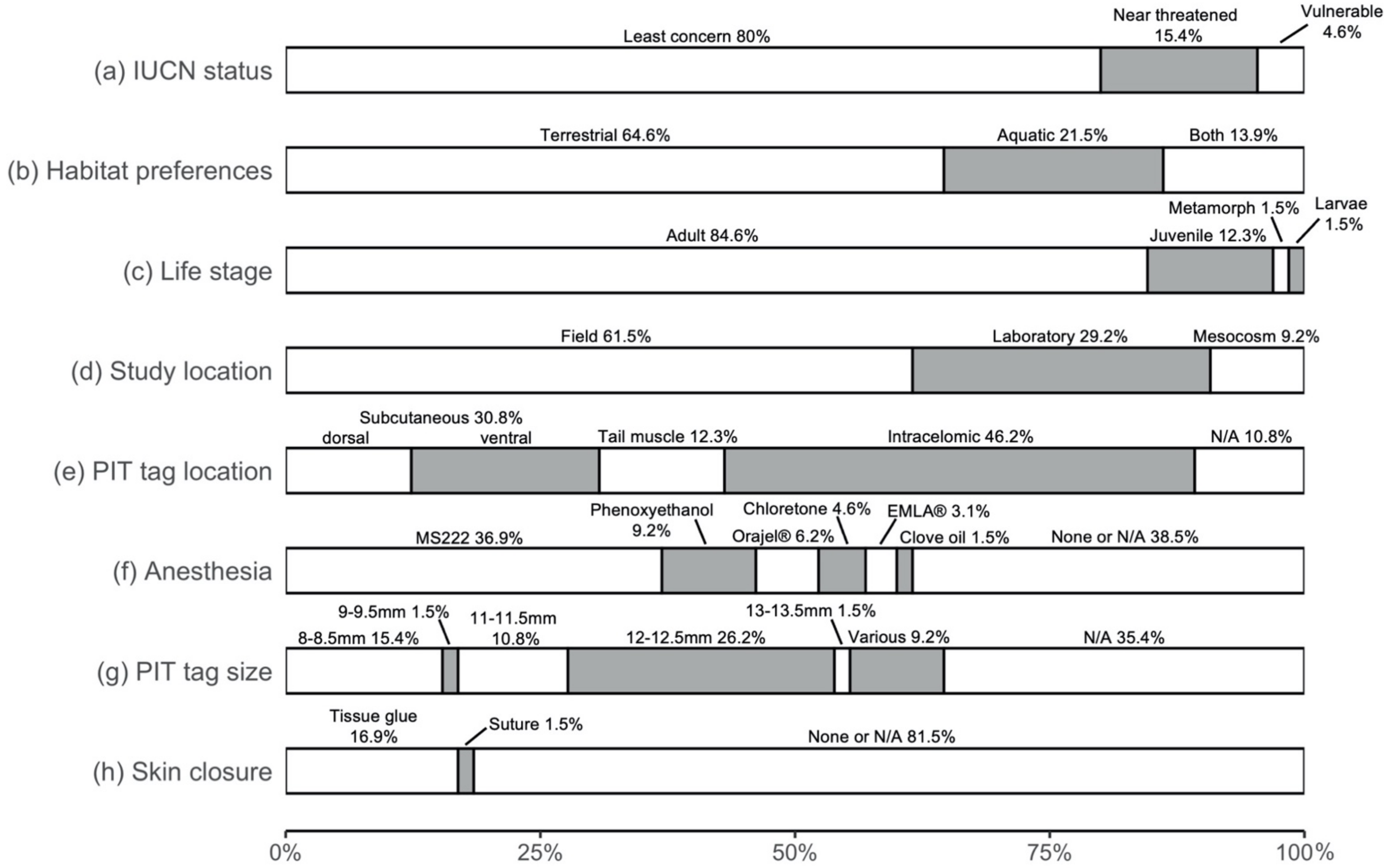


Figure 2. Bar graph demonstrating the proportion of (a) species grouped according to IUCN status, (b) species grouped according to habitat preferences, (c) urodele life stages, (d) study location, (e) anatomical location of PIT tag, (f) anesthetic methods, (g) PIT tag size, and (h) methods of skin closure, used on the 65 experiments reviewed. N/A: not available.

Regarding PIT tagging methods, the most common implanting location was the celomic cavity (30/65), followed by subcutaneous (20/65) and into the tail musculature (8/65) (Fig. 2e). Subcutaneous PIT tags were either inserted dorsally (8 experiments) or ventrally (12 experiments). The location of tag implantation was not mentioned in seven experiments. Additionally, we were uncertain about the true location of tags in two studies classified as subcutaneous, but where the authors later mentioned ambiguous sentences, such as referring to tags into the "body cavity". PIT tags were most frequently implanted under anesthesia (40/65) whereas anesthesia was either not used or not mentioned for the rest of experiments (25/65) (Fig. 2f). Anesthesia methods included the topical use of the following agents: tricaine methanesulfonate (MS222; 24 experiments), 2-phenoxyethanol (6 experiments), Orajel® (20% benzocaine; 4 experiments), chloretone in water (3 experiments), EMLA® cream (2.5% lidocaine and 2.5% prilocaine; 2 experiments) and clove oil (1 experiment). PIT tags were implanted in all body locations irrespective of the use of anesthesia, but anesthesia was predominantly used in intracelomic and ventral subcutaneous implants (Fig. 3).

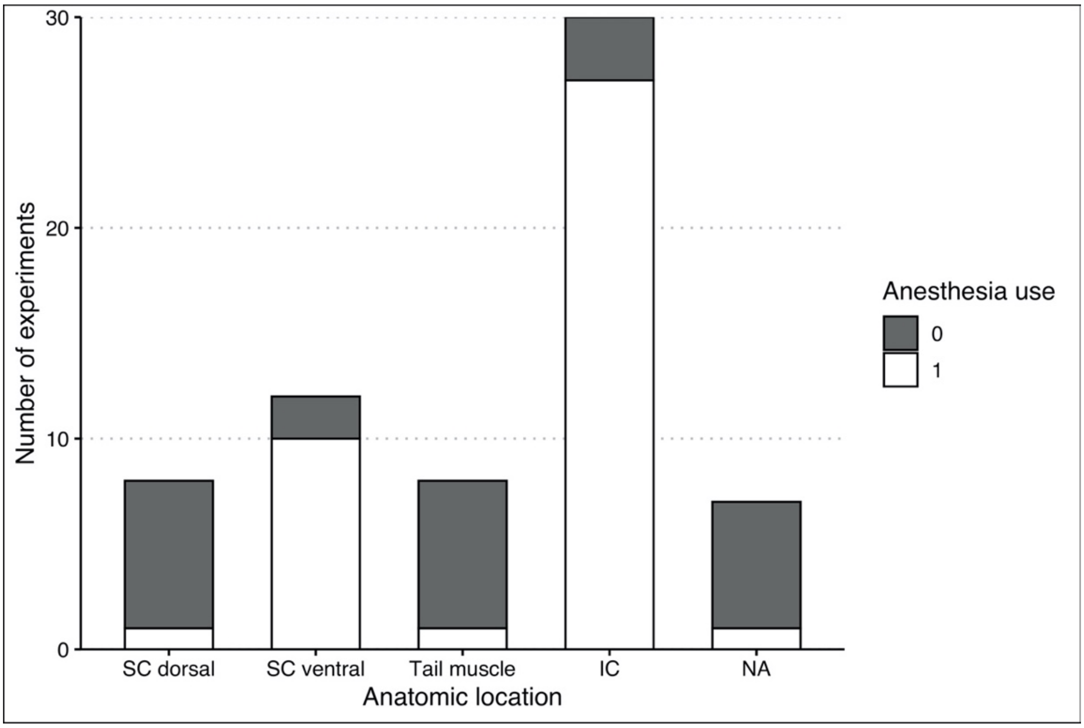


Figure 3. Bar graph showing the number of experiments that used anesthesia in each anatomical location where passive integrated transponders were implanted in urodeles. SC: subcutaneous; IC: intracelomic; NA: not available; 0: anesthesia was not used or not mentioned; 1: anesthesia was used during the tagging procedure.

Experiments used diverse PIT tag sizes, with 12-12.5 mm tags being more frequently used (17/65), followed by 8-8.5 mm (10/65), 11-11.5 mm (7/65), 9-9.5 mm and 13 mm tags (1/65 each) (Fig. 2g). Numerous experiments did not mention tag sizes (23/65), accounting for 35.38% of all experiments. Six experiments used various tag sizes depending on the length of the individual, however, the number of animals marked with each tag size was not specified and results were combined for all tag sizes. The ratio of tag length to animal length (tag:urodele length) could be calculated in 33 experiments, ranging from 0.018 to 0.137. Methods of skin closure after PIT tagging were not commonly used or reported (53/65). Eleven experiments mentioned the use of tissue adhesive and a single experiment used traditional sutures for wound closure, but they were removed three days after tagging (Fig. 2h). We could only identify five experiments where sterility was ensured throughout the PIT tagging procedure, in which tags were injected with sterile needles in four cases while sterile tags were surgically inserted with a sterile technique in one experiment. The remaining experiments were divided between injected tags where sterility was not mentioned (24/65), and surgically inserted tags where sterility of all materials (e.g., PIT tags, scalpel blades, etc.) was not ensured or where these had been rinsed with disinfectants (21/65). We were not able to determine sterility conditions for 15 experiments. Injection with a needle was the only method used to implant PIT tags into the tail musculature, whereas both injection and surgical insertion were used for all other anatomical locations.

Information on tag rejection was only available for 29 experiments and ranged from 0 to 45.45% (mean = 5.3%, median = 0%). No tag rejection was detected in 29.23% of experiments (19/65). Survival of urodeles in laboratory and mesocosm setups ranged from 44.4 to 100% (mean = 95.58%), with most experiments reporting 100% survival (15/25) and four mesocosm experiments lacking information about survival. In field studies, survival could only be calculated in one experiment where all individuals were recaptured alive after PIT tagging. Nevertheless, recapture rates were available for 29 field experiments, ranging from 3 to 100% (mean = 36.48%, median = 30%). Additionally, two out of the four mesocosm studies that did not report survival described recapture rates of 49% and 80%, which could be interpreted as a proxy for survival.

Data analysis

The final model for the predictors of PIT tag rejection was based on 21 experiments from 16 urodele species within 15 unique publications. None of the hypothesized factors were significant predictors of PIT tag rejection ($p > 0.05$).

Experimental PIT tagging

Survival was 100% for all species throughout the experiment. PIT tag retention was 100% in *S. salamandra* and *P. waltl*. Wounds in *S. salamandra* progressed to a proliferative stage 7 days post-tagging, were remodeling 14 days post-tagging and were considered mature 21 days after tagging. In *P. waltl*, wounds also reached a proliferative stage 7 days post-tagging but this stage extended until the next control, 14 days after tagging. At this time point, two individuals showed wounds coated with a whitish filamentous material, compatible with fungal hyphae. In the following control (21 days post-tagging), four animals were considered to have mature wounds whereas two individuals continued on a proliferative stage and showed crusty lesions. The two newts that showed fungal-like growth on the past control, presented large wounds in an inflammatory stage, suggesting a step back in the healing process probably due to wound infection. On the last control, 28 days post-tagging, these two individuals showed an advanced proliferative stage while the rest of newts had mature wounds.

In *C. asper*, overall PIT tag retention was 33.3%. Three animals lost tags after 7 days and there was one additional tag loss each at 14, 21 and 28 days. PIT tag retention was significantly different between *C. asper* and the other species (Fig. 4). At day 7 post-tagging, all newts were considered to be in an inflammatory stage of wound healing and presented larger wounds than the other species. After 14 days, wounds appeared to be in a proliferative stage in four of the remaining tagged newts while one showed marked inflammation and a cystic lesion filled with whitish material, suggestive of infection and/or necrosis. Interestingly, wound maturation had already started in the newts that had lost their tags. In the following control (21 days post-tagging), the individual that presented wound infection had lost its tag and another animal still presented a proliferative wound. The rest of the animals presented mature wounds. On the final control (28 days post-tagging), the animal with proliferative wounds had lost its tag while the remaining tagged Pyrenean brook newts had mature wounds. Tissue adhesive remained attached in most individuals from the three species for up to 14 days.

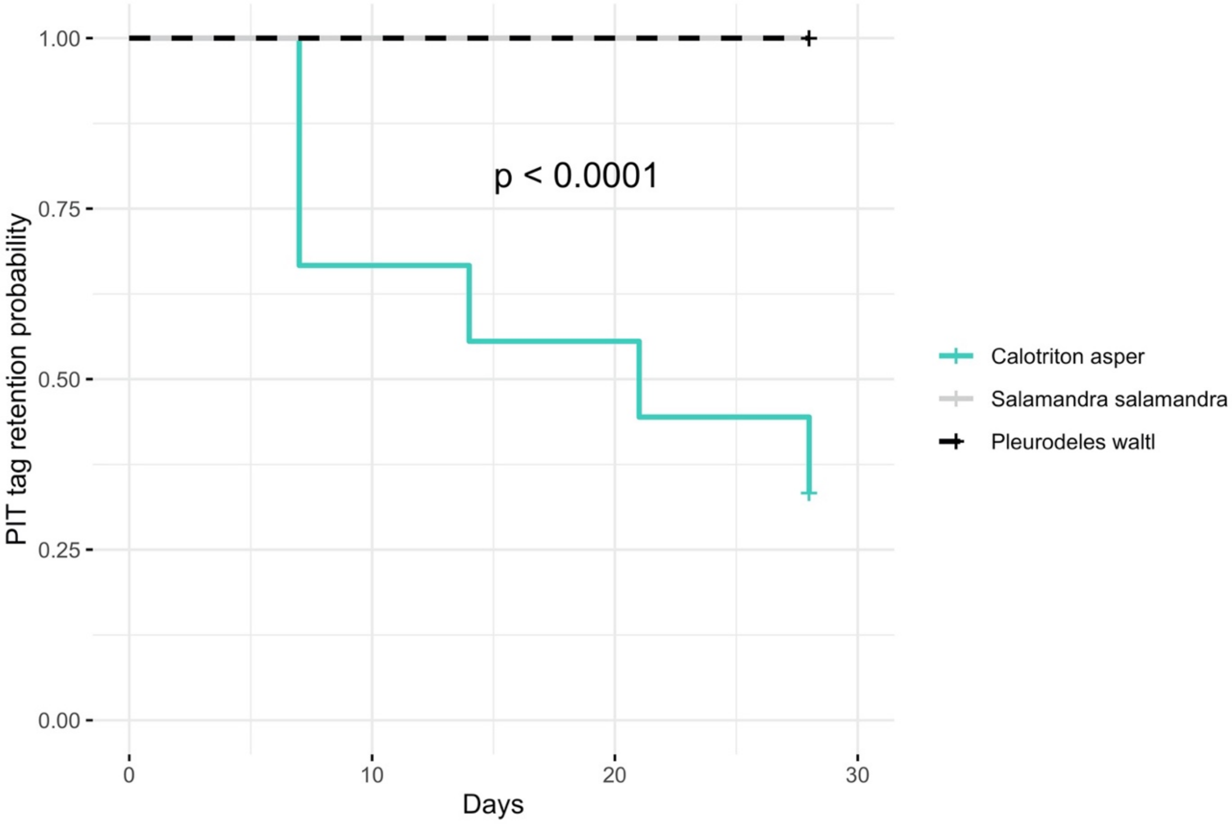


Figure 4. Kaplan–Meier analysis and survival plot showing PIT tag retention over 28 days in *Calotriton asper* (n=9), *Salamandra salamandra* (n=18) and *Pleurodeles waltl* n=8). Tag retention rates were significantly lower in *C. asper* compared with the other species (X^2=23.9; df=2, p=6e^{-06}).

Discussion

The findings of our study evaluated the extent of use and effectivity of PIT tags for marking urodeles, including study characteristics, urodele species, PIT tagging methods and overall findings. Moreover, our work allowed testing potential predictors of PIT tag rejection and offered a PIT tagging method that proved reliable and ready-to-use for *S. Salamandra* and *P. waltl*. Here, we critically analyze our findings in order to assist population monitoring programs and to improve individual welfare.

Limited validation studies and potential biases in population studies

The results of our systematic review provide a comprehensive picture of the use of PIT tags in urodeles over a 30-year period. In the past decades, the recognition of global amphibian declines and the emergence of devastating amphibian diseases have boosted the study of this group of vertebrates (Scheele, Pasmans, et al., 2019; Stuart et al., 2004). In parallel, PIT tag technology has increased steadily in amphibian research due to its advantages over traditional marking methods in population monitoring (Gibbons & Andrews, 2004). The general growth of this field of research is reflected in the increase in scientific production identified by our literature review over the past two decades. Despite this development in research, we found limited studies focusing on the validation of PIT tagging methods for particular urodele species.

Similar to other marks, PIT tags are not exempt from disadvantages such as tag loss or impacts on individual survival. Without proper validation of specific protocols for PIT tag implantation, it is, therefore, possible to obtain inaccurate estimates of population parameters that are relevant for amphibian conservation. Through our systematic search, we could only identify 19 studies where the main focus was to describe and evaluate the performance of PIT tags in urodeles. These results are concerning since they indicate that many population studies are performed without prior knowledge of the limitations of this marking technique. Moreover, while controlled laboratory studies offer the best evidence of PIT tagging performance, they tended to be constrained in sample size and study duration. In our laboratory trial as well as in other works (Moon et al., 2022), PIT tag losses have been documented up to a month after implantation, suggesting that shorter experiment durations may underestimate tag losses. A cautious approach would be to set the end of the experiment when skin wounds are completely healed, minimizing the possibility of tag loss. We found that mean PIT tag rejection across studies was relatively low (5.3%), but some experiments reported alarming rejection rates, reaching 45.5%. For field experiments, these numbers may be significantly higher since they can only estimate minimum PIT tag losses, when free PIT tags are found in the field – a challenging task.

We acknowledge that significant publications on PIT tag use in urodeles may have been missed by our search due to the inherent limitations of systematic reviews. Grey literature and valid literature produced in languages other than English were excluded by the platforms that we used to search the literature. The specific search terms and search fields used to find publications restricted the retrieval of literature that did not include our terms. Nevertheless, studies focusing on method validation were the most commonly captured by our search and we are thus confident that our results offer a valid representation of the use of PIT tags in urodeles. Overall, our data indicates that current information on urodele population dynamics based on CMR studies using PIT tags could be significantly biased, potentially overestimating population sizes while underestimating the threat status of some species.

The use of PIT tags was restricted to a small proportion of urodele diversity

There are currently 701 species of extant urodeles described, representing 70 different genera and 9 families (IUCN, 2021). Our review found that PIT tags have been used in almost all families (7/9 families). However, only 17 genera and 29 species were included in the reviewed studies, representing solely 24.3% and 4.1% of urodele diversity, respectively. When comparing the range of total length between all extant urodeles (20-1,360 mm) and the species represented by our review (87.5-978 mm), it is also clear that PIT tags have not been used in the entire range of urodele sizes. Moreover, the majority of experiments were performed on adult animals, whereas juvenile and pre-metamorphic stages were hardly studied. These life stages can be significantly smaller in body size than adults and, therefore, PIT tags may not be suitable marking techniques for them.

Urodeles are distributed throughout most of the Holarctic, with a hotspot of diversity in the United States and few species occurring below the equator in South America. This was consistent with our findings that most studies were conducted in the United States. Nevertheless, it was surprising that many European countries were overlooked in our review as well as the East Asia region, North Africa and South America. These results show an important gap of knowledge on the use of PIT tags in urodele species native to these areas. Of particular concern, only three experiments were conducted on threatened urodele species. Considering that there are 400 threatened urodele species and the potential of PIT tags to assist population monitoring, it is crucial to validate PIT tagging methods in a greater number of species in order to inform conservation programs.

PIT tag implantation procedures were imprecise and not standardized

We detected a striking lack of detail in the description of the PIT tagging procedures and methods in the reviewed studies. The majority of studies did not offer complete reports of fundamental details including PIT tag size, its anatomical placement in the animal, the use of anesthesia, level of sterility or the use of skin closure methods. It is, therefore, difficult to draw comparisons and conclusions about the performance of different implantation procedures using this incomplete data. Nevertheless, these factors could strongly influence the probability of PIT tag rejection as well as impact individual welfare as reported for other ectothermic species (Gibbons & Andrews, 2004; Lyon et al., 2019; Musselman et al., 2017).

Inadequate standardization in the use of specific methods during PIT tagging protocols was also concerning. In particular, we detected a great inconsistency in the use of anesthesia and in the selection of anesthetic methods. Anesthesia may be indicated in PIT tagging procedures to achieve adequate muscle relaxation or to reduce pain and discomfort of the animals. Implantation of a PIT tag into the celomic cavity can be considered equivalent to intra-celomic surgery and should involve the use of anesthesia as well as maximum levels of sterility and muscle and skin closure, which were not ensured across the reviewed studies. Additionally, we identified the use of anesthetic methods that may require further evaluation in amphibians since high mortality and inconsistent anesthetic depth have been reported, namely Orajel® and EMLA® cream (Baitchman & Stetter, 2014). On the other hand, may require additional permits, special equipment and trained personnel, which can prevent its use under field conditions. The selection of an acceptable anatomical location for PIT tag placement, among others, could help avoiding the use of anesthesia.

The selection of the size of the PIT tag was also not standardized and inconsistent with the size of the urodele species under study. Currently, there are no evidence-based guidelines for the minimum animal size for PIT tagging and rules-of-thumb such as the "2% rule" for fish (Winter, 1996) have been strongly opposed (R. S. Brown et al., 1999; Jepsen et al., 2005). However, it is clearly important to minimize PIT tag size in relation to animal body size in order to reduce the probability of PIT tag rejection and to improve animal welfare (Jepsen et al., 2005). Altogether, PIT tagging is a procedure that entails important risks for the animals and we recommend that it is always conducted under the supervision of a trained veterinary surgeon and following the appropriate regulations of each country.

PIT tags need to be validated for each particular species and implantation method

Using data from the reviewed studies, we were not able to identify predictors of PIT tag rejection in urodeles. Conversely, studies in fishes have identified several factors that can greatly influence PIT tag retention, such as anatomical location or the use of sutures (Musselman et al., 2017). Due to the inconsistency in reporting the methods for PIT tag implantation, we could only include 21 out of 65 experiments in our final model, which may have influenced our ability to identify significant predictors. These results suggest that the effectivity of PIT tags may be highly specific to the species and method used and further highlight the need for conducting controlled studies before marking free-living populations.

In the present study, we contributed to this need by evaluating the performance of a PIT tagging method without anesthesia in three European species of urodeles. Our protocol presents some advantages for field studies such as that no anesthesia is required. Moreover, we demonstrated that Vetbond® or equivalent tissue adhesives are useful for wound closure, possibly contributing to wound healing and to PIT tag retention in the field. Our results showed significant differences in the effectivity of PIT tags between species. On one hand, PIT tags have been demonstrated as reliable marks in both fire salamanders and sharp-ribbed newts. Conversely, our PIT tagging method is not a suitable technique for marking Pyrenean brook newts, both in terms of reliability and animal welfare.

Lesions in *C. asper* were considerably larger and took over a month to heal while the PIT tag was still in place. The appearance of lesions and the rapid healing after PIT tag loss, are indicative of a foreign body reaction to the PIT tag (Kastellorizios et al., 2015). *Calotriton asper* was the smallest species studied (i.e., total length 160 mm compared to > 280 mm in the other two species), suggesting that body size may be important for PIT tag retention. Despite all PIT tags being retained in *P. waltl*, delayed healing and wound infection in some individuals raise welfare concerns when using our protocol. *Pleurodeles waltl* newts have an average total length of 300 mm and wounds were markedly different from those of *C. asper*. However, both species were PIT tagged during an aquatic phase and it is plausible that delayed healing is related to the aquatic environment. Further studies should test our PIT tagging method during a terrestrial phase to determine its safety in *P. waltl*.

Conclusions

In this quantitative systematic review, we critically analyzed the use of PIT tags in urodeles on a worldwide scale, providing valuable information related to publication numbers, species, methods and reported success of this marking technique, as well as attempted to identify predictors of PIT tag loss. In particular, we identified a striking lack of standardization in reporting the protocols used for PIT tagging these species, together with unpredictable patterns of tag loss. We recommend a greater emphasis on reporting implantation methods, ensuring animal welfare and performing species and protocol-specific laboratory trials before using PIT tags in the field. We also described a PIT tagging technique without anesthesia that can be reliable for marking *S. salamandra*, *P. waltl*, and potentially other urodele species. Urodeles are suffering dramatic declines worldwide and capture-mark-recapture studies with reliable marks are essential tools for population and disease ecology. Optimization of individual marking techniques, such as PIT tags, is therefore crucial for evidence-based management and conservation of endangered urodele species.

Chapter 2

Human disturbance and temperature drive amphibian chytrid fungus in Catalonia, Northeastern Spain

Maria Puig Ribas
Xavier Fernández Aguilar
Johan Espunyes
Laura Carrera Faja
Ignasi Marco
Oscar Cabezón

Prepared according to the guidelines of Animal Conservation

Chytridiomycosis, caused by the fungal pathogens *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal), poses a major threat to amphibian biodiversity worldwide. Although Bd is widespread in Europe, its epidemiology remains poorly understood at local scales, particularly within diverse multi-host communities. Here, we present a five-year field study (2019 - 2023) to assess chytrid fungi occurrence and associated environmental drivers in 3,253 wild amphibians from 14 species across 41 sites in Catalonia, Northeastern Spain. Using duplex qPCR, we detected Bd in 141 individuals (4.3%) from eight species and found no evidence of Bsal. Infections were geographically widespread, with significant differences in prevalence among species. *Alytes obstetricans* larvae showed the highest infection prevalence (26.6%), supporting their role as potential long-term reservoirs. *Hyla meridionalis* also exhibited high prevalence (14.3%) and substantial infection loads, suggesting it may function as a Bd-tolerant reservoir species. In contrast, most other species showed low or no infection, including urodeles and alpine specialists. Although we did not observe overt disease in any amphibian, chytridiomycosis-related mortality was suspected in Bd-positive post-metamorphic *A. obstetricans*, aligning with known vulnerability during metamorphosis. Using negative binomial models, we identified anthropogenic disturbance and maximum summer temperature as significant predictors of Bd occurrence. These findings support the growing evidence that human environmental modification and climate-related factors interact to influence pathogen distribution and persistence. The apparent endemicity of Bd and absence of Bsal in Catalonia's amphibian communities underscore the need for continued surveillance, particularly focused on likely reservoir species, and targeted conservation strategies. Understanding species-specific responses to infection and roles in disease dynamics, along with key environmental drivers, is essential to mitigate disease risk and preserve amphibian biodiversity.

Introduction

Emerging infectious diseases are increasingly recognized as key threats to biodiversity conservation (Daszak et al., 2000). Amphibian chytridiomycosis, caused by the fungal agents *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal), is responsible for the greatest loss of biodiversity associated with any pathogen ever recorded (Berger et al., 1998; Martel et al., 2013; Skerratt et al., 2007). Both pathogens have been directly linked to the decline of at least 501 amphibian species, with 90 species confirmed or presumed extinct (Scheele, Pasmans, et al., 2019). Despite over 20 years of intensive research since the discovery of Bd in 1998 (Berger et al., 1998), effective management strategies suitable for use in wild amphibian populations are still lacking. As a result, amphibians are currently the most threatened vertebrates on Earth, with over 41% of species at risk of extinction and ongoing population declines caused by these agents (IUCN, 2024; Stuart et al., 2004).

Bd and Bsal are obligate non-hyphal chytridiomycete fungi that infect the stratified keratinizing epidermis of amphibians, specifically the skin of post-metamorphic individuals and the mouthparts of tadpoles (Longcore et al., 1999; Martel et al., 2013). Both pathogens have life cycles that include motile aquatic zoospores as a dispersal stage and exhibit strong temperature dependence, with optimal growth ranging from 17–25°C for Bd and 10–15°C for Bsal (Longcore et al., 1999; Martel et al., 2013). Growth is possible outside these ranges at reduced rates, but mortality occurs at >28ºC for Bd and >25ºC for Bsal (Berger et al., 2004; Martel et al., 2013). Both pathogens exhibit an exceptionally broad host range – Bd alone can infect over 700 species (Olson et al., 2013) – yet responses to exposure and infection vary significantly across species, populations and life stages (Scheele et al., 2017; Searle, Gervasi, et al., 2011; Tobler & Schmidt, 2010).

Infection dynamics and outcomes are shaped by a complex interplay of host, pathogen and environmental factors. Host species identity and life stage are key determinants (Blaustein et al., 2018), with larvae typically exhibiting asymptomatic localized infections, while recently metamorphosed amphibians suffer the most severe effects (Berger et al., 1998). Among pathogen-related factors, virulence varies across Bd lineages and strains (Blaustein et al., 2018), and infection intensity is a major factor influencing individual and population-level outcomes (Briggs et al., 2010; Voyles et al., 2009). Bioclimatic environmental variables, particularly temperature and humidity, are crucial mediators of chytrid fungi epidemiology by affecting pathogen survival and transmission rates (Blaustein et al., 2018). These factors can also be influenced by latitude and altitude and shift seasonally, especially in temperate areas. Moreover, several studies suggest that anthropogenic disturbances play a significant role in shaping the occurrence of chytrid fungi by facilitating pathogen introduction and spread, as well as altering ecosystem functioning (Alvarado-Rybak et al., 2021; Bacigalupe et al., 2017).

Central to the understanding of pathogen dynamics and population outcomes is the variation in host defenses among species, populations and life stages. Animal defenses against infection involve two distinct but complementary mechanisms: tolerance and resistance. Tolerance refers to the host's ability to carry a given pathogen burden with limited detrimental effects on fitness/survival, whereas resistance is the host's ability to prevent infection or limit infection intensity after exposure (Råberg et al., 2007). Within a single amphibian species, tolerant and resistant individuals may be crucial long-term survivors contributing to the persistence of amphibian populations. Conversely, pathogen-tolerant reservoir species or life stages can facilitate pathogen maintenance, posing challenges for effective disease mitigation (Scheele et al., 2017). Overall, the devastating impact of chytridiomycosis is driven by its introduction into naïve host populations, the environmental persistence of infectious zoospores, and the presence of such amphibian reservoirs that sustain a high force of infection for susceptible hosts (Berger et al., 2016).

In Europe, chytridiomycosis has led to significant mortality in multiple species, driving population declines and local extinctions. Bd had particularly severe impacts in *Alytes obstetricans* populations in Central Spain, Portugal, and the Western French Pyrenees (Bosch et al., 2001; Clare, 2014; Clare et al., 2016; Rosa et al., 2013). Similarly, Bsal introduction had catastrophic consequences for *S. salamandra* populations in Central Europe (Martel et al., 2013). Although Bd is now widespread across Europe (Allain & Duffus, 2019), research in its landscape epidemiology and host-pathogen dynamics within multi-host amphibian communities remains limited. Catalonia (NE Spain) covers an area of 32,108 km^2 and boasts remarkable ecological diversity. Its landscapes range from alpine ecosystems in the Pyrenees, reaching elevations of 3,143 masl, to Mediterranean habitats spanning coastal ranges and lowlands. This diversity is also reflected in its amphibians, with 15 species adapted to various habitats and altitudes, including two endemic species (*Calotriton asper* and *C. arnoldi*). Despite its ecological significance, little research has focused on the dynamics of chytrid fungi in Catalonia's amphibian populations (but see Obon et al., 2013; Martínez-Silvestre et al., 2020, 2023). A notable gap in research is the absence of studies on *A. obstetricans* in the Catalan Pyrenees, despite the severe mortality events and the potential role of tadpoles as reservoirs in other montane systems (Clare et al., 2016; Walker et al., 2010). Additionally, in 2018, an outbreak of Bsal chytridiomycosis was detected in *Montnegre i el Corredor* Natural Park, 1,000 km from its nearest known occurrence in northern Europe (Martel et al., 2020). However, no further investigations were conducted to assess the presence of Bsal in surrounding areas or across Catalonia following the outbreak. Overall, long-term and region-wide studies on multi-host communities remain scarce, limiting our understanding of chytridiomycosis epidemiology at a more local scale. Consequently, there is a pressing need to analyze the distribution patterns of chytrid fungi and evaluate the influence of environmental factors and host species on infection dynamics.

In this study, we assessed chytrid fungi occurrence in amphibians from Catalonia over five years to evaluate its epidemiology across a broad latitudinal, altitudinal and taxonomic range. Using duplex qPCR assay results, we analyzed variations in infection prevalence and intensity among amphibian hosts and modeled Bd prevalence to identify environmental and anthropogenic factors influencing pathogen occurrence. This knowledge may provide sustainable prevention and mitigation strategies for high-risk populations, a key research priority in amphibian conservation.

Methods

Ethics statement

Animal capture, manipulation and sampling protocols were approved by *Departament d'Acció Climàtica, Alimentació i Agenda Rural* (Generalitat de Catalunya, Spain) under the license SF/0013/23. Additional permits were also obtained for developing scientific activities within protected areas: *Zona Volcànica de la Garrotxa* Natural Park (NP) (2023PNATZVGAUT005), *Aiguamolls de l'Empordà* NP (2023PNATAAEAUT025), *Capçaleres del Ter i del Freser* NP (FUE-2023-03010204), *Cadí-Moixeró* NP (9015-117182/2023) and *Alt Pirineu* NP (9015-117558/2023). All methods are reported following ARRIVE guidelines https://arriveguidelines.org.

Study area and survey protocol

From 2019 to 2023, we sampled amphibians at 41 sites from Catalonia, NE Spain (Fig. 1). Sampling sites were selected taking into account areas of high amphibian diversity and abundance, various bioregions and altitudes (0 - 2,360 masl), protected areas (i.e., NPs) and baseline data on chytrid occurrence (Martínez-Silvestre et al., 2020). Catalonia is home to 15 amphibian species – 9 anurans and 6 urodeles – all of which are protected regionally (Departament Acció Climàtica & Generalitat de Catalunya, 2023). One urodele species, *C. arnoldi,* is classified as Critically Endangered by the IUCN and is currently subject to several conservation management strategies, including a captive breeding program (IUCN SSC Amphibian Specialist Group, 2022; Life Tritó Montseny, n.d.). For this study, we sampled post-metamorphic amphibians from 14 species representing all seven extant families found in Catalonia: *Salamandra salamandra, Pleurodeles waltl, Calotriton asper, Triturus marmoratus, Lissotriton helveticus, Alytes obstetricans, Discoglossus pictus, Pelodytes punctatus, Hyla meridionalis, Pelophylax perezi, Rana temporaria, Pelobates cultripes, Epidalea calamita* and *Bufo spinosus*. Additionally, we opportunistically sampled *A. obstetricans* tadpoles. Due to the conservation status of *C. arnoldi* and the recent detection of Bsal in *Montnegre i el Corredor* NP (Martel et al., 2020), this species and certain areas were excluded from sampling in the present study. As most amphibians are inactive during winter in Catalonia, we performed surveys in spring and summer, coinciding with their breeding season.

Animal capture and sampling

Amphibians were located through calls and visual encounters after sunset and caught by hand or using dipnets, in the case of aquatic species and tadpoles. After capture, each animal was contained in a clean plastic bag and handled with a fresh pair of nitrile gloves for sampling. We then obtained a skin swab (Snappable Ps + cotton, Deltalab®) from each amphibian by firmly drawing it five times each along both sides of the ventral abdomen, both ventral hind limbs and drink patch, and the plantar surface of both hind feet, for a total of 30 strokes (Hyatt et al., 2007). In urodeles, the 30 strokes were divided between the ventral abdomen and the plantar surface of all four limbs. In tadpoles, their mouthparts were swabbed by inserting a fine-tip swab (Aluminium + cotton, Deltalab®) into the mouth and twirling it several times. All animals were thoroughly examined for signs of disease before being released back at their capture site. Skin swabs were stored at -20ºC within the first 24 h after collection until DNA extraction.

Strict disinfection and biosecurity protocols were followed to minimize cross-contamination of samples and the spread of pathogens within and between field sites. Any equipment in contact with the animals was either single-use (e.g., gloves, bags, swabs) or was thoroughly disinfected with 1% Virkon® S (Zotal Laboratorios, Sevilla, Spain) between uses, including personal clothing and equipment.

Chytrid fungi diagnostics

We extracted DNA from skin swabs using 65 μL of PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, USA) following Hyatt et al. (2007). We conducted a duplex qPCR assay to simultaneously detect Bd and Bsal and quantify infection intensity in each animal sample (Blooi et al., 2013). A synthetic double-stranded 750 bp gBlocks® (Integrated DNA Technologies, USA) gene fragment containing the target PCR sequences of both pathogens was used as a positive control and quantitation standard.

Standard dilutions in log10 increments (10^{-1} to 10^{6} copies; in duplicate) were included in each qPCR plate to create a standard curve, allowing us to quantify the genomic equivalents (GE) per qPCR reaction. Negative controls (nuclease-free water) were also included in each plate. A sample was considered positive if the gene copy number was above 1. All qPCR assays were run on an Applied Biosystems™ 7500 Real-Time PCR System.

Bd prevalence and infection loads by host species

We calculated Bd prevalence from the ratio of positive animals to the total number of samples within a species with 95% Wilson confidence intervals. Differences in Bd prevalence among positive species were assessed using Fisher exact tests and pairwise comparisons with Benjamini-Hochberg correction to control for the false discovery rate. Log-transformed Bd loads were compared between species using a Kruskal-Wallis test. We also explored whether Bd prevalence at each sampling site varied significantly across years and whether there was a monotonic trend over time using mixed-effects models (with sampling site as a random effect) and the Mann-Kendall test, respectively.

Modeling Bd infection risk across Catalonia

We used an information-theoretic approach to compare multiple models and identify the best predictors of Bd infection occurrence in post-metamorphic amphibians in Catalonia. *Alytes obstetricans* larvae data were removed from this analysis as they were not homogeneously sampled across sites. In the statistical modeling, we used eight bioclimatic, geographic, and anthropogenic factors as predictors, with Bd counts by species and sampling events as the response variable. We intentionally separated counts by species to account for the independent variability introduced by the amphibian species in the approach. Total counts with less than five animals sampled were also removed from the analyses to improve reliability, resulting in a dataset of 156 observations with a total count of 2,731 individuals sampled. Explanatory variables included anthropogenic disturbance, amphibian species richness, bioregion, habitat type, altitude, mean temperature, summer mean maximum temperature, and precipitation (see Supplementary Information for a detailed description of each variable and data sources). Geospatial variables were extracted from raster layers in buffers of 5 km around sampling points using “raster”, “sp”, “sf” and “geosphere” packages in R (Hijmans, 2024, 2025; Pebesma & Bivand, 2025; Pebesma, 2025).

We initially explored data to investigate records, distributions, imbalances and the relationship among the response variables and predictors (Zuur, Ieno, & Elphick, 2010). Since no significant differences between years or trends over time were detected in the exploratory analyses, year of sampling was not included as a predictor. We standardized the continuous predictors using the “scale” function in R and log-transformed the variable “anthropogenic disturbance” to improve the assumption of data normality. To avoid collinearity between explanatory factors, we used the variance inflation factor (VIF) with a correlation cut-off coefficient ≥ 0.7. Accordingly, we excluded “mean temperature”, “precipitation” and “elevation” from subsequent analyses.

Because our data exhibited strong overdispersion, an aggregated sampling design, and a large proportion of zeros, we first fitted zero-inflated negative-binomial mixed-effects models (nbinom1 family, log link) with a random intercept for sampling site using glmmTMB (Brooks *et al.*, 2017). We then used likelihood-ratio tests to compare each full model to a simpler nested model without the zero-inflation component and assessed the contribution of the site random effect. Neither factor significantly improved model fit, and most predictors exhibited minimal within-site variation. Consequently, we excluded both terms and refitted a fixed-effects-only negative binomial models (nbinom1, log link) for the count of positives, including the natural logarithm of the total individuals sampled as an offset to control for sampling effort.

The most parsimonious models were identified using the corrected Akaike Information Criterion (AICc) for small sample sizes in a manual forward-stepwise procedure. Among the candidate set of models, any model with a ΔAICc < 2 relative to the best-fitting model was considered as having substantial support (Johnson & Omland, 2004). To validate the final models, the simulated residuals, the dispersion and uniformity of the residuals, the presence of outliers, and the zero-inflation were examined using the DHARMa package (Hartig, 2024) (Figures S1-S2). All statistical analyses were performed using R version 4.1.4 (R Core Team, 2024) and significance thresholds set at $p \leq 0.05$.

Results

Patterns of chytrid fungi occurrence

Over the study period, we collected skin swabs from 3,253 wild amphibians from 14 species across Catalonia (Fig. 1, Table 1). Infection with Bd was detected in 141 individuals from seven different species, including 55 *A. obstetricans* larvae, with an overall prevalence of 4.33 (95% CI 3.69 - 5.09). Infection with Bd was detected at 12 of the 41 study sites, indicating a substantial geographical distribution across Catalonia. No significant differences in prevalence between years or trends over time were detected. In contrast, Bsal was not detected at any site throughout the study period.

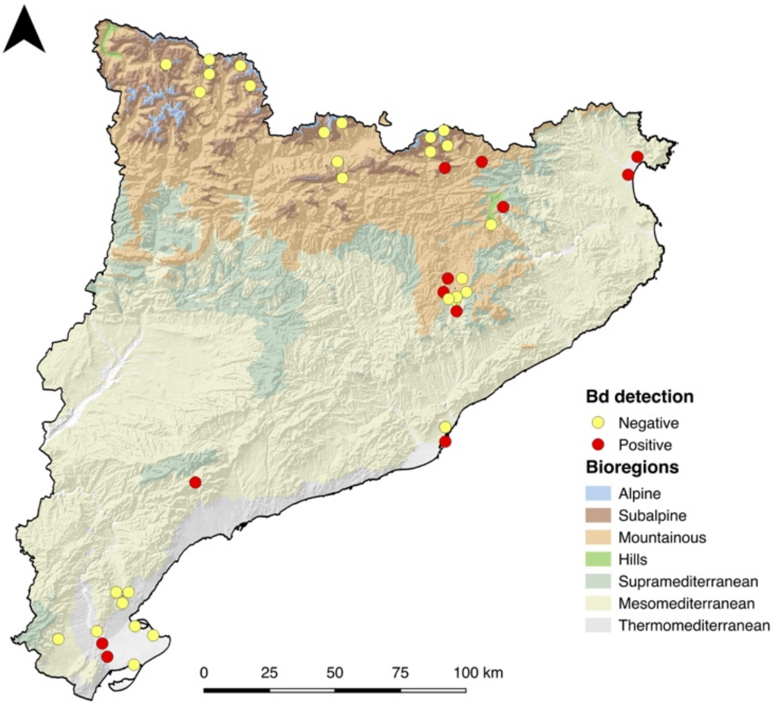


Figure 1. Map showing the distribution of *Batrachochytrium dendrobatidis* (Bd) infection in amphibians studied in Catalonia (NE Spain) from 2019 to 2023. Bd-positive (red dots) and Bd-negative (yellow dots) sampling sites, along with the main bioregions, are depicted.

Among the seven Bd-positive amphibian species, *A. obstetricans* larvae showed the highest Bd infection prevalence with 26.57%, followed by *H. meridionalis* with 14.34%, *P. perezi* with 6.79%, *D. pictus* with 2.56%, *E. calamita* with 1.69%, *A. obstetricans* with 1.21%, *T. marmoratus* with 1.11% and *C. asper* with 0.28% (Table 1). Among the positive species, significant differences were observed between *A. obstetricans* larvae and all other species, including *A. obstetricans* adults. *Hyla meridionalis* prevalence also had significant differences with all species except for *D. pictus*, and *P. perezi* had significant differences with *C.asper* and *A. obstetricans* adults (Fig. S4). Infection loads in Bd-positive animals ranged from 1 to 70,383 GE (median=52 GE) (Fig. 2, Table 1). Although some individuals exhibited high infection loads, most post-metamorphic amphibians sampled appeared healthy and showed no signs of disease. Depigmentation of the mouthparts was observed in 17 of the 51 Bd-positive *A. obstetricans* larvae, despite appearing otherwise healthy. The highest Bd infection load was detected in a deceased *A. obstetricans* metamorph exhibiting signs of dysecdysis. Moreover, another Bd-positive individual was also found dead, and the only three positive *A. obstetricans* detected were metamorphs with high infection loads (Fig. 2). No significant differences were detected in infection loads between Bd-positive species.

Table 1. Infection of *Batrachochytrium dendrobatidis* (Bd) across amphibian species sampled in Catalonia from 2019 to 2023. The table presents the species, family, sample size, number of Bd-positive individuals, prevalence (%), 95% confidence interval (CI), median genomic equivalent (GE), and maximum GE detected. Different letters indicate significant differences in prevalence between positive species.

Species	**Sample size**	**Bd positive**	**Prevalence (%)**	**95% CI**	**Median GE**	**Max. GE**
Alytes obstetricans larvae	207	55	26.57 ^{a}	21.02 - 32.97	55	12,580
Hyla meridionalis	265	38	14.34 ^{b}	10.63 - 19.07	188	63,000
Pelophylax perezi	589	40	6.79 ^{c}	5.03 - 9.12	22	5,880
Discoglossus pictus	39	1	2.56 ^{bcd}	0.13 - 13.18	6	6
Epidalea calamita	59	1	1.69 ^{cd}	0.09 - 8.99	1	1
Alytes obstetricans	247	3	1.21 ^{d}	0.41 - 3.51	812	70,383
Triturus marmoratus	90	1	1.11 ^{cd}	0.06 - 6.03	88	88
Calotriton asper	715	2	0.28 ^{d}	0.08 - 1.01	6	12
Lissotriton helveticus	28	0	0	0.00 - 12.06	0	0
Pleurodeles waltl	78	0	0	0.00 - 4.69	0	0
Salamandra salamandra	123	0	0	0.00 - 3.03	0	0
Bufo spinosus	87	0	0	0.00 - 4.23	0	0
Pelobates cultripes	3	0	0	0.00 - 56.15	0	0
Pelodytes punctatus	10	0	0	0.00 - 27.75	0	0
Rana temporaria	713	0	0	0.00 - 0.54	0	0
Total	**3,253**	**141**	**4.33**	**3.69 - 5.09**	**52**	**70,383**

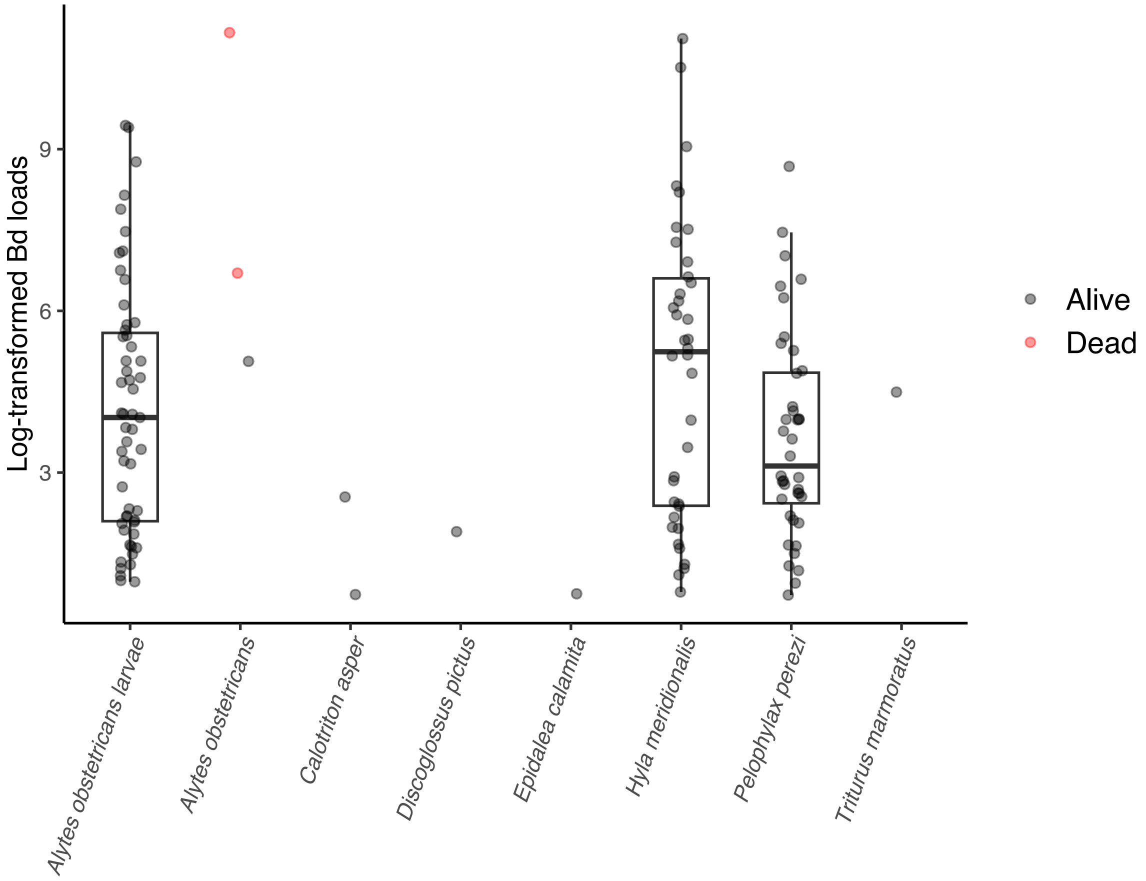


Figure 2. *Batrachochytrium dendrobatidis* (Bd) log-transformed infection loads across amphibian species sampled in Catalonia from 2019 to 2023. Individual data points are overlaid on boxplots (for species with >3 observations) to show the distribution of Bd loads. Black points represent live individuals, while red points highlight Bd-positive individuals found dead during sampling.

Patterns of chytrid fungi occurrence

Among all the evaluated models (Table S3), the best model for explaining Bd occurrence across Catalonia included the variables "mean maximum summer temperature" ($\beta = 0.813$, SE = 0.392, $z = 2.07$, $p = 0.038$) and "anthropogenic disturbance" ($\beta = 0.948$, SE = 0.208, $z = 4.57$, $p < 0.001$) as significant predictors (AIC = 179.6, BIC = 191.8, logLik = -85.8, deviance = 171.6, df.resid = 152). In this model, each one-unit increase in scaled maximum summer temperature was associated with a 2.25-fold increase in the expected number of Bd-positive individuals (IRR = 2.25, 95% CI 1.05–4.86), and each one-unit increase in scaled anthropogenic disturbance with a 2.58-fold increase (IRR = 2.58, 95% CI 1.72–3.88). A second model within ΔAICc < 2 included "mean maximum summer temperature", "anthropogenic disturbance" and "species richness", but only anthropogenic disturbance was a significant predictor (Table S4). The average model between the two top models had “mean maximum summer temperature” and “anthropogenic disturbance” as significant predictors (Table 2). The top-ranked negative-binomial model explained 21.4% of the deviance (McFadden’s pseudo-$R^2 = 0.214$) relative to the offset-only null, indicating a substantial improvement in fit. When averaging across the two models with ΔAICc < 2, the normalized model-averaged McFadden’s pseudo-R^2 was 0.217, demonstrating that accounting for model-selection uncertainty yielded the same explanatory power.

Table 2. Model-averaged parameter estimates (full-average) for the top two selected models. Estimates are accompanied by standard errors (SE), adjusted SE, z-values and p-values; significance codes: *** $p < 0.001$, * $p < 0.05$.

Predictor	**Estimate**	**SE**	**Adj. SE**	**z-value**	**p-value**
(Intercept)	- 4.803	0.4702	0.4739	10.134	$< 2 \times 10^{-16}$***
Anthropogenic disturbance	0.984	0.2262	0.2279	4.315	1.6×10^{-5} ***
Mean max. summer temperature	0.832	0.4161	0.4194	1.983	0.0473*
Species richness	0.111	0.1745	0.1752	0.635	0.5255

Discussion

Our study provides valuable insights into the occurrence and epidemiology of chytrid fungi in amphibian populations across Catalonia, NE Spain. The findings highlight the endemic nature of Bd in the region, with significant variation in infection prevalence among species. These differences suggest varying levels of resistance and tolerance across species and life stages, providing an initial step toward identifying potential infection reservoirs. Additionally, our statistical modeling identifies anthropogenic disturbances and maximum summer temperatures as key predictors of Bd occurrence, reinforcing the growing concern that climate change and human activities have a role in shaping disease dynamics in amphibian communities.

Bd Distribution and Endemicity in Catalonia

The detection of Bd at 12 of the 41 surveyed sites indicates that the pathogen is well established in Catalonia, infecting amphibians across diverse habitats and elevations. Despite its broad geographical distribution, Bd prevalence remained relatively low and stable throughout the study period, suggesting an endemic state rather than an emerging epidemic event. These findings align with previous studies in Spain and other European regions, where Bd is widespread but exhibits spatial and temporal variability in infection dynamics (Lips, 2016). In Europe, large-scale epidemics and mass mortalities have been restricted to montane areas of the Iberian Peninsula and the French Pyrenees (Bosch et al., 2001; Clare, 2014; Rosa et al., 2013).

Notably, our results indicate that Bd infection is currently absent from Catalonia's alpine and subalpine ecosystems, contrasting with the findings of Martínez-Silvestre et al. (2020), who reported infections in *C. asper* at one of our surveyed sites in the Western Catalan Pyrenees. Pathogen fade-out may occur when the local density of susceptible hosts is too low to sustain transmission or when environmental conditions reduce the survival of free-living pathogen stages. However, the latter is unlikely in Pyrenean areas, as Bd is known to survive and grow at temperatures below 4ºC (Piotrowski et al., 2004). A more plausible explanation is that the host community has contributed to Bd's disappearance at this site, likely due to the absence of pathogen-tolerant reservoirs. The current amphibian assemblage at this site is largely composed of *C. asper* and *R. temporaria,* with only a single *A. obstetricans* adult and no larvae ever detected during our surveys. These two species, thus, appear unable to support long-term pathogen persistence (see additional discussion on species patterns below). Additionally, we cannot rule out the possibility that the initial introduction of Bd triggered a chytridiomycosis outbreak, leading to the local decline or near-extirpation of *A. obstetricans*, as documented in other montane areas. The loss of the most susceptible host may have favored more resistant species, ultimately leading to pathogen fade-out.

On the other hand, our results confirmed the presence of Bd at sites where the pathogen had been previously detected (Martínez-Silvestre et al., 2020; Martinez-Silvestre et al., 2021). The persistence of Bd likely involves the presence of competent, pathogen-tolerant reservoirs and environmental conditions conducive to its survival. However, historical data were unavailable for most of our study sites (38 out of 41), and we documented Bd infection at 10 locations for the first time. Aside from the two deceased metamorphs, for which chytridiomycosis is a likely cause of mortality, no overt outbreaks have been reported before or during our study period in Catalonia. Instead, the low prevalence observed appears more compatible with sporadic and cryptic mortality without population declines, indicating a state of host-pathogen coexistence similar to that described in other European regions (Spitzen-Van Der Sluijs et al., 2014). Longitudinal studies examining seasonal and multi-year infection dynamics will be essential to further clarify the long-term impacts of Bd on amphibian populations in Catalonia. In the following sections, we further discuss factors associated with host species, environmental factors and anthropogenic influences to gain a deeper understanding of Bd infection dynamics in Catalonia.

The absence of Bsal in our study area is encouraging, as this pathogen has been responsible for severe declines in *S. salamandra* populations in other parts of Europe (Martel et al., 2013). Although we were unable to sample the index site and cannot confirm its complete eradication, our results suggest that Bsal has not further spread into amphibian populations across Catalonia. Nevertheless, continued surveillance remains crucial due to its potential for introduction and spread.

Host-Specific Patterns of Bd Infection

Our results reveal substantial interspecific variation in Bd prevalence and infection intensity, emphasizing the importance of host species in disease dynamics. *Alytes obstetricans* larvae exhibited the highest prevalence, significantly differing from the rest of the studied species. Although not statistically significant, Bd loads in larvae were also relatively high despite the absence of generalized clinical disease. These findings suggest that *A. obstetricans* larvae exhibit a degree of tolerance to Bd infection, aligning with previous research identifying them as key reservoirs for Bd persistence (Clare et al., 2016). At high altitudes, *A. obstetricans* larvae undergo prolonged developmental periods and may overwinter for at least one year. These long-lived tadpoles with high infection prevalence can act as a continuous reservoir, sustaining a Bd infection source year-round – similar to patterns observed in other Bd systems (Hollanders et al., 2024; Rachowicz & Vredenburg, 2004). Moreover, this species is distributed throughout Catalonia, found across a broad range of habitats from sea level to over 2500 masl, further enhancing its potential as a reservoir.

Conversely, we rarely detected Bd infections in postmetamorphic *A. obstetricans*, with only three metamorphs testing positive – two of which were found dead and had high Bd loads. Given the high prevalence detected in larvae, these findings suggest that Bd infections are cleared during metamorphosis or lead to mortality at this stage. The high susceptibility of *A. obstetricans* to chytridiomycosis is well-documented in the literature (Bosch, Martínez-Solano, & García-París, 2001; Rosa et al., 2013; Clare, 2014), with newly metamorphosed individuals recognized as the most vulnerable stage in susceptible species (Berger et al., 1998). Once metamorphosed, adults become fully terrestrial, and opportunities for Bd exposure are probably limited to mating and when males approach water during egg hatching. These life history traits may also explain the low prevalences observed in adults. While we did not observe large mortality outbreaks, our findings support previous literature indicating that this species is highly susceptible and may succumb to Bd (Bosch, Martínez-Solano, & García-París, 2001; Rosa *et al.*, 2013; Clare, 2014).

We also documented significantly higher Bd prevalence and substantial infection intensities in *H. meridionalis* despite the absence of clinical disease. Such high prevalences were unexpected, given that aquatic species are generally more exposed to Bd infectious stages (Burrowes et al., 2017; Scheele, Pasmans, et al., 2019), while *H. meridionalis* has an arboreal lifestyle. This suggests that, even if exposure is rare, it tends to result in successful infections, indicating relatively low species resistance to Bd. These findings offer a novel contribution to the literature and identify this species as a potentially Bd-tolerant host that could play a significant role in Bd maintenance.

Although not statistically significant, *P. perezi* had the third-highest prevalence while showing lower Bd loads than *A. obstetricans* larvae or *H. meridionalis*. Considering its aquatic lifestyle, exposure to Bd zoospores is likely recurrent, which may indicate that this species has a comparatively higher resistance to infection. The potential role of *Pelophylax* spp. as Bd reservoirs has been proposed in other European countries (Baláž et al., 2014). While our results do not fully support a similar role for *P. perezi* in Catalonia, its widespread distribution – only absent in alpine areas – renders it a relevant species for future Bd research and surveillance efforts.

The other amphibian species studied exhibited very low prevalence or absence of Bd infection. This could indicate that they are either resistant to infection or highly susceptible to disease, meaning that infected individuals are rarely, if ever, found alive. Individual mortality has been reported in several of these species, including *Bufo* spp., *S. salamandra*, *D. pictus*, *R. temporaria* and *P. perezi* (Bosch & Martínez-Solano, 2006; Clare et al., 2016; Martinez-Silvestre et al., 2021). Since Bd outcomes are largely dependent on infection intensity (Blaustein et al., 2018), any species may experience disease and mortality if pathogen load reaches a critical threshold. However, such events are likely rare, as no mortality outbreaks have ever been reported for these European species. Additionally, previous research has documented asymptomatic infections in *E. calamita* and *D. pictus* in Catalonia (Montori et al., 2019), and some authors suggest that *D. pictus* could act as a Bd supershedder (Martínez-Silvestre et al., 2023). Instead, we believe the low occurrence of Bd infections in these species supports the hypothesis that they are relatively resistant hosts and unlikely to serve as effective reservoirs. Consistent with our findings, urodeles appear to exhibit greater resistance to Bd infection and disease across multiple studies (Scheele, Pasmans, et al., 2019; Van Rooij et al., 2015). Nevertheless, the small sample size for some species may obscure true patterns and differences, warranting caution when interpreting our results.

Environmental and Anthropogenic Drivers of Bd Occurrence

Our statistical modeling identified two significant predictors of Bd presence: maximum summer temperature and anthropogenic disturbance. Temperature is widely recognized as the most important environmental factor influencing Bd occurrence in the wild (K. A. Murray et al., 2011; Woodhams et al., 2008; Xie et al., 2016). Laboratory studies indicate that Bd thrives in moderate temperatures and is generally intolerant of temperatures exceeding 28–30°C, leading to the expectation that higher temperatures would reduce Bd prevalence (Piotrowski et al., 2004). However, our results show a positive correlation between Bd prevalence and maximum temperatures, suggesting that factors beyond simple thermal limits *in vitro* influence disease dynamics (Flechas et al., 2017; Rohr & Raffel, 2010). One potential explanation is that macroclimatic temperature measurements do not accurately capture the thermal conditions of amphibian microhabitats, where Bd may persist despite high air temperatures (Kearney et al., 2014). Many amphibians exhibit behavioral thermoregulation by seeking cooler, moist refuges—such as leaf litter, shaded streams, or burrows—that maintain conditions favorable for Bd, even during hot periods when air temperatures peak (Richards-Zawacki, 2010; Rowley & Alford, 2013). Another possibility is that increased temperature variability, rather than absolute temperature, influences infection patterns by inducing physiological stress in amphibians, weakening immune defenses and facilitating Bd infection (Raffel et al., 2006; Rollins-Smith et al., 2011). These findings highlight the need for further research into microclimate conditions and host behavior to elucidate the complex relationships between temperature and chytrid dynamics in natural populations. While our observational data do not allow for direct causal inference, our analysis supports the hypothesis that climate change could facilitate Bd persistence and transmission, particularly in temperate regions (Venesky, Raffel, et al., 2014; Xie et al., 2016).

Anthropogenic disturbance emerged as another key driver of Bd occurrence, supporting previous research on the role of human activities in the pathogen's epidemiology (Adams et al., 2010; Alvarado-Rybak et al., 2021; Bacigalupe et al., 2017, 2019). Our model incorporated an index reflecting various forms of human environmental alteration, with variation representing a gradient from relatively wild and inaccessible areas to densely populated or agriculturally dominated areas. However, we could not determine the relative contribution of specific human activities to Bd prevalence. Human-assisted movements of infected amphibians, contaminated water, sediments or objects are known to drive Bd distribution, introducing or reintroducing the pathogen into previously uninfected areas (Fisher et al., 2009; Liu et al., 2013; O'Hanlon et al., 2018; Schloegel et al., 2009). Additionally, human activities degrade natural ecosystems, reducing habitat quality for amphibians and increasing other stressors that may weaken hosts immunity and promote Bd infection and persistence (Becker et al., 2016; Blaustein et al., 2018; McCoy & Peralta, 2018). Given the ongoing expansion of human activities in Catalonia, conservation strategies should incorporate measures to mitigate anthropogenic impacts on amphibian populations.

Conclusions

Our study highlights the intricate relationships between host species, environmental factors, and anthropogenic disturbances in shaping Bd epidemiology in Catalonia. The identification of *A. obstetricans* larvae and *H. meridionalis* as key hosts suggests that targeted surveillance of these species could enhance monitoring efforts. Furthermore, understanding species-specific susceptibility and the potential reservoir role of certain hosts remains crucial for mitigating disease impacts. While infection dynamics vary across species, landscapes, and seasons, our findings indicate that climate change and anthropogenic disturbances contribute to Bd introduction and persistence, underscoring the need for integrated conservation strategies. As human activities and climate shifts continue to reshape ecosystems, proactive management, long-term monitoring, and preventative measures—particularly against the potential spread of Bsal—will be essential to safeguard amphibian biodiversity.

Supplementary Information

Explanatory variables used in the models

Anthropogenic disturbance

To quantify the level of anthropogenic disturbance at each sampling point, we constructed an index based on multiple variables reflecting human population density and anthropogenic environmental alterations. We performed a Principal Component Analysis (PCA) and used the first principal component (PC1) as a composite index of anthropogenic disturbance. The variables included in the PCA were:

- Land Cover: percentage of urban land use within a 5 km buffer around the sampling point, obtained from the Institut Cartogràfic i Geològic de Catalunya (ICGC) database. Source: https://www.icgc.cat/ca/Ambits-tematics/Territori-sostenible/Cobertes-del-sol
- Road Density: density of roads within a 5 km buffer around the sampling point, extracted from the Centro Nacional de Información Geográfica (CNIG). Source: https://centrodedescargas.cnig.es/CentroDescargas/index.jsp
- Distance to Populations: linear distance from the sampling point to the nearest human settlement.
- Population Density: population density of the comarca (administrative region) where the sampling was performed, obtained from Institut d'Estadística de Catalunya (IDESCAT). Source: https://www.idescat.cat/indicadors/?id=aec&n=15227
- Total Nitrogen: a proxy for livestock-related environmental impact, calculated as the sum of nitrogen input from livestock farms within a 5 km buffer around the sampling point, based on farm records and species type from the Registre d'Explotacions Ramaderes (Generalitat de Catalunya). Source: https://analisi.transparenciacatalunya.cat/Medi-Rural-Pesca/Registre-d-explotacions-ramaderes/7bpt-5azk/about_data

The PCA was performed on the standardized values of these variables and PC1 was retained as the anthropogenic disturbance index, as it explained the largest proportion of variance among the dataset (Tables S1 and S2).

Table S1. Importance of each principal component describing anthropogenic disturbance.

	PC1	PC2	PC3	PC4	PC5
Standard deviation	1.7168	1.2042	0.70230	0.32031	0.08308
Proportion of variance	0.5895	0.2900	0.09864	0.02052	0.00138
Cumulative proportion	0.5895	0.8795	0.97810	0.99862	1.0000

Table S2. Principal Component Analysis (PCA) loadings for variables reflecting anthropogenic alterations to the environment.

Variable	PC1	PC2	PC3	PC4	PC5
PopDens	-0.472074	0.470357	-0.118096	0.350839	0.647207
Sum_Nit	-0.202634	-0.717549	-0.472318	0.468968	0.033399
Dist_pop	0.417213	0.311918	-0.833244	-0.183612	0.025119
Urb_percent	-0.524623	0.336926	-0.191758	0.148828	-0.743189
Road_dens	-0.535593	-0.230377	-0.178610	-0.775318	0.164461

Amphibian species richness

Count of the amphibian species detected in each sampling point, extracted from observational data from fieldwork and from Natural Park's registers.

Bioregion

Broad ecological zones that categorize the territory based on climatic and vegetation characteristics, including general thermality index, weighted thermality index, continentality, annual ombrothermal index, ombrothermal index of the two driest months of the year, mean maximum temperature of the coldest month and mean minimum temperature of the warmest month of the year. This classification describes six distinct bioregions: Marsh, Mesomediterranean, Thermomediterranean, Mountainous, Subalpine, and Alpine. Data were obtained from Ministerio para la Transición Ecológica y el Reto Demográfico (MITECO). Sources: https://www.miteco.gob.es/ca/biodiversidad/servicios/banco-datos-naturaleza/informacion-disponible/memoria_mapa_series_veg_descargas.html

Rivas-Martínez S, Penas Á, Díaz González TE, Cantó P, del Río S, Costa JC, Molero J. Biogeographic units of the Iberian Peninsula and Balearic Islands to district level. A concise synopsis. The Vegetation of the Iberian Peninsula: Volume 1, 2017:131-188.

Habitat type

Categorical variable describing the type of aquatic habitat of the sampling point, obtained from fieldwork. Categories: Lake, Pond, Road, Stream and Wetland.

Altitude

Continuous variable representing the altitude in meters of each sampling site. levation data were extracted from a Digital Elevation Model (DEM) with a 5x5 meter resolution, provided by the Institut Cartogràfic i Geològic de Catalunya (ICC). Source: http://www.icc.es/cat/Home-CCCC/Normes-i-estandards/Especificacions-tecniques/Model-d-elevacions-del-terreny-de-Catalunya-15x15-m-MET-15-v2.0-5x5-m-MET-5-v1.0

Mean temperature

Continuous variable representing the average air temperature in ºC at each sampling site. It is calculated as the daily mean of the air temperature at 2 meters above the ground level, averaged over the entire study period (2019-2023). Data were obtained from the Copernicus ERA5-Land dataset with a resolution of 9 km. Source: https://cds.climate.copernicus.eu/cdsapp#!/dataset/reanalysis-era5-land?tab=overview

Summer mean maximum temperature
Continuous variable representing the average of the daily maximum air temperatures in °C during the summer months (July–August) over the period 2019–2023. This variable is also derived from the air temperature at 2 meters above ground level. Data were obtained from the Copernicus ERA5-Land dataset with a resolution of 9 km. Source: https://cds.climate.copernicus.eu/cdsapp#!/dataset/reanalysis-era5-land?tab=overview

Precipitation
Continuous variable representing the sum of annual precipitation in mm at each sampling site averaged over the study period (2019-2023). It includes all forms of precipitation (rain, snow, and other moisture) accumulated over a year. Data were obtained from the Copernicus ERA5-Land dataset with a resolution of 9 km. Source: https://cds.climate.copernicus.eu/cdsapp#!/dataset/reanalysis-era5-land?tab=overview

Model selection and diagnostics

Table S2. Top candidate models (ΔAICc < 4) ranked by AICc. Fixed-effect predictors and model selection statistics are shown.

Model rank	Model predictors	df	logLik	AICc	ΔAICc	Weight
1	Tmax_summer + Anth_Ind	4	-85.802	179.9	0.00	0.358
2	Tmax_summer + Anth_Ind + Sp_rich_Ind	5	-84.939	180.3	0.41	0.292
3	Anth_Ind + Sp_rich_Ind	4	-86.933	182.1	2.26	0.116
4	Anth_Ind	3	-88.114	182.4	2.52	0.102
5	Anth_Ind + Sp_rich_Ind + Type	8	-83.374	183.7	3.86	0.052

Table S3. Summary of the two best-supported nbinom1 GLMM models (ΔAICc < 2) for Bd-positive counts, showing model components and parameter estimates (Estimate ± SE, z-value, p-value).

Model rank	Components	Predictor	Estimate	SE	z-value	p-value
1	Pos ~ Tmax_summer + Anth_Ind	(Intercept)	-4.7349	0.4376	-10.820	$< 2 \times 10^{-16}$ *
		Tmax_summer	0.8128	0.3921	2.073	0.0382 *
		Anth_Ind	0.9481	0.2076	4.566	4.97×10^{-6} *
2	Pos ~ Tmax_summer + Anth_Ind + Sp_rich_Ind	(Intercept)	-4.8857	0.4949	-9.872	$< 2 \times 10^{-16}$ *
		Tmax_summer	0.8552	0.4427	1.932	0.0534 ·
		Anth_Ind	1.0271	0.2400	4.280	1.87×10^{-5} *
		Sp_rich_Ind	0.2476	0.1844	1.343	0.1793

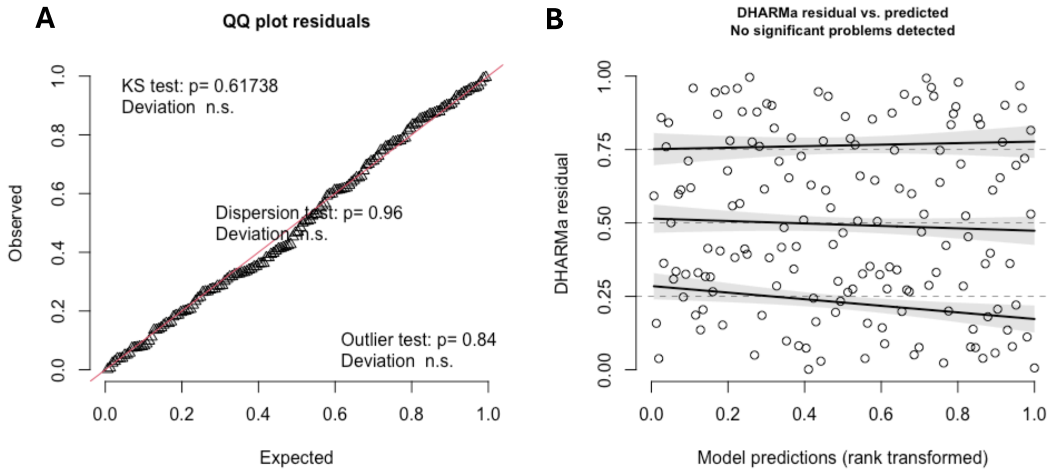


Figure S1. Model residual diagnostics of the best model selected (Model rank 1). (A) Quantile-quantile (QQ) plot of residuals comparing observed vs. expected residual distributions for model validation. The red line represents the theoretical expectation under a well-fitting model. Statistical tests (Kolmogorv-Smirnov test, dispersion test, and outlier test) indicate no significant deviations, suggesting an appropriate residual distribution. (B) Residuals vs. predicted values plot showing scaled DHARMa residuals plotted against rank-transformed model predictions. The black lines represent smoothed trends, and the shaded regions indicate confidence intervals. The model shows no significant deviations, with only a few potential outliers (red asterisks). Overall diagnostics suggest that the model assumptions are met, with no major issues detected.

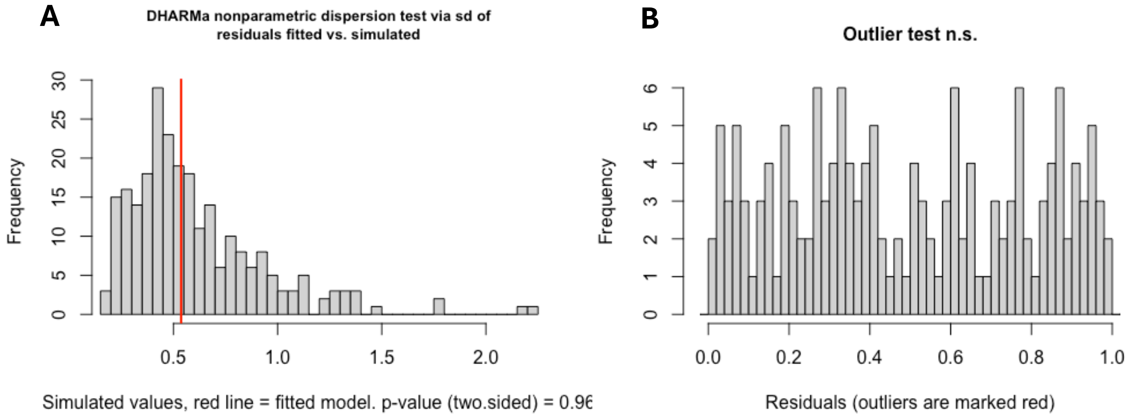


Figure S2. DHARMa dispersion test for residuals and residual outliers for the best model selected (Model rank 1). Histogram of simulated dispersion values compared to the observed model dispersion (red vertical line). The two-sided p-value (0.96) indicates no significant overdispersion or underdispersion, suggesting that the model's variance structure is appropriate. This confirms that the model correctly accounts for variability in the data. No residual outliers are detected.

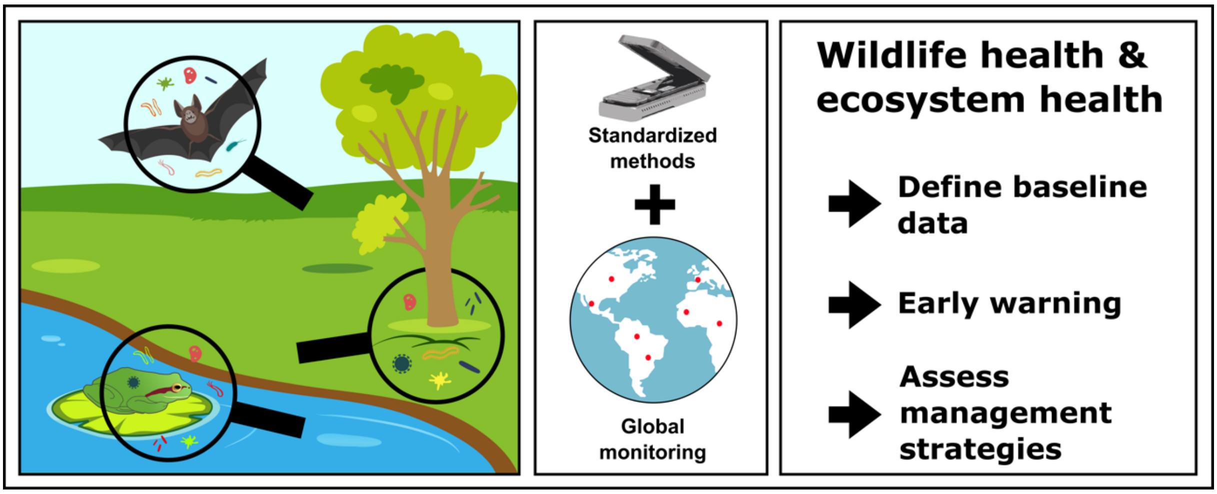
Species differences in Bd infection prevalence

Comparisons	p. Fisher	p.adj. Fisher
Alytes obstetricans larvae vs Alytes obstetricans	**1.47E-17**	**1.37E-16**
Alytes obstetricans larvae vs Calotriton asper	**4.35E-36**	**1.22E-34**
Alytes obstetricans larvae vs Discoglossus pictus	**3.18E-04**	**8.90E-04**
Alytes obstetricans larvae vs Epidalea calamita	**3.25E-06**	**1.14E-05**
Alytes obstetricans larvae vs Hyla meridionalis	**1.07E-03**	**2.30E-03**
Alytes obstetricans larvae vs Pelophylax perezi	**1.66E-12**	**1.16E-11**
Alytes obstetricans larvae vs Triturus marmoratus	**8.06E-09**	**3.76E-08**
Alytes obstetricans vs Calotriton asper	1.10E-01	1.81E-01
Alytes obstetricans vs Discoglossus pictus	4.46E-01	5.68E-01
Alytes obstetricans vs Epidalea calamita	5.77E-01	6.46E-01
Alytes obstetricans vs Hyla meridionalis	**1.00E-08**	**4.00E-08**
Alytes obstetricans vs Pelophylax perezi	**4.37E-04**	**1.11E-03**
Alytes obstetricans vs Triturus marmoratus	1.00E+00	1.00E+00
Calotriton asper vs Discoglossus pictus	1.47E-01	2.29E-01
Calotriton asper vs Epidalea calamita	2.12E-01	2.97E-01
Calotriton asper vs Hyla meridionalis	**1.52E-20**	**2.13E-19**
Calotriton asper vs Pelophylax perezi	**2.11E-12**	**1.18E-11**
Calotriton asper vs Triturus marmoratus	3.00E-01	4.00E-01
Discoglossus pictus vs Epidalea calamita	1.00E+00	1.00E+00
Discoglossus pictus vs Hyla meridionalis	3.97E-02	6.95E-02
Discoglossus pictus vs Pelophylax perezi	5.03E-01	6.01E-01
Discoglossus pictus vs Triturus marmoratus	5.15E-01	6.01E-01
Epidalea calamita vs Hyla meridionalis	**3.67E-03**	**7.34E-03**
Epidalea calamita vs Pelophylax perezi	1.63E-01	2.40E-01
Epidalea calamita vs Triturus marmoratus	1.00E+00	1.00E+00
Hyla meridionalis vs Pelophylax perezi	**7.38E-04**	**1.72E-03**
Hyla meridionalis vs Triturus marmoratus	**1.33E-04**	**4.14E-04**
Pelophylax perezi vs Triturus marmoratus	3.14E-02	5.86E-02

Figure S4. Pairwise comparisons of *Batrachochytrium dendrobatidis* (Bd) prevalence among positive amphibian species. The table displays species comparisons, unadjusted Fisher's exact test *p*-values (p.Fisher), and adjusted *p*-values (p.adj.Fisher) after multiple testing correction. Significant comparisons (p.adj.Fisher < 0.05) indicate differences in Bd prevalence between species. Significant differences in adjusted p-values are highlighted in bold.

Chapter 3

Microbiome as an indicator of amphibian health



Chapter 3.1

Improving the assessment of ecosystem and wildlife health: microbiome as an early indicator

Maria Puig Ribas
Manuel García-Ulloa
Johan Espunyes
Oscar Cabezón

Current Opinion in Biotechnology (2023) 81:102923

Human activities are causing dramatic declines in ecosystem health, compromising the functioning of the life-support system, economic activity, and animal and human health. In this context, monitoring the health of ecosystems and wildlife populations is crucial for determining ecological dynamics and assessing management interventions. A growing body of evidence indicates that microbiome provides a meaningful early indicator of ecosystem and wildlife health. Microbiome is ubiquitous and both environmental and host-associated microbiomes rapidly reflect anthropogenic disturbances. However, we still need to overcome current limitations such as nucleic acid degradation, sequencing depth, and the establishment of baseline data to maximize the potential of microbiome studies.

Introduction

Earth's ecosystems are largely and increasingly dominated by humans through land transformation, resource exploitation, modifications in biodiversity, climate change, and alterations in biogeochemical cycles (Vitousek et al., 1997). Particularly, the loss of biodiversity has been identified as one of the main threats and challenges for the coming decades (CBD, 2020). The current rate of species extinction is estimated to be 100 to 1,000 times higher than historical rates and it is estimated to further increase by 10 to 100 times over the next 50 years (Mace et al., 2005). These human impacts are causing dramatic declines in ecosystem health, compromising the functioning of the life-support system, the sustainment of economic activity, and animal and human health (Rapport et al., 1998). It is now clear that we cannot escape the responsibility and necessity of restoring and managing our planet. In this context, monitoring the health of ecosystems and wildlife populations is crucial for determining ecological dynamics, prioritizing active management, and assessing the efficacy of management interventions. Nevertheless, the majority of health assessments and symptoms of ecosystem dysfunction are retrospective. Despite years of research, we still rely heavily on alterations that manifest late in the disturbance process such as changes in animal abundance and richness (Ceballos et al., 2020), loss of interactions and functional diversity (Graham et al., 2006; Myers et al., 2007), decline in ecosystem services (Halpern et al., 2012) or pathogen emergence (Jones et al., 2008; Keesing & Ostfeld, 2012). We urgently need to develop reliable prospective indicators of wildlife and ecosystem health with early warning capabilities. One such promising indicator is the microbiome, described as the interacting community of commensal, symbiotic and pathogenic microorganisms within a body space or other environment, together with the whole spectrum of molecules that they produce and mobile genetic elements (i.e., phages, viruses, extracellular DNA) (G. Berg et al., 2020).

Microbiome reflects ecosystem disturbance

A growing body of research points out that microbiome provides a meaningful early indicator of ecosystem disturbance (Astudillo-García et al., 2019). First, microbiome is ubiquitous in terrestrial, freshwater and marine habitats, as well as in the animals and plants living in them. Therefore, unlike most traditional indicators (e.g., macroinvertebrate communities), microbiome approaches are potentially applicable to any ecosystem, including extreme or highly degraded environments. In ecosystems, environmental microbiome is a key component performing essential ecological processes such as primary production, nutrient cycling and retention, and influencing plant diversity and host-associated microbiomes (Sehnal et al., 2021; Sunagawa et al., 2015; Wagg et al., 2014).

Crucial for signaling ecosystem health, microbiome is highly sensitive to environmental disturbances, including anthropogenic impacts (Rocca et al., 2019). Recently, Rillig et al. (2019) showed that the complexity, composition and overall abundance of soil microbiomes declined along with the number of global change factors, including resource availability, abiotic factors, toxic compounds and microplastics. Soil microbiomes have also shown to shift in response to anticipated consequences of climate change, including elevated atmospheric carbon dioxide, elevated temperature, permafrost thawing, drought, floods, and wildfires (Jansson & Hofmockel, 2020). One of the primary anthropogenic stressors driving ecosystem degradation worldwide is habitat loss as a consequence of land use change (Millennium Ecosystem Assessment, 2005), which also influences the soil microbiome (Díaz-Vallejo et al., 2021). Detectable changes in environmental microbiomes of terrestrial, freshwater and marine ecosystems are also induced by pollution through chemicals and light (Premke et al., 2022), mine drainage (Jin et al., 2022), oil spills (Mugge et al., 2021), heavy metals (Dell'Anno et al., 2003), microplastics (Lear et al., 2021) and sewage waters from urban centers (Nogales et al., 2011). The latter is not only an example of pollution but also illustrates how the encroachment of human and domestic animal microbiomes and pathogens can alter environmental microbiomes. Biological invasions, either by microorganisms, plants or animals, are one of the main drivers of global change and generate, as well, shifts in the microbiome of native ecosystems (Malacrinò et al., 2020).

The examples above demonstrate that environmental microbiomes are generally not resistant to anthropogenic disturbances. However, microbiomes could be resilient to environmental perturbations, quickly recovering their initial composition and hampering our ability to detect transient shifts (Fig. 1). Encouragingly, Allison and Martiny (Allison & Martiny, 2008) showed that, within a few years, microbial composition still differed from that of undisturbed communities, thus allowing realistic sampling intervals for ecosystem monitoring. Furthermore, microbial communities are probably not functionally redundant and disturbances often impact the rates of ecosystem processes (Allison & Martiny, 2008). This suggests that we can detect shifts in community composition and function as two complementary axes of variation (Galbraith & Convertino, 2021). Overall, conditions altering the composition of environmental microbiomes may severely impact the structure, function and natural balance of ecosystems, and increase human and animal exposure to pathogens. Despite the evidence that microbiome can be used to detect change processes in ecosystems, its use as an indicator has mainly been evaluated for marine ecosystems, particularly for coral reef ecosystems (Aylagas et al., 2017; Glasl et al., 2019; Roitman et al., 2018). Therefore, microbiome still holds an unexplored diagnostic capacity to predict ecosystem alterations and guide management interventions, especially for terrestrial ecosystems.

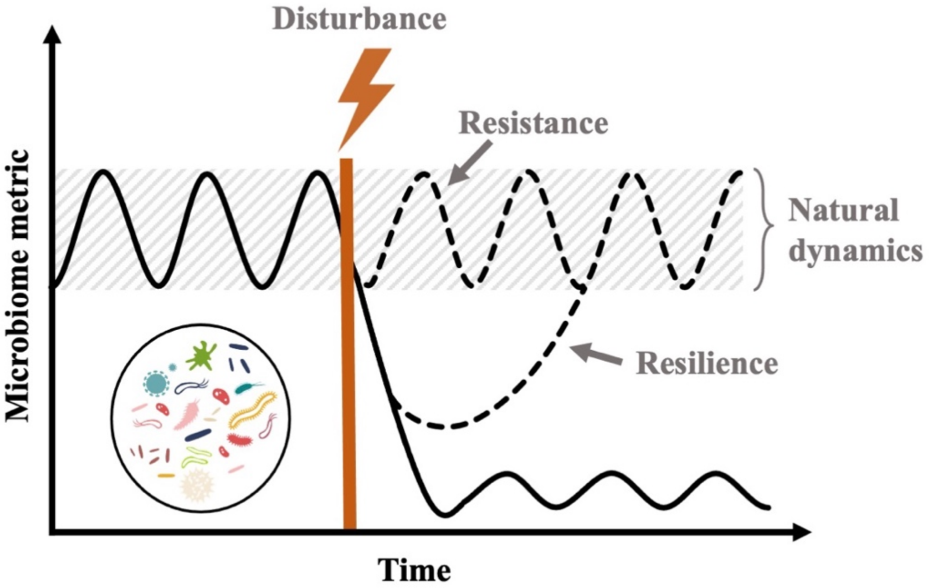


Figure 1. Graphical representation of microbiome dynamics. The horizontal streaky bar represents normal oscillations or baseline data over time for a particular microbiome metric (e.g., diversity). Disturbance events (orange vertical bar) can alter environmental conditions and trigger shifts in microbiome (solid black line), leading to alternative stable states that significantly deviate from the natural state. Resistance is the ability of a community to withstand a disturbance without change. Resilience is the rate at which the original state is regained after a disturbance event. Only after the establishment of these baseline intervals of normality, we can begin to understand microbiome responses to disturbance or stress. Adapted from Glasl et al., (2017).

Wildlife population health and bioindicator species

Tracking the microbiome of wildlife populations has potential human, animal and ecosystem health applications. In an era of global change, we are witnessing major outbreaks of emerging diseases in humans (Baker et al., 2022). The majority of emerging infectious disease threats originate from wildlife, and outbreak risk is increased by ecological degradation (Evans et al., 2020; Jones et al., 2008). Over recent years, some progress has been made in monitoring wildlife microbiomes in order to predict, prevent, and control zoonotic disease emergence. For example, in 2009, the U.S Agency for International Development (USAID) launched the Emerging Pandemic Threats Program (EPT-1) which has been followed by a new generation of investments (EPT-2, https://www.usaid.gov/ept2). Nevertheless, important steps still need to be taken to strengthen the monitoring of population trends and health for wild reservoir species, to identify novel infectious agents, and to better understand the links between ecosystem degradation and zoonotic spillover.

Concurrently, infectious diseases have been identified as a growing threat to wildlife conservation, contributing to the global loss of biodiversity (Cunningham et al., 2017; Daszak et al., 2000). Amphibian chytridiomycosis is perhaps the most striking example of the impact of diseases on biodiversity. Since its discovery in the 1990s, chytridiomycosis has been directly linked to the decline of over 500 amphibian species, the extinction of 90, and continues to threaten amphibian diversity globally (Scheele, Pasmans, et al., 2019). Disease threats to biodiversity share common anthropogenic drivers with disease outbreaks in humans (Daszak et al., 2000). Despite the need to monitor the health of declining wild populations, little progress has been made on the use of microbiome as an indicator of wildlife population health and most pathogen threats still remain undiscovered.

Microbiome has been linked to metabolic functions, aiding digestion, nutrition, development, and immune defenses in animal hosts (Sehnal et al., 2021; Turnbaugh et al., 2007; Williams et al., 2018). Shifts in microbial community composition may thus lead to dysfunction of host machinery, contributing to the development of a broad range of infectious and non-infectious diseases. Animals interact with their environment extensively and environmental characteristics strongly shape microbiome composition and influence microbiome functionality. Anthropogenic disturbances also leave traceable changes in hosts' microbiomes, suggesting that host-associated microbiomes may be realistic indicators of individual and population health in wildlife species. In amphibians, shifts in skin microbiome as a result of habitat degradation and environmental pollutants can increase the susceptibility and the outcomes of disease (Jiménez & Sommer, 2017). Similarly, impacts on the fecal microbiome of polar bears have been detected as a result of global change, suggesting that it could be incorporated into health assessments for conservation practices (Watson et al., 2019). In honey bees, changes in the structure and function of the gut microbiome were detected after pesticide exposure, which appeared to reflect the overall health of the colony (Kakumanu et al., 2016). Moreover, monitoring microbiomes could significantly improve ethical and practical aspects of wildlife sampling, replacing procedures such as physical and chemical restraint, blood sampling or tissue samples (e.g., toe clipping in amphibians). Indeed, microbiome studies can be performed in non-invasive samples, and in elusive, protected, too-large or too-small species (Sehnal et al., 2021). Importantly, these microbiome alterations seem to precede clinical manifestations of disease processes. For example, despite healthy appearance, coral microbiome communities under anthropogenic stress resembled those of diseased individuals, potentially being at a tipping point towards disease (Ziegler et al., 2016). This shows that wildlife microbiomes could serve as a reliable early warning system, advising management interventions before disease outbreaks or further biodiversity losses.

More broadly, wildlife microbiomes can also be used to signal ecosystem degradation and inform overall ecosystem health. Host-associated microbiomes are influenced by environmental conditions and can reflect stressors and processes of change. It is clear, however, that not all wildlife species are equally capable of signaling ecosystem health. Therefore, we need to identify suitable bioindicator and biomonitor species, which respond predictably to environmental change and quality (Markert & Wünschmann, 2011). Wildlife species that have disproportionate effects on the ecosystem relative to their abundance are interesting targets since they may respond earlier to changes. Coral microbiomes can be one such bioindicator/biomonitor of the health of coastal marine ecosystems, since corals are the backbone of reef systems, provide food and shelter for many species, and are tied to the changes in reef health (Roitman et al., 2018). The gut microbiome of several filter-feeding species is especially sensitive to the environment and also holds potential for aquatic ecosystems (Lawson et al., 2022). In terrestrial ecosystems, honey bee microbiomes have been suggested as good proxies for ecosystem health status (M. M. Cunningham et al., 2022). Other promising indicators are top predators or species with narrow ecological niches as they can indicate changes in the trophic network and respond rapidly to minimal shifts in habitat conditions (Watson et al., 2019). Bats are another interesting taxon that, despite having received great interest as reservoirs for zoonotic agents, have not been explored in ecosystem health assessments. Their diversity of species, broad distribution, feeding habits, and ability to fly point out their inherent capacity to signal ecosystem alterations. In fact, new studies demonstrate that the fecal bacterial microbiome of insectivorous bats can reflect habitat degradation and propose innovative uses of microbiome monitoring (Lobato-Bailón et al., 2023).

Future needs for microbiome studies

Since the advent of next-generation sequencing technologies, microbiome studies rely on metagenomics to collect taxonomic and functional data from microbial communities (Temperton & Giovannoni, 2012). These molecular technologies make studying microbial communities a cost-effective alternative to traditional methods and enable a more integrative approach. However, a careful balancing act between resource allocation, sample size and sequencing depth must be performed in order to achieve meaningful results. Firstly, a large amount of genetic material should be recovered in order to achieve a complete community sampling. With variable results, multiple buffer solutions and rapid freezing, often in liquid nitrogen, are the two most common methods implemented in order to avoid high degrees of nucleic acid degradation in fresh samples (Natarajan et al., 2021; Seelenfreund et al., 2014). Even in the best-case preservation scenario, partial fragmentation and loss of genetic material are inevitable at the nucleic acid extraction step regardless of the method (Ballare et al., 2019; Vo & Jedlicka, 2014); commercial kits are fairly standardized and scalable but provide moderate to low yields, while phase separation methods such as phenol-chloroform generate high yields but require manual precision and often carry contaminants to downstream processes. Secondly, sufficiently high sequencing depth must be met to provide a full recovery of the community, a need that rapidly increases with the amount and diversity of the target sequences (Gweon et al., 2019). The question of how much sequencing depth is enough is often difficult to answer, particularly for exploratory studies targeting low diversity taxa and those involving non-model organisms, given the lack of reference studies. Thus, sequencing the totality of the genetic material may only be suitable for select cases of high-quality, low-diversity samples with previous references. Despite its known methodological biases (J. P. Brooks et al., 2015), researchers have turned to selectively sequence a gene or small subset of genes which serve as a proxy for the entire community composition or a subset of its functionality. For instance, most taxonomic studies focus on deep amplicon sequencing of the 16S rRNA gene from prokaryotes, which nowadays can easily be recovered in its entirety through long-read sequencing technologies (Jovel et al., 2016; Kai et al., 2019). Contrastingly, viral diversity is often difficult to study, since viruses do not have a universal marker gene and are often found in low abundance in healthy individuals (Khot et al., 2020). Targeted sequencing of functional genes is also tricky, as there are not many standardized kits available and the scope of the intended scientific questions should be rather narrow by design. Recently, Nanopore adaptive sequencing emerged as a promising tool for the study of low abundance sequences through various enrichment approaches (Martin et al., 2022), although its full potential is still under study and development (Cheng et al., 2022; Danilevsky et al., 2022) (Fig. 2).

Besides methodological constraints, the use of microbiome indicators still entails some other relevant limitations. Microbiome responses to stress do not have a straightforward interpretation and we cannot infer health or disease based solely on the direction of the change (i.e. increases or reductions in abundance, diversity, and function). There is a growing consensus that occurrence and in some cases even degree of anthropogenic disturbance often manifests as one of the following microbiome alterations: 1) overall decrease in alpha diversity, 2) increase in beta diversity, 3) decrease in beneficial and/or increase in pathogenic or disease-associated bacteria (sometimes can occur along an increase in alpha diversity), 4) increase in the dominance of generalist over specialist bacteria and 5) increase in the abundance of microbial resistance genes (Amato et al., 2020; Bornbusch & Drea, 2021; Jiménez & Sommer, 2017; Malik et al., 2022; Murray et al., 2020; Preuss et al., 2020; Ramey, 2021; Rocca et al., 2019; Shi et al., 2019; Sugden et al., 2020; Wang et al., 2020). These parameters and changes may represent useful microbiome indicators of wildlife and ecosystem health. However, the magnitude and direction of these shifts are difficult to generalize across different ecosystems, resulting in both increases and reductions of particular species, microbial biomass and activity (Jansson & Hofmockel, 2020). A clear example is seen in arctic and sub-arctic ecosystems experiencing permafrost thawing where increases in alpha diversity are commonly reported (Jansson & Hofmockel, 2020; Seitz et al., 2022). Most probably, this means that we need to develop specific microbiome indicators and be aware of their particular responses in each ecosystem and species, and probably for each threatening process. Local changes imply that local authorities and environmental agencies should become more involved in microbiome studies, whereas now they mostly attract the interest of international institutions.

Microbiomes naturally are dynamic, their composition and function oscillate within certain intervals, even in undisturbed ecosystems and in healthy hosts (Faust et al., 2015). Therefore, we need comprehensive analyses of the temporal and spatial variation of each unique microbial community and the subsequent identification of baseline reference data in order to correctly interpret microbiome changes. Only after the establishment of these intervals of normality, we can begin to timely detect microbiome responses to disturbance or stress (Fig. 1). Considering that human impacts are widespread throughout the planet, are we too late to establish baseline data for the microbiomes of wildlife hosts and ecosystems? Probably. However, we can define health based on reference data by comparing different ecosystems and wildlife populations. Systems capable of maintaining ecological integrity, ecological processes and services can act as proxies of pristine and unperturbed communities, and will constitute our best-guess estimations for baseline microbiomes. Importantly, the dynamic nature of microbiomes also allows for signaling positive changes in ecosystem and wildlife health. Most scientific attention has focused on studying microbiome responses to disturbance; however, it may be equally valuable as an indicator of improvement in health parameters. Particularly, microbiome could help monitor the outcome of management interventions to restore ecosystem functionality or re-establish healthy wild animal populations. Finally, further challenges remain for the broadscale application of microbiome as a health indicator, particularly in remote areas or developing countries. Metagenomic tools require advanced technical and computational skills and, despite they have become cheaper over the past years, they still entail significant costs. Therefore, these methods may not be readily available in remote areas and can be prohibitively expensive in low-income countries (De León et al., 2023). Moreover, in order to detect changes in microbiome indicators, it is likely that long-term datasets are needed, requiring long-term commitment and funding, and being even more difficult to achieve in such regions. Unfortunately, most biodiversity hotspots and the highest rates of environmental change occur in such remote or economically poorer regions, hampering many real-world applications for microbiome studies.

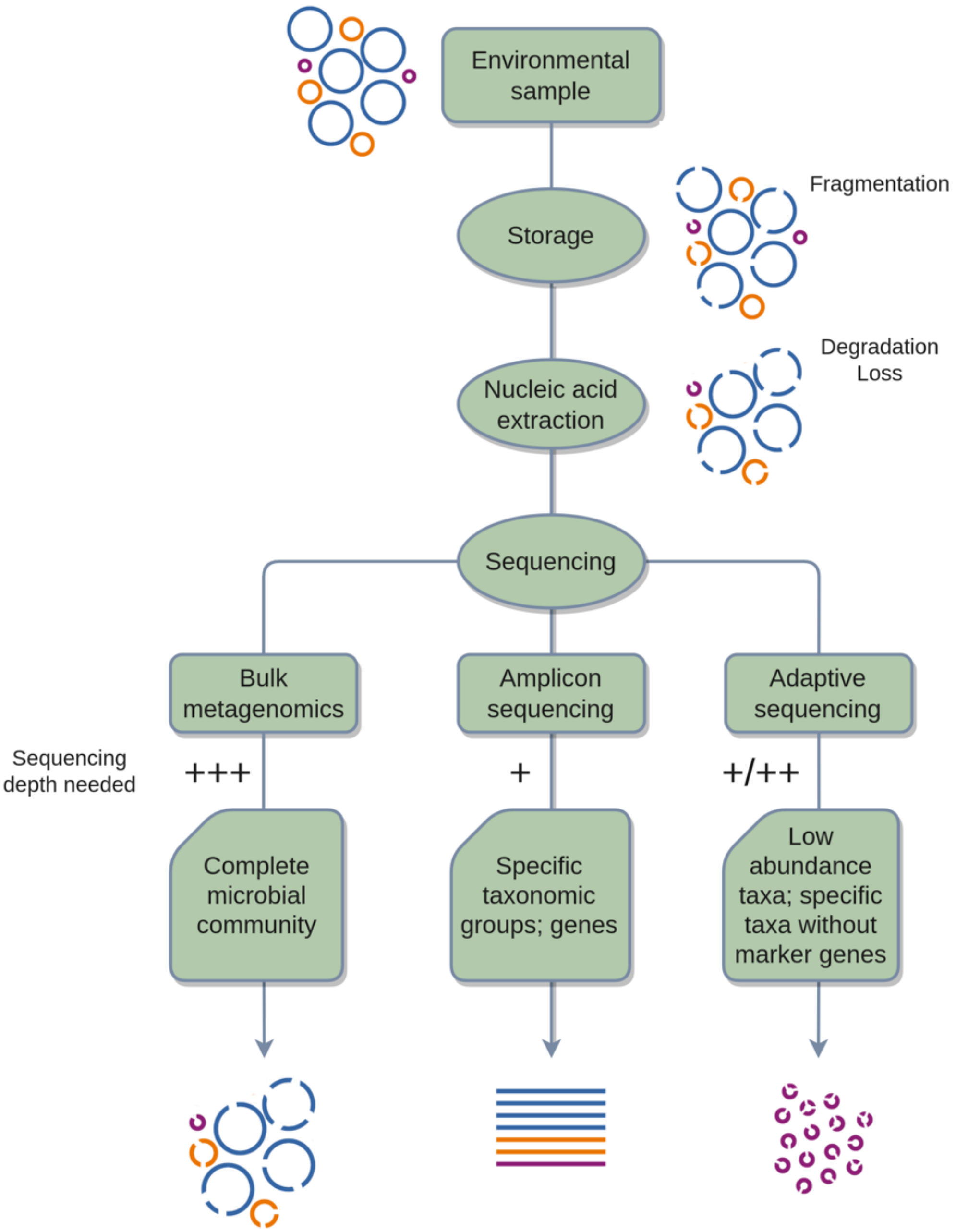


Figure 2. Common drawbacks and constraints in the collection of nucleic acid data from environmental samples. The composition of microbial community samples becomes altered when subjected to laboratory procedures. For instance, nucleic acid

material often gets fragmented when stored, and further degraded and partially lost during the extraction step. In exploratory studies, the decision of which type of sequencing to use is strongly constrained by the complexity of the sample and the resources available to afford the necessary sequencing depth to achieve meaningful results.

Conclusion

Human activities are having dramatic impacts throughout the planet and we urgently need reliable early-warning indicators for monitoring the health of wildlife populations and ecosystems. Microbiome, both environmental and host-associated, can be one such promising indicator since it is ubiquitous, performs key processes in ecosystems and hosts, and reflects anthropogenic disturbances. Changes in the microbiome tend to occur earlier in the disturbance process compared to common health indicators, allowing effective monitoring of wildlife and ecosystem health as well as the outcomes of management interventions. Nevertheless, microbiome potential as a health indicator remains largely unexplored in terrestrial ecosystems. Important limitations still remain for the widespread application of microbiome as a health indicator including biotechnological tools, interpretation of shifts in selected parameters, understanding baseline and altered microbiome dynamics, and logistical constraints. Overcoming current limitations will require establishing clear methods and best-guess baselines, increasing funding, and fostering international interdisciplinary collaboration. Future efforts in this direction will maximize the potential of microbiome for monitoring wildlife and ecosystem health.

Chapter 3.2

Skin microbiome variation among hosts and *Batrachochytrium dendrobatidis* infection in *Hyla meridionalis* and *Pelophylax perezi*

Maria Puig Ribas
Carla Tort
Mariette Viladomat Jasso
Lourdes Migura-Garcia
Oscar Cabezón

Under Review in Molecular Ecology

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), is a key driver of global amphibian declines. The amphibian skin microbiome, which may include Bd-inhibitory bacteria, plays a crucial role in defense against infection, influencing host susceptibility and disease outcome. In this study, we investigated the skin microbiome and Bd infection in two frog species, *Hyla meridionalis* (Hm) and *Pelophylax perezi* (Pp), from northeastern Spain through full-length 16S rRNA sequencing. We found that microbiome composition significantly differs between frog species, with Pp harboring greater bacterial richness. Asymptomatic Bd infection did not significantly alter microbiome diversity in either species, although specific bacterial taxa were differentially abundant in infected Hm. Interestingly, Hm showed a higher abundance of putative Bd-inhibitory bacteria despite carrying higher Bd loads than Pp, suggesting that the microbiome's protective role may not be straightforward. Lower pathogen loads in Pp hint at a potential link between microbial richness and disease resistance. Our findings indicate that amphibian skin microbiomes do not necessarily shift in response to Bd infection in asymptomatic hosts but may play a role in species-specific mechanisms of tolerance and resistance. Furthermore, we detected potentially pathogenic bacteria of public and animal health concern in amphibian skin, highlighting their role as zoonotic pathogen carriers. Our results contribute to understanding the variation of amphibian skin microbiome and its relationship with Bd infection, offering a foundation for probiotic bioaugmentation approaches to mitigate chytridiomycosis. Moreover, tracking amphibian microbiomes could serve as an early indicator of ecosystem health and emerging public health threats.

Introduction

Amphibians are the most threatened vertebrates on Earth, with over 41% of species at risk of extinction (IUCN, 2024). A major driver of global amphibian declines is chytridiomycosis, a disease caused by the chytrid fungi *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal)(Longcore et al., 1999; Martel et al., 2014). These multi-host pathogens have been linked to the decline of over 500 amphibian species and the extinction of 90, representing the greatest loss of biodiversity due to disease ever recorded (Scheele, Pasmans, et al., 2019). Nevertheless, the outcomes of exposure and infection vary significantly between host species, populations and life stages (Grogan et al., 2023). These differences are explained by environmental, pathogen and host factors. Among host factors, the immune response is key in determining variations in resistance and tolerance to pathogens.

The pathogenicity of chytrid fungi is based on their capacity to infect and disrupt the vital functions of amphibian skin. Consequently, the skin and its microbiome are the first line of defense and such constitutive immune components are crucial determinants of host susceptibility and disease outcomes (Bernardo-Cravo et al., 2020; Grogan et al., 2023). Amphibian skin microbiome can contain bacteria that repel, prevent colonization and/or inhibit chytrid fungi growth by producing antifungal metabolites (Grogan et al., 2018; Woodhams et al., 2015). Microbiota can also directly compete with chytrid fungi for resources, enhance host immune responses and support physiological functions (Grogan et al., 2018). Understanding the composition and variation of skin microbiomes among species and populations is, therefore, crucial for anticipating chytridiomycosis susceptibility and outbreak emergence. Additionally, identifying protective microbial communities may allow manipulation of the skin microbiome in susceptible amphibians. Probiotic bioaugmentation has been advocated as one of the few sustainable strategies for chytridiomycosis prevention and treatment, promoting amphibian conservation (Bletz et al., 2013; Harris, Brucker, et al., 2009; Harris, Lauer, et al., 2009; Kueneman et al., 2016).

Differences in the diversity and composition of skin microbial communities can be explained by both amphibian intrinsic and extrinsic characteristics (Reviewed by Bernardo-Cravo et al., 2020; Jiménez & Sommer, 2017). Intrinsic characteristics include host factors such as species identity, life stage, sex, genetics, immune status, behavior or diet. Extrinsic characteristics represent biotic and abiotic environmental traits including sympatric species and community composition, habitat or climatic conditions that serve as a source pool of bacterial exposure. In addition, habitat loss, exposure to pollutants or other anthropogenic disturbances are also known to alter skin microbiome, besides having other health and fitness consequences (Jiménez & Sommer, 2017). More generally, factors affecting the overall health status of the host, such as the presence of potential pathogens, can lead to shifts in skin microbiota.

As pathogens with a niche restricted to skin, chytrid fungi can conceivably disturb the diversity and composition of amphibian cutaneous microbiota. Previous studies found that Bd infection altered the skin microbiome of frogs under both experimental and field conditions (Jani & Briggs, 2014; Walke et al., 2015). By sampling pre-epizootic populations, Jani et al. (2017) showed that bacterial community composition in frog skin was more closely associated with Bd infection loads than with disease outcomes. The authors, therefore, suggested that differences in microbiome composition were not conferring prior resistance, and were a consequence rather than a cause of Bd establishment and load. Altogether, there is good evidence that Bd infection triggers detectable changes in skin microbiome composition and function in diverse amphibian species. However, distinguishing between microbiome-induced resistance and Bd-induced disturbance when evaluating differences in microbiome is not straightforward. Inferring cause or effect requires longitudinal studies replicated across multiple scenarios, and both responses are not mutually exclusive (Jani & Briggs, 2014).

Regardless of whether microbiome alterations precede or result from infection, studying the relationship between Bd and skin microbiomes in multiple amphibian species is essential to improve chytridiomycosis monitoring and management. Moreover, establishing baseline data and tracking wildlife microbiomes can have broader applications for human, domestic animal and ecosystem health (Ribas et al., 2023). Monitoring amphibian-associated microbiomes might help to foresee zoonotic disease outbreaks as amphibians can harbor pathogens significant to public health (Mettee Zarecki et al., 2013; Mitchell, 2011), and thrive in human-modified environments. Amphibians are highly sensitive to environmental change and monitoring their microbiomes has been proposed as an early indicator of ecosystem degradation and overall ecosystem health (Ribas et al., 2023).

In this study, we analyzed the skin microbiome of two European frog species, *Hyla meridionalis* (Hm) and *Pelophylax perezi* (Pp), inhabiting an urban environment in northeastern Spain using high-throughput amplicon sequencing of the full-length 16S rRNA gene. Our goals were to 1) describe and compare the diversity and composition of skin bacterial communities among frog species and environmental (water) microbiota, 2) evaluate the prevalence of putative Bd-inhibitory bacteria in both frog species, 3) analyze whether Bd infection is associated with variations in skin microbiome diversity and composition and 4) assess the presence of potentially pathogenic bacteria of human and animal health concern in amphibian skin. To our knowledge, this is the first study reporting Bd infection, describing skin microbiome and analyzing their associations in these species.

Methods

Study site

The amphibian species under study were based at Montjuïc Park (41º36’71’’ N, 2º16’47’’ E) in Barcelona city, Northeastern Spain. This urban habitat comprises a main pond connected to a series of 30 smaller ponds. All ponds are human-made and contain abundant aquatic vegetation, dominated by water lilies. Around the ponds, the park includes paved footpaths, grass pitches, and numerous tree, bush and plant species. The park sustains populations of three anuran species: *Hyla meridionalis* (Hm, Family Hylidae), *Pelophylax perezi* (Pp, Family Ranidae) and *Alytes obstetricans* (Family Alytidae). Bd infection has been previously detected through qPCR (Blooi et al., 2013) in post-metamorphic individuals of the first two species without clinical signs of disease (Ribas M.P., unpublished results).

Field sampling

Animal capture, manipulation and sampling protocols were approved by Departament d'Acció Climàtica, Alimentació i Agenda Rural (Generalitat de Catalunya, Spain) under the license SF/0144/24.

Field sampling took place on the 20th of May 2024, coinciding with the reproductive season of the study species and to limit the impact of seasonality on microbiome and infection data. We focused our study on adult individuals of two frog species: Hm and Pp. Frogs were located through calls and visual encounters. Hm have mainly an arboreal lifestyle and were captured by hand from the nearby vegetation, whereas Pp are generally aquatic species and were captured using dipnets. Each animal was contained in a new plastic bag and handled with a fresh pair of nitrile gloves to prevent pathogen transmission and sample contamination. We swabbed the skin of each frog in duplicate using sterile dry swabs (Snappable Ps + cotton, Deltalab) according to a standardized protocol that targets the ventral region and limbs (Hyatt et al., 2007). Frogs were thoroughly examined for signs of disease before being released back at their capture site. Skin swabs were placed in sterile 1.5 mL cryovials and stored within the first 6h after collection at -20ºC until DNA extraction.

To characterize the microbial community of the aquatic environment, we collected 200 mL of pond water in triplicate. Each sample was filtered through a 0.45 µm Whatman® filter and DNA was extracted within the first 24h.

Chytrid fungi diagnostics

We extracted DNA from the first skin swab using 65 µL of PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, USA) following Hyatt et al. (2007). Extractions were diluted 1:5 with nuclease-free water before qPCR assay to reduce the effects of potential inhibitors (Boyle et al., 2004; Hyatt et al., 2007). To determine chytrid infection status and quantify pathogen loads, we performed a duplex qPCR to simultaneously detect Bd and Bsal on each animal sample (Blooi et al., 2013). A synthetic double-stranded 750 bp gBlocks® (Integrated DNA Technologies, USA) gene fragment containing the target PCR sequences of both pathogens was used as a positive control and quantitation standard. Standard dilutions in log10 increments (10^{-1} to 10^{6} copies; in duplicate) were included in each qPCR plate to create a standard curve, allowing us to quantify the gene copy number in samples. Negative controls (nuclease-free water) were also included in each plate. A sample was considered positive if the gene copy number was above 1. To determine final pathogen loads, obtained copy numbers were multiplied by the dilution factor (i.e., 5). All qPCR assays were run on an Applied Biosystems™ 7500 Real-Time PCR System.

16S rRNA Amplicon sequencing and library preparation

We extracted DNA from duplicate skin swabs and filters using the QIAmp PowerFecal Pro DNA Kit (QIAGEN, Germany), following the manufacturer's instructions. The duplicate swab of 12 Bd-positive and 12 Bd-negative Hm, and 7 Bd-positive and 7 Bd-negative Pp was used for skin microbiome analysis. Negative samples included in the microbiome study were randomly selected from all qPCR negative swabs.

Bacterial 16S rRNA was amplified by PCR using the SQK-16S114.24 Barcoding Kit (Oxford Nanopore Technologies, ONT, UK) containing the 27F/1492R primer set, which enabled the amplification of the entire 1500 bp 16S rRNA gene. Each 50 µL reaction contained 25 µL of LongAmp Hot Start Taq 2x Master Mix (New England Biolabs, USA), 10 ng of genomic DNA sample adjusted to 15 µL with nuclease-free water and 10 µL of barcoded primers at 1 µM each. The temperature and cycling conditions were as follows: a denaturation step of 95 °C for 1 min; followed by 25 cycles at 95 °C for 20 secs, 55 °C for 30 secs and 65 °C for 2 min; and a final extension at 65 °C for 5 min. Sample DNA was quantified using a Qubit® 4 Fluorometer and the dsDNA High Sensitivity Assay (Invitrogen, USA) before and after PCR amplification.

Library was prepared following the manufacturer's protocol for the ONT SQK-16S114.24 Barcoding Kit. Briefly, 10 ng/µL of the full-length 16S product of each sample was used as starting material and the final library with 50 fmol of DNA was loaded on a new R10.4.1 flow cell (FLO-MIN114, ONT). Real-time base-calling was performed on MinION™ Mk1C with the MinKNOW™ software (v.24.02.16) (both ONT), with Q < 8 (default threshold) for data acquisition and basecalled with Dorado (v. 7.3.11)(ONT).

Bacterial community and statistical analyses

Nanopore raw reads were analyzed with Spaghetti pipeline (https://github.com/adlape95/Spaghetti). Adapters and barcodes were trimmed with Porechop (v 24.5.0), reads were filtered by length (1.200-1.800 bp) with Nanofilt (v.2.8.0) and quality check with Nanostat (v 1.6.0). Chimera removal was carried out with ycard (v 1.0.0) (Marijon et al., 2020) and taxonomic assignment with minimap2 (v. 2.26) (Li, 2021), using Silva 132 database. Finally, filtering and alignment were performed with Python scripts for obtaining taxonomy and abundance tables of single reads (included in the pipeline) (Latorre-Pérez et al., 2021). In addition, Nanopore data were collapsed to the final assignments and filtered with a minimum of 3 sequences represented in 20% of the samples.

Alpha and beta diversity were estimated by the number of single reads by phyloseq package (v. 1.46) (McMurdie & Holmes, 2013) and were studied at the genus level between sample types. Before estimation of the alpha diversity indexes, samples were rarefied to a depth of 85,511 reads to correct for the sequencing depth. We estimated the Alpha diversity of each sample by the observed feature index to represent richness and the Shannon index. Differences in alpha diversity measures between frog species and water were assessed using Kruskal-Wallis tests, followed by Dunn's post-hoc tests with Bonferroni correction. Within frog species, differences between Bd-positive and Bd-negative individuals were assessed using Wilcoxon rank-sum tests. We also analyzed the correlation between Bd loads and both alpha diversity variables using simple linear regression models and log-transformed pathogen loads (zero values excluded).

Beta diversity was represented by Principal Coordinates Analysis (PCoA) using the Bray-Curtis dissimilarity between samples. Permutational multivariate analysis of variance using distance matrices (PERMANOVA) test was performed with vegan package (v 2.4-6) (Oksanen & Stevens, 2007) to assess significant differences in microbial communities among host species and Bd infection status. Abundance profiles and taxonomic signatures were carried out with MicrobiomeStat package (v 1.2.0) (Lu et al., 2023). Differential analysis was performed to evaluate differential genera among amphibian species and Bd infection status within each species with DESeq2 (v 1.42) (Love et al., 2014), with post-counts as a method for estimation. Custom figures were created using ggplot2 (v. 3.5.1), ggrepel (v. 0.9.5), gridExtra (v. 2.3), plotly (v. 4.10.4) and ggsignif (v. 0.6.4). Core microbiome (taxa shared by 100% of samples) at the genus level was identified using a Venn diagram obtained through https://www.interactivenn.net. Water was not included in the beta diversity, differentially abundant taxa and core microbiome analysis due to the low sample replication.

Prevalence of Bd infection was estimated from the ratio of positive to the total number of samples with Wilson confidence intervals of 95%. Differences in prevalence among species were assessed using a Pearson Chi-squared test. Log-transformed Bd loads were compared among species using Student's t-tests. All data visualization and statistical analyses were performed using R version 4.1.4 (R Core Team, 2024) and significance thresholds set at $p \leq 0.05$.

Putative Bd-inhibitory bacteria analysis

Taxonomically assigned sequences from the Spaghetti pipeline were extracted for mapping against the database of putative Bd-inhibitory bacteria (Woodhams et al., 2015), using minimap2 with default parameters. The resulting files were filtered with a strict threshold to include only high-quality alignments, keeping only mapped sequences with a minimum alignment length of 500 bp, a minimum alignment of 80% of the total target sequences and a mapping quality score of 60 (highest mapQ).

The relative abundance and the proportion of taxa classified as 'putative Bd-inhibitory' were calculated for each animal and environmental (water) sample. We, then, assessed differences in the relative abundance and proportion of putative Bd-inhibitory taxa among sample types (amphibian species and water) using Kruskal-Wallis tests, followed by Dunn's posthoc tests with Bonferroni correction. Differences in infection status within frog species were assessed using Wilcoxon rank-sum tests. Additionally, we examined the relationship between Bd loads and both relative abundance and proportion of Bd-inhibitory bacteria variables using simple linear regression models and log-transformed pathogen loads (zero values excluded).

Potentially pathogenic bacteria analysis

Bacteria of public and animal health concern were selected from our reads database based on the bacterial diseases listed by the European Centre for Disease Prevention and Control (ECDC, 2024), the bacterial priority pathogens list and bacteria of potential health concern by the World Health Organization (WHO)(Lightfoot, 2003; WHO, 2024) and the World Organization for Animal Health list of notifiable diseases (WOAH, 2024a, 2024b) (Supplemental Information: Table S2). Barplots of the selected bacteria's relative abundance were built for Hm, Pp and water samples with R software using the above packages.

Results

We collected a total of 95 skin swabs in duplicate from the two frog species (Hm = 46 and Pp = 49). We found evidence of Bd infection via qPCR in 19 individuals, resulting in an overall infection prevalence of 20% ($CI_{95\%}$: 13.19-29.14), and species prevalence of 26.09% (12/46, $CI_{95\%}$: 15.60-40.26) and 14.29% (7/49, $CI_{95\%}$: 7.10-26.67) for Hm and Pp, respectively. Differences in Bd prevalence among amphibian species were not statistically significant. The median pathogen load was 39.25 copies (range 4.18 – 10,700.62 copies) and pathogen loads were significantly higher in Hm than in Pp ($p = 0.0082$, Supplemental Information: Fig. S1). All animals were Bsal-negative and evaluated as clinically healthy.

The entire 16S rRNA gene of the skin microbiota was sequenced for 38 individuals, 24 Hm (12 Bd-positive, 12 Bd-negative) and 14 Pp (7 Bd-positive and 7 Bd-negative), and 3 water samples (Supplemental Information: Table S1). Long-read sequencing samples using the Nanopore MinION platform produced an average of 7.58 Gb of estimated bases and 5,120,000 raw reads. After filtering and quality control, sequence length had an average of 1,485.40 bp and quality control of 10.23. Once the reads were aligned against the Silva reference database, a total of 3,182 taxa collapsed at the genus level. Furthermore, by filtering with a frequency greater than three and 20% prevalence among samples, a total of 500 taxa were obtained at the genus level. The percentage of taxonomic assignment at the genus level was an average of 99.3% between all three sample groups (Supplemental Information: Table S1).

Taxonomic composition

Proteobacteria was the most abundant phylum (> 86%) in all frog species, regardless of infection status, as well as in water samples (Fig. 1). Cyanobacteria, Firmicutes, and Bacteroidota were also present at high abundances across all groups, albeit to a different extent depending on the type of sample (0.26 - 4.11%). Interestingly, Campilobacterota was the second most abundant phylum in water (8.9%) but was only marginally found in Pp (0.75%) and Hm (0.02 %) samples.

At the genus level (Fig. 2), remarkable differences were observed among frog species. On one hand, *Erwinia* (23.63%), *Bordetella* (13.63%), *Achromobacter* (9.91%), *Advenella* (9.6%), *Pelistega* (7.26%) and *Pseudomonas* (7.05%) were the most abundant genera in Hm. On the other hand, *Pseudomonas* (31.91%) was the most abundant genus in Pp with all other bacterial genera found at less than 5% of relative abundance. Water samples showed a divergent pattern, being dominated by *Limnohabitans* (52.36%), followed by *Hydrogenophaga* (12.21%), *Pseudarcobacter* (6.92%), *Rhodoferax* (3.69%) and *Pseudomonas* (3.62%).

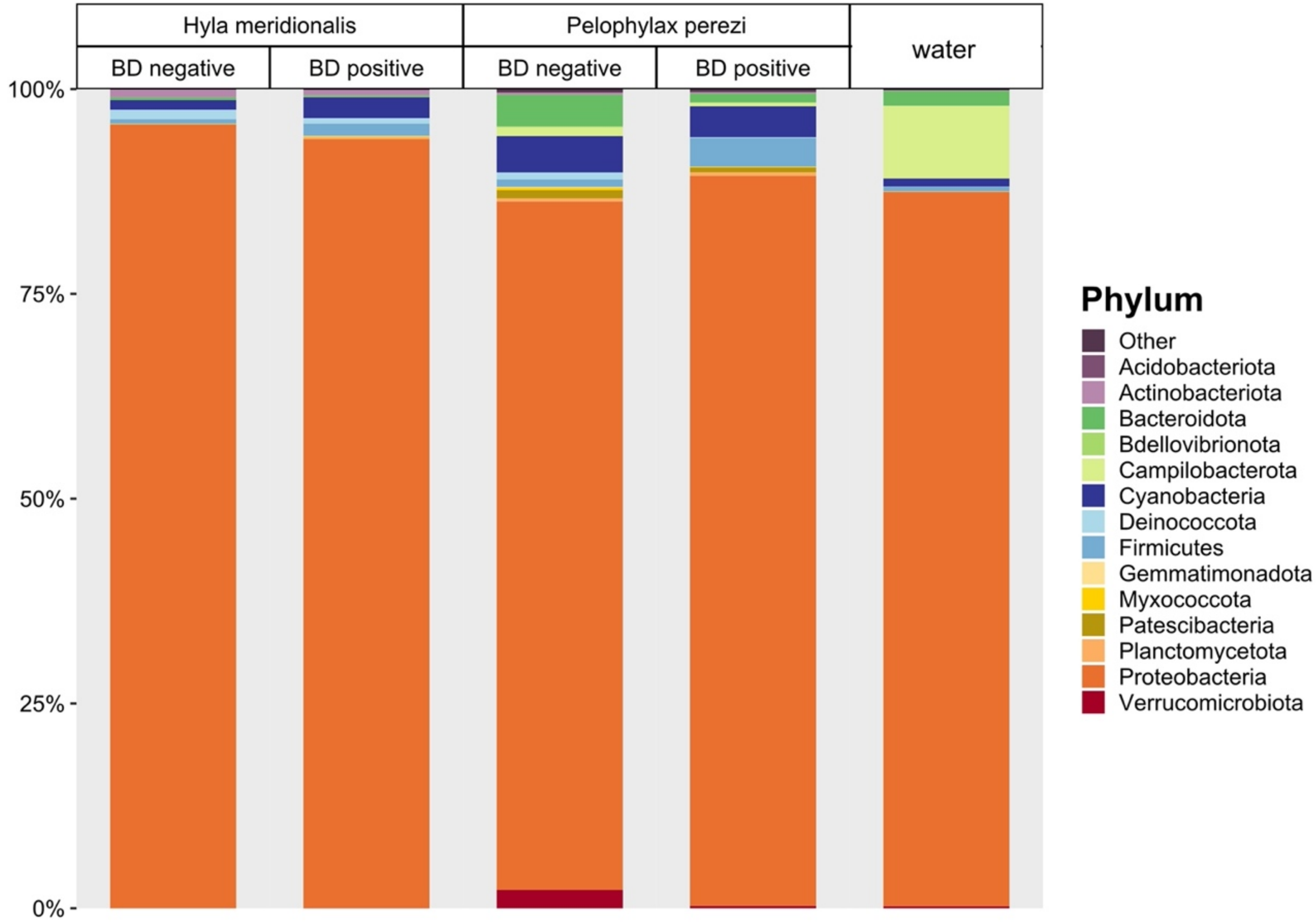


Figure 1. Relative abundance of the top 15 most abundant bacterial phyla across frog species (*Hyla meridionalis* and *Pelophylax perezi*), infection status (Bd negative and Bd positive), and in the water environment. BD = *Batrachochytrium dendrobatidis*.

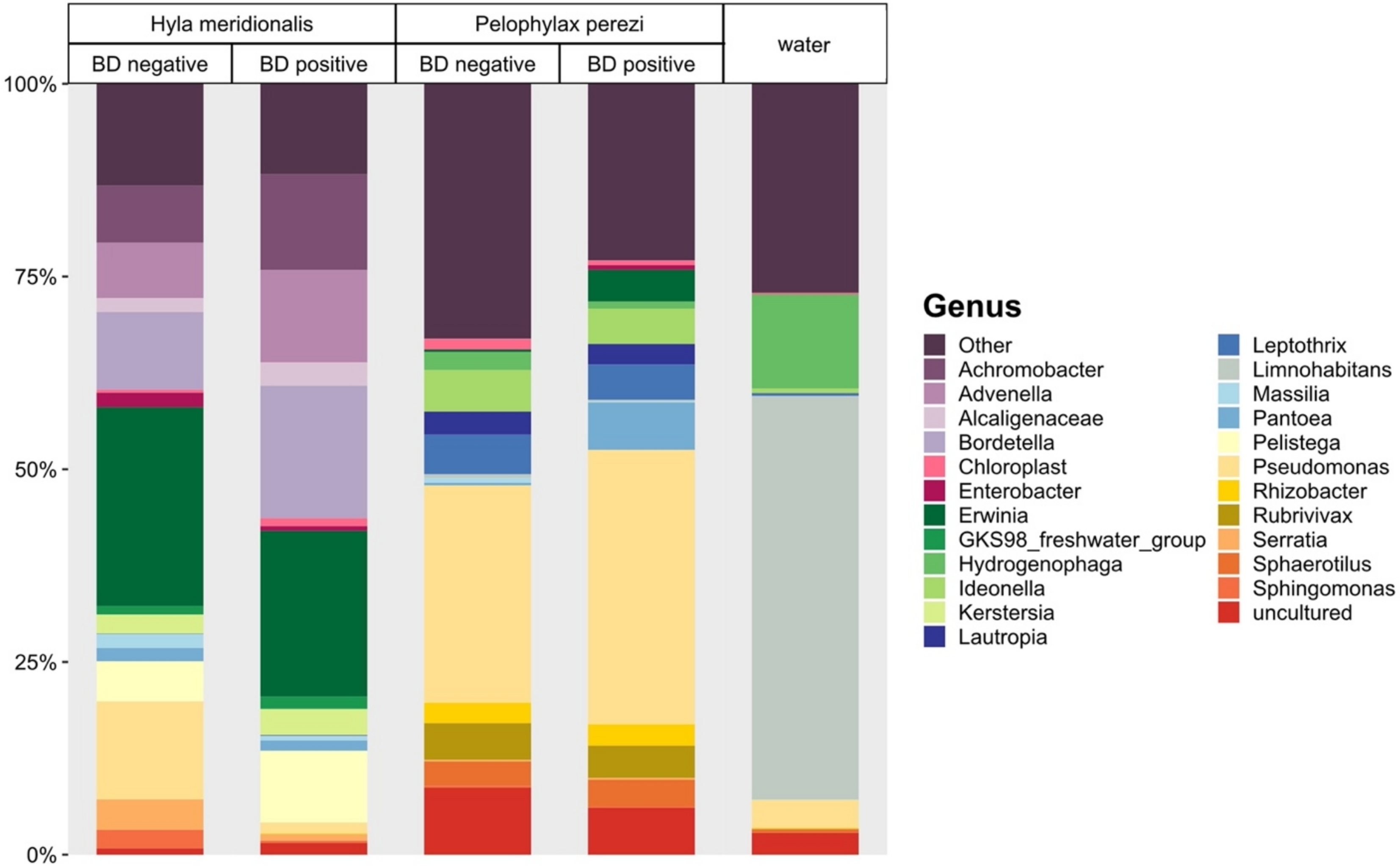


Figure 2. Relative abundance of the top 25 most abundant bacterial genera across frog species (*Hyla meridionalis* and *Pelophylax perezi*), infection status (Bd negative and Bd positive), and in the water environment. Bd = *Batrachochytrium dendrobatidis*.

Alpha diversity

Bacterial richness ranged from 92 to 664 reads per sample and Shannon index values from 0.0897 to 4.3132. Observed feature index was significantly different between Hm and Pp (Z = -2.7098, p = 0.0101) (Fig. 3). However, differences were non-significant between either frog species and water and when testing Shannon diversity indices. Alpha diversity measures were not significantly different between Bd-positive and Bd-negative individuals within frog species and were not correlated with Bd loads (Supplemental Information: Fig. S2 and S3).

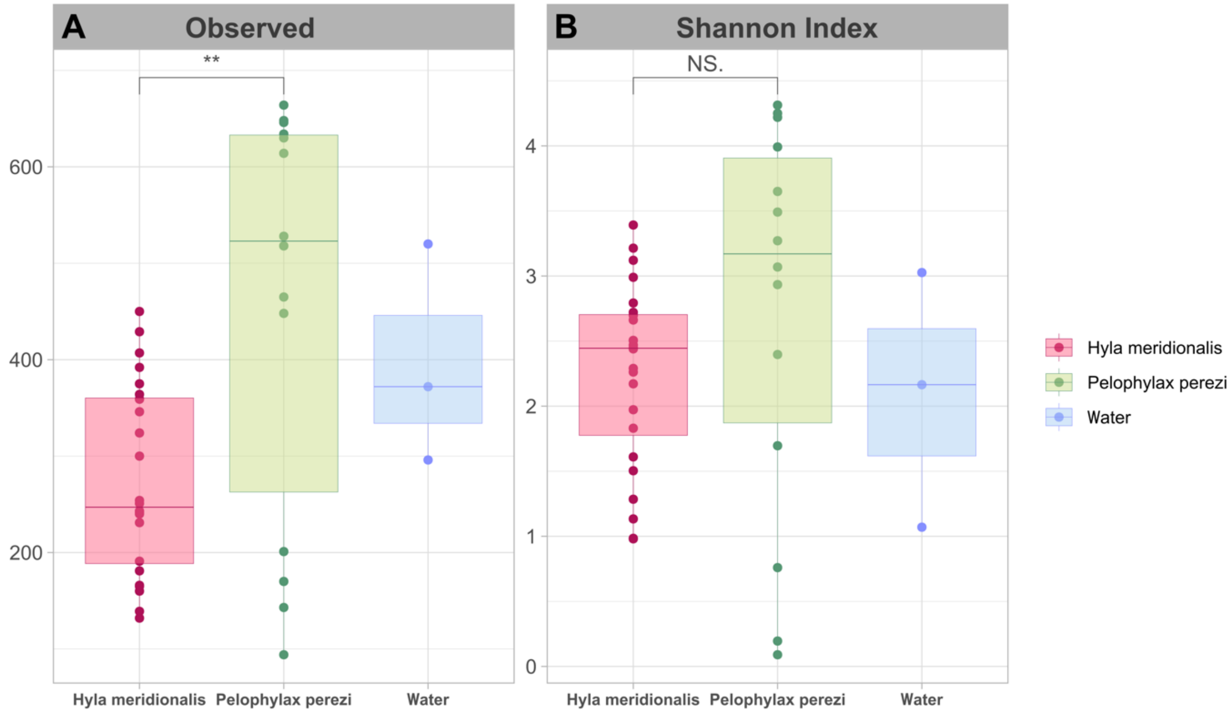


Figure 3. Alpha diversity measures of *Hyla meridionalis, Pelophylax perezi,* and environmental (water) samples at the genus taxonomic level. The observed feature index was used to measure bacterial richness (**A**) and the Shannon index was used as a combined measure of richness and evenness (**B**). ** = statistically significant differences ($p \leq 0.05$); NS = non-significant differences ($p > 0.05$) (Kruskal-Wallis rest).

Beta diversity

Beta diversity of bacterial communities was significantly different among frog species (PERMANOVA F(1,36) = 16.90, R^2 = 0.3195, p = 0.001)(Fig. 4). Frog species identity explained 31.95 % of the total variation in beta diversity. Despite not being included in the statistical analysis, environmental (water) samples consistently clustered together with Pp samples. Bd infection did not influence the composition of bacterial communities within either frog species.

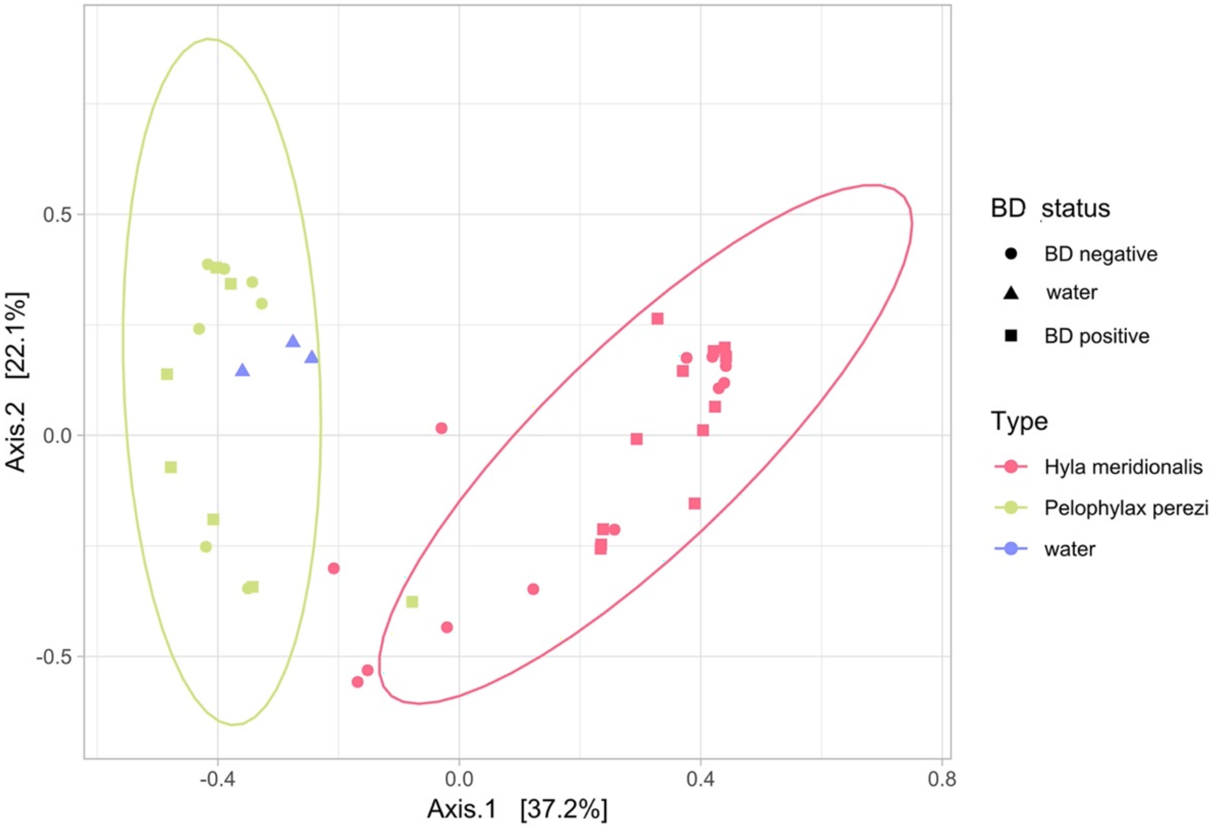


Figure 4. Principal Coordinate Analysis (PCoA) plot of skin bacterial communities of *Hyla meridionalis, Pelophylax perezi,* and environmental (water) samples, based on Bray-Curtis distances at the genus taxonomic level. Each point represents the bacterial community of an individual amphibian or a water sample. PERMANOVA results showed that species but not *Batrachochytrium dendrobatidis* infection status influenced microbiome beta diversity. BD = *Batrachochytrium dendrobatidis*.

Differential abundance analyses

Based on DESeq2 analysis, several bacterial genera were identified as being significantly different among frog species (Fig. 5), and Bd-positive and Bd-negative Hm (Fig. 6). In contrast, no differences were observed for Bd-positive and Bd-negative Pp. Among frog species, 228 bacterial genera were identified as the most significant contributors to explaining the observed microbial community. Of these, 130 were differentially more abundant in Pp, while 98 were more abundant in Hm. *Alcaligenaceae* (log2 fold change: 11.84), *Pelistega* (11.63), *Advenella* (11.48), *Achromobacter* (11.12), *Bordetella* (10.87), and GKS98 freshwater group (10.63) were the top differentially abundant genera in Hm. On the other hand, *Rubrivivax* (-8.15) and *Sphaerotilus* (-7.69) were the most differentiated genera in Pp. Within Hm, *Dongia* (5.27) and *Bacillus* (3.14) were more abundant in Bd-positive individuals; while *Vibrio* (-5.33), *Marinobacter* (-5.07), *Rickettsiella* (-4.85), *Psychrobact*er (-4.58), *Pseudomonas* (-4.52), *Tatumella* (-3.24) and *Chronobacter* (-3.13) were more abundant in Bd-negative animals.

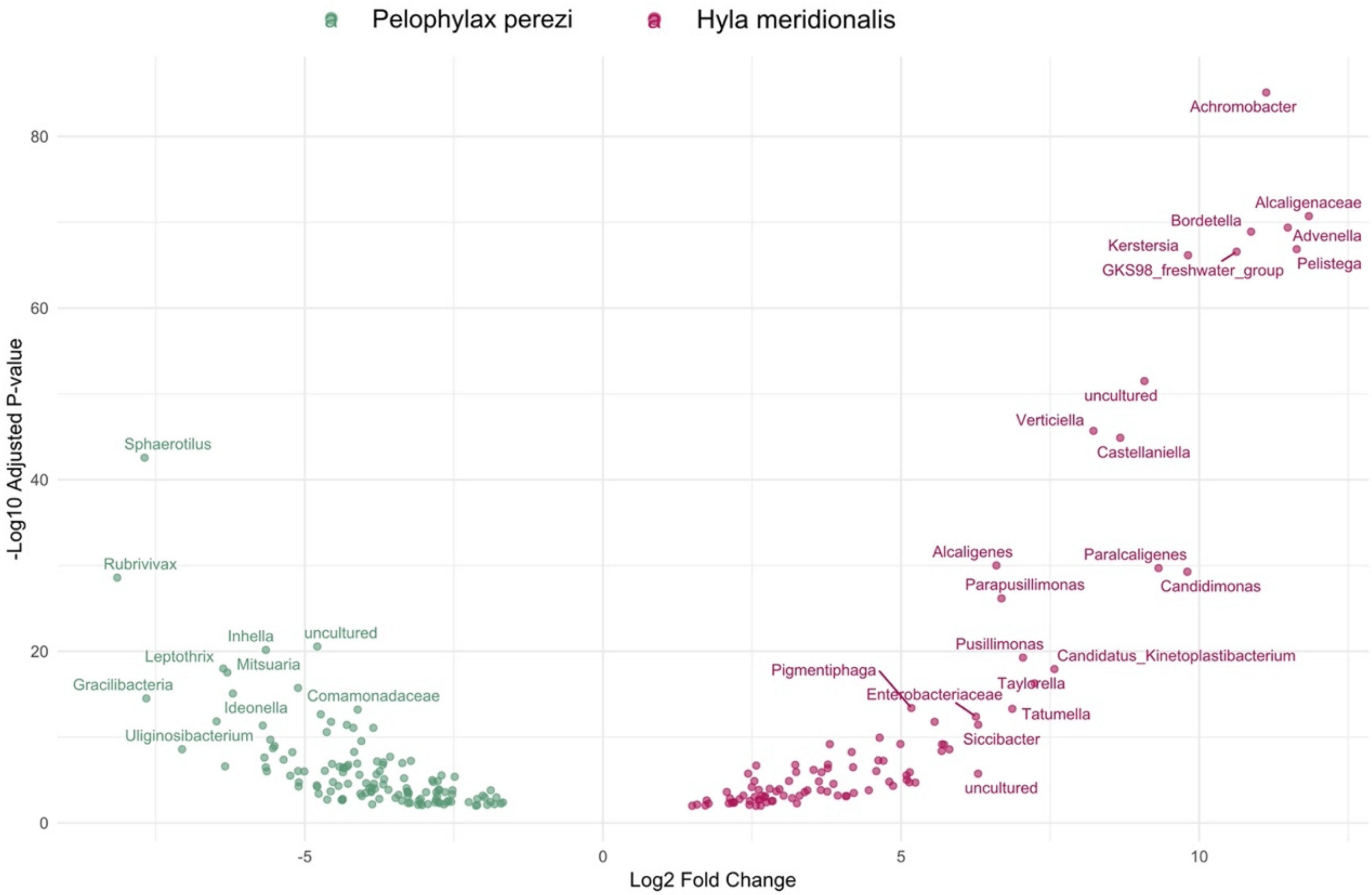


Figure 5. Volcano plot of significantly differentially abundant bacterial genera (p adj < 0.01) between *Hyla meridionalis* and *Pelophylax perezi*. The x-axis represents the magnitude of differences between the two species (Log2 Fold Change), with bacteria on the left being significantly more abundant in *P. perezi* and those on the right in *H. meridionalis*. The y-axis represents the statistical significance of these differences (-Log10 Adjusted P-value).

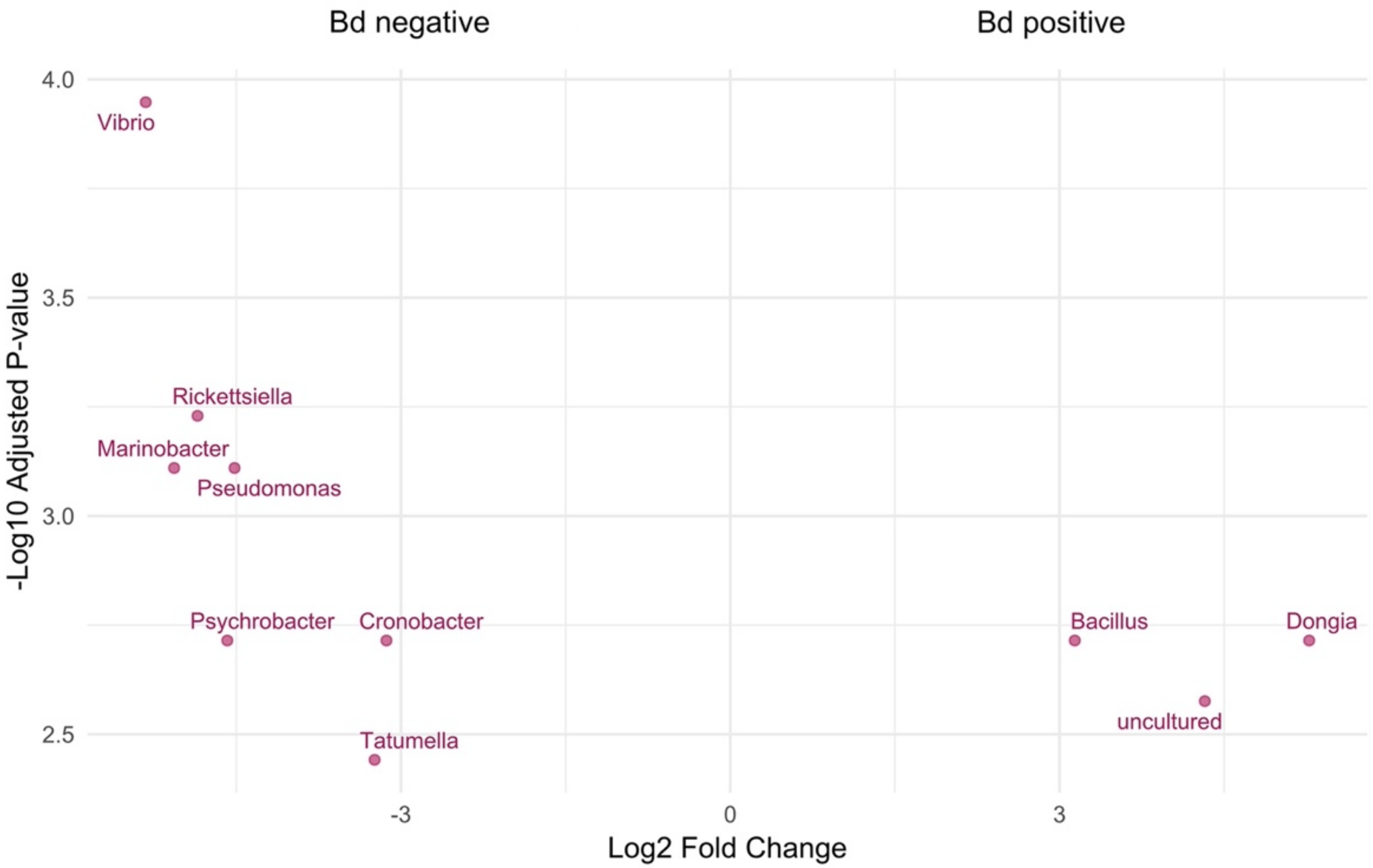


Figure 6. Volcano plot of significantly differentially abundant bacterial genera (p adj < 0.01) between *Batrachochytrium dendrobatidis* (Bd)-positive and Bd-negative *Hyla meridionalis*. The x-axis represents the magnitude of differences (Log2 Fold Change), with bacteria on the left being more abundant in Bd-negative and those on the right in Bd-positive *H.meridionalis*. The y-axis represents the statistical significance of these differences (-Log10 Adjusted P-value).

Core microbiome

Core community analysis showed 461 genera shared across all four sample types studied (Hm and Pp, Bd-positive and Bd-negative), while no genera exclusive to one scenario were detected (Fig. 7). Five genera were exclusive to Hm and four to Pp. No genera were exclusive to Bd-positive or Bd-negative individuals across frog species.

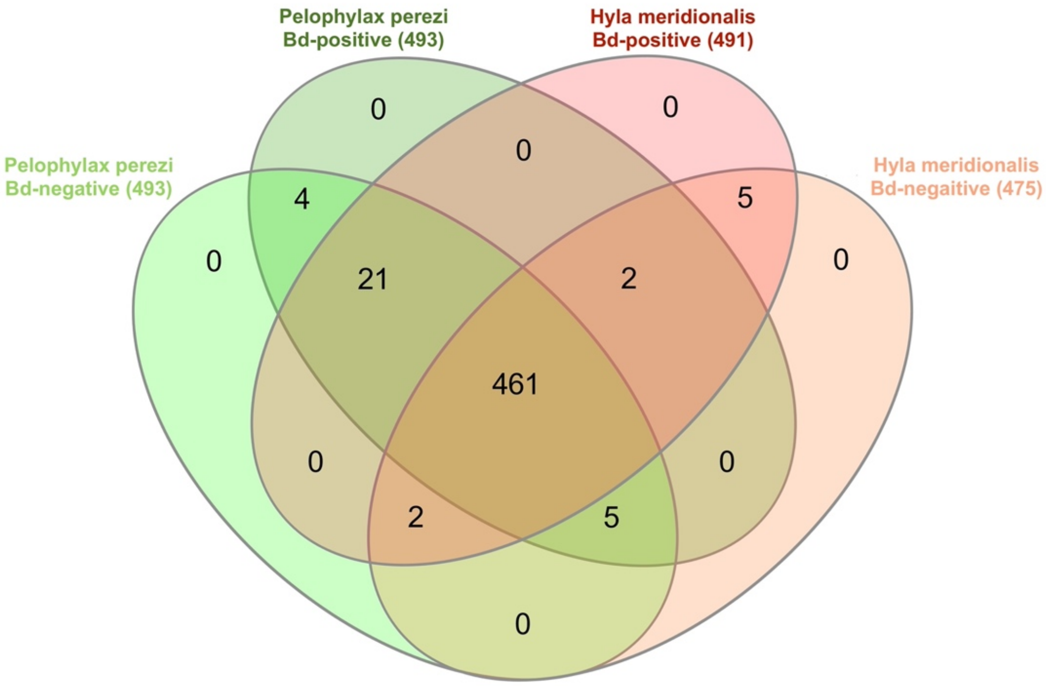


Figure 7. Venn Diagram of bacterial genera shared across and exclusive to each sample type. Sample types include frog species (*Hyla meridionalis* and *Pelophylax perezi*) and *Batrachochytrium dendrobatidis* (Bd) infection status (Bd-positive and Bd-negative), resulting in four scenarios studied.

Putative Bd-inhibitory bacteria

Most samples, including animal and environmental (water) samples, exhibited an approximate proportion of putative Bd-inhibitory taxa of 50%, but the relative abundance of these taxa varied across samples, from 0.70% to 99.78% (Fig. 8; Supplemental Information: Fig. S4). The relative abundance of Bd-inhibitory taxa was significantly higher in Hm than in Pp ($Z = 2.7734$, $p = 0.0083$). Conversely, no significant differences were found in relative abundance between either frog species and water and in the proportion of Bd-inhibitory taxa. Neither the relative abundance nor the proportion of putative Bd-inhibitory bacteria significantly affected the Bd infection status of the studied frog species. We identified a significant correlation between Bd loads and the relative abundance of Bd-inhibitory bacteria when both frog species were analyzed together (n = 19, $R^2 = 0.24$, F (1, 17) = 5.415, p = 0.0326); however, this association was not significant when analyzing the pathogen loads for each species separately (Supplemental Information: Fig. S5). The relationship between Bd loads and the proportion of Bd-inhibitory bacteria was not statistically significant in either analysis.

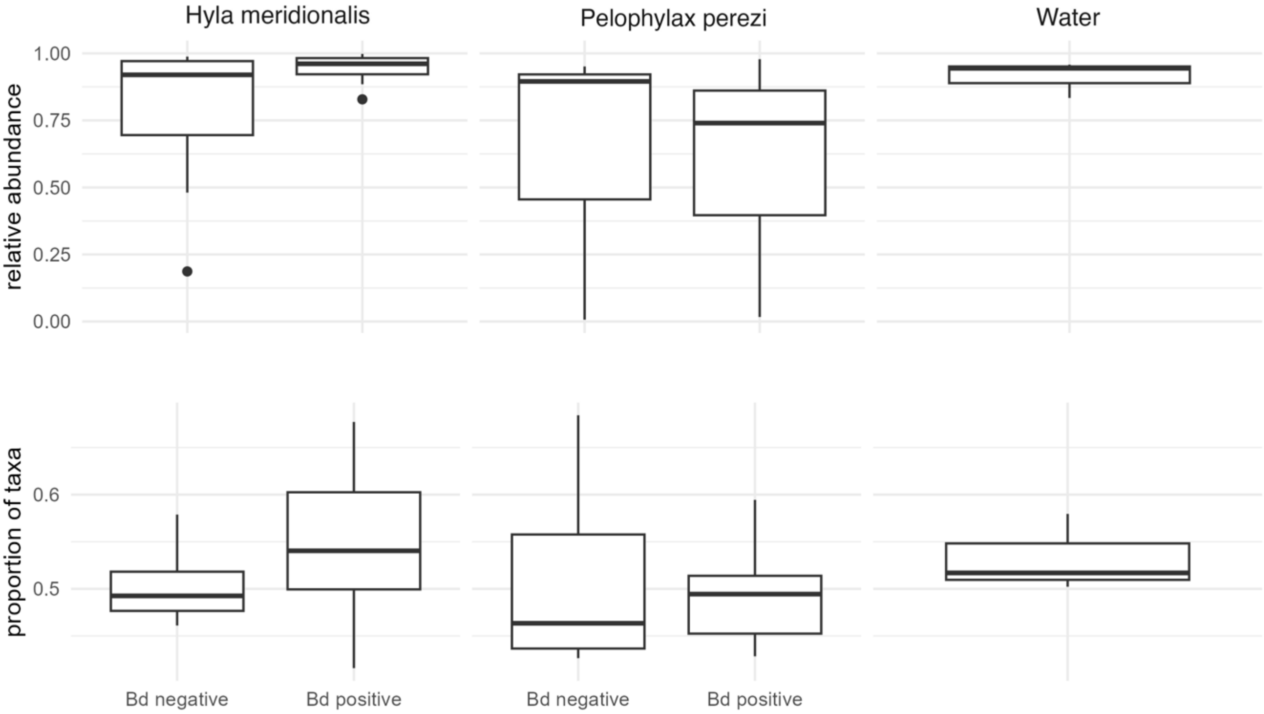


Figure 8. Boxplots showing the relative abundance (top) and proportion (bottom) of putative *Batrachochytrium dendrobatidis* (Bd)-inhibitory bacteria in the skin microbiome of Bd-positive and Bd-negative *Hyla meridionalis* and *Pelophylax perezi* and in environmental (water) microbiome. Significant differences are only observed in the relative abundance among amphibian species ($p < 0.05$).

Potentially pathogenic bacteria

We identified 26 potentially pathogenic bacterial species in our study samples, as well as unassigned species from the genera *Aeromonas, Bordetella, Citrobacter, Clostridium, Enterobacter* and *Serratia*. Potentially pathogenic bacteria represented 8.68% of Hm skin microbiome, whereas only 1.54% of Pp skin microbiome and 0.24% of water microbiome (Fig. 9). The most abundant bacteria in Hm were *Bordetella sp.* (4.06 %), *B. pertussis* (1.52%) and *Serratia fonticola* (1.13%), followed by *Enterobacter sp.* (0.44%)*, Serratia sp.* (0.43 %) and *B. bronchiseptica* (0.3%). Conversely, the most abundant bacteria in Pp were *Clostridium sp.* (0.62%) and *Aeromonas veronii* (0.31%), with all other species <0.2% of relative abundance. For water, *Aeromonas veronii* was the most abundant species (0.11%), followed by other *Aeromonas spp.* (all <0.04% of relative abundance).

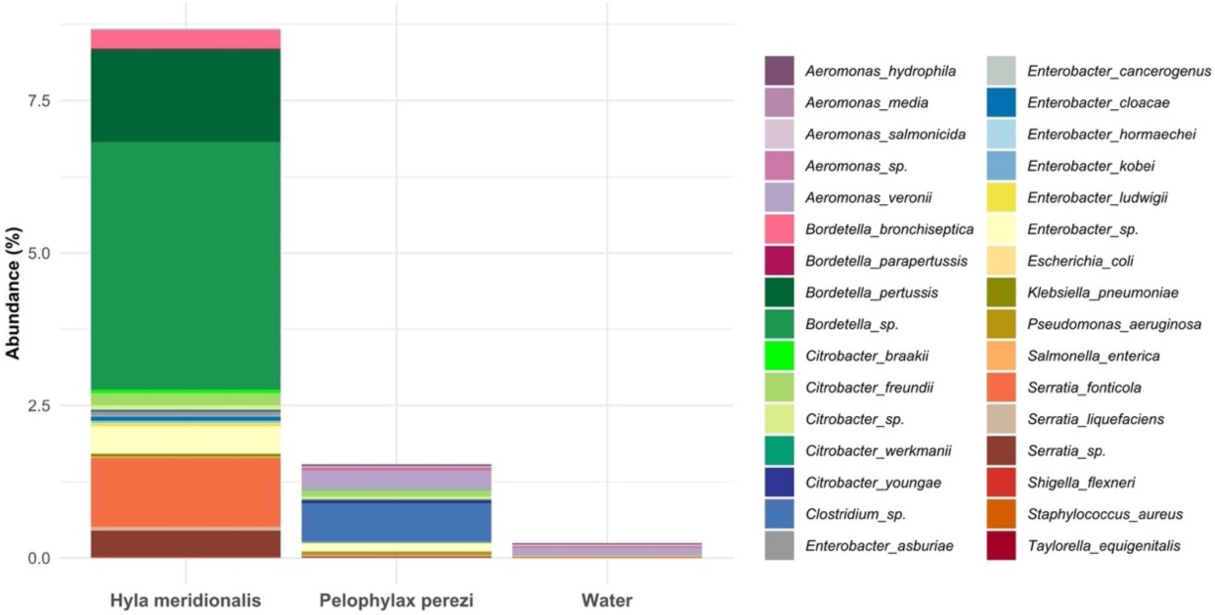


Figure 9. Relative abundance (%) of the potentially pathogenic bacteria identified in the skin microbiome of *Hyla meridionalis* and *Pelophylax perezi,* and in environmental (water) samples.

Discussion

In this study, we evaluated the variation in skin microbiome among two European frog species and its relationship with asymptomatic Bd infection. Although high-throughput sequencing techniques are increasingly used to investigate amphibian skin microbiome, most studies focus on tropical species (Abarca et al., 2018; Bletz et al., 2017; Jiménez et al., 2019; Mutnale et al., 2021; Rebollar et al., 2016; Schmeller et al., 2022), or temperate species from North America (Goodwin et al., 2022; Kruger, 2020; Kueneman et al., 2014; Muletz Wolz et al., 2018; Ramírez-Barahona et al., 2023). Many of these studies are starting to focus their attention on non-susceptible hosts or populations in coexistence with Bd. Nevertheless, little is known about skin microbiome variations among European amphibian species and in response to Bd infection, with studies only conducted on a single highly susceptible species (Bates et al., 2022). For the first time, we report Bd infections without disease or mortality in Hm and Pp from northeastern Spain. By exploring the differential responses to asymptomatic Bd infection and variations in skin microbiome among these species, we contribute to a better understanding of the microbiome's protective role in chytridiomycosis.

Skin microbiome varies with host species

We found distinctly different skin microbiomes between sympatric Hm and Pp. In agreement with other studies, we show that the skin bacterial communities of our study species were replete with Proteobacteria, followed by Firmicutes and Bacteroidota (Abarca et al., 2018; Bletz et al., 2017; Kueneman et al., 2014; Schmeller et al., 2022; Smith et al., 2023). Moreover, the relatively high abundance of *Pseudomonas*, particularly in Pp, is congruent with the findings reported f other frog species around the world (Bletz et al., 2017; Kueneman et al., 2014; Schmeller et al., 2022). While few bacterial taxa were exclusive to each species (5 genera exclusive to Hm and 4 to Pp), we show significant differences in alpha diversity, beta diversity and differentially abundant taxa among frog species. This provides additional evidence that host species identity plays a crucial role in shaping the skin microbiome's community composition (Abarca et al., 2018; Kueneman et al., 2014; McKenzie et al., 2012; Rebollar et al., 2016).

Nevertheless, microbiome variation among species may be more influenced by host ecology, behavior and microhabitat selection than by host phylogeny (Bletz et al., 2017; Smith et al., 2023). Microhabitat preferences may expose amphibians to different environmental microbial pools, which affect the colonization and maintenance of the skin microbiota. Across studies, arboreal frog species appear to have the least bacterial diversity (alpha diversity metrics) compared to terrestrial or aquatic species (Bletz et al., 2017; Kueneman et al., 2014). Similarly, our aquatic species, Pp, harbored significantly greater bacterial richness than the arboreal Hm. Although water was included in the statistical analysis, the influence of the environmental pool appears evident in our beta diversity study (Fig. 4), where water samples consistently cluster together with Pp.

The differentially abundant genera in each frog species also suggest a substantial influence of microhabitat on skin microbiota. In Hm, the main significantly enriched genera belonged to the family Alcaligenaceae, which has consistently been associated with arboreal frog species (Bletz et al., 2017). Members of this family are found in water, soil and animal samples, but there is evidence that genera such as *Achromobacter* and *Advenella* have evolved in close association with plants (Kuzmina et al., 2022; Nascimento et al., 2021). This could explain their enriched abundance in the skin microbiota of arboreal frogs. Conversely, the differentially abundant genera *Sphaerotilus* and *Rubrivivax* in Pp belong to the family Comamonadaceae. This family was also abundant in the water samples of our study (genera *Limnohabitans* and *Hydrogenophaga*), reflecting the aquatic lifestyle and close association of Pp with stagnant water.

Both host and environmental factors probably drive the community composition of the skin microbiome of amphibians. Unfortunately, our study design did not allow us to investigate the influence of large-scale climatic and ecological variation. Our study species are broadly distributed across northeastern Spain, as well as other regions of the Iberian Peninsula and France. Replicating sampling in different geographic locations and habitats in the future would allow the examination of patterns in community composition across sites and elucidate the natural variation in skin microbiome within species. Additionally, environmental sampling should be extended to include various sample types (e.g. water, sediment, soil) and measurements to assess their relationship with host microbiomes. Regardless, our study lays the foundation for future research on skin microbiomes of European amphibian communities.

Asymptomatic Bd infection is not associated with major shifts in skin microbiome

Neither Bd infection status nor infection loads were linked to major differences in the cutaneous bacterial community composition of our study species. This was consistent across microbiome metrics, including alpha and beta diversity and core microbiome analyses. While differential abundance analysis was also unremarkable within Pp, significantly enriched bacterial taxa were identified between Bd-positive and Bd-negative Hm. However, these differential genera do not appear to follow a distinct pattern of characteristics that would explain their abundance in either infected or uninfected amphibians. Furthermore, we observed no differences in putative Bd-inhibitory bacteria among infected and uninfected individuals within a frog species. Overall, skin microbiome showed little variation that could explain differential risk or response to Bd infection at an individual level. Other studies found a similar lack of association between Bd infection and bacterial communities in species with asymptomatic infections or populations with a long history of pathogen exposure (endemic infections)(Belden et al., 2015; Kruger, 2020; Longo et al., 2015). Given that both host-associated microbiomes and Bd infections are dynamic throughout seasons and over time (Estrada et al., 2019; Longo & Zamudio, 2017), longitudinal studies may be necessary to provide greater insight into these relationships.

On the other hand, variations in microbiome composition may explain distinct responses or defense mechanisms against Bd infection among host species. Animal defenses against infection involve two complementary mechanisms: resistance and tolerance. Resistance refers to the host's ability to limit pathogen establishment or infection loads, whereas tolerance defines the ability to minimize the resulting pathology. Although Bd infections were asymptomatic in both of our study species, Pp harbored significantly lower pathogen loads than Hm. Moreover, despite aquatic species being generally more exposed to Bd infectious stages

(Burrowes et al., 2017; Scheele et al., 2019), we observed similar prevalence in both species. Altogether, this suggests that Pp exhibits greater resistance to Bd, while the high pathogen loads coupled with the absence of pathology in Hm point towards a greater tolerance. Differences in resistance and tolerance among amphibian species can arise from variations in immune function, including both innate and acquired immune components (Grogan et al., 2018). Skin microbiome is part of the constitutive innate immunity against Bd and field studies have demonstrated consistently different skin microbial communities between Bd susceptible and resistant/tolerant species (Grogan et al., 2018; Rebollar et al., 2016). Notably, skin microbiome has been proposed as a major determinant of species resistance, as certain bacteria can prevent colonization and inhibit Bd growth (Grogan et al., 2023).

One characteristic of the skin microbiome that may account for the higher resistance of Pp to Bd infection observed in our study is bacterial richness. Richer microbial communities are generally considered more resistant and resilient to pathogen invasion (Lozupone et al., 2012; Rocca et al., 2019). This principle also seems consistent in Bd-amphibian systems, with studies demonstrating a correlation between higher microbial richness and reduced Bd growth or enhanced host-pathogen coexistence (Jani et al., 2017; Piovia-Scott et al., 2017). Higher microbial richness might confer resistance by increasing competition for resources and space with Bd and/or due to a greater probability that key protective bacteria are present. However, we would also have expected a greater proportion or abundance of Bd-inhibitory bacteria in Pp, whereas our results show that these bacteria are significantly more abundant in Hm. Although this pattern may be in response to Bd colonization as a mechanism to fight infection (Muletz-Wolz et al., 2019; Walke et al., 2017), the lack of correlation with Bd loads observed in our study suggests baseline differences in the skin microbiome among these frog species rather than supporting this hypothesis. More likely, our results highlight the limitations of using 16S rRNA sequence similarity to predict Bd-inhibitory function. Woodhams et al. (2015) database is based on antifungal properties detected on bacteria cultured *in vitro* but this may not reflect the *in vivo* expression of inhibitory functions. Further work will be required to determine how the putative Bd-inhibitory bacteria identified in the present study interact with Hm and Pp in their natural environments.

Amphibian skin microbiome contains potentially pathogenic bacteria

By specifically searching for selected bacteria species, we show that amphibians can harbor important potential pathogens of human and animal health concern in their skin microbiome. Moreover, these bacteria appear in greater abundance in amphibian hosts than in environmental samples (water), highlighting their potential role as carriers. Of particular concern is our finding that Hm carries *B. pertussis* and *B. parapertussis* which can cause significant morbidity and mortality in children (Nieves & Heininger, 2016). Nevertheless, most microbiome studies in amphibians do not report the presence of such pathogens as they are solely focused on amphibian health. Many amphibians thrive in urban and suburban environments (Hamer & McDonnell, 2008; Konowalik et al., 2020), increasing the opportunities for exposure to human and domestic animal pathogens. Although amphibians may not be the primary hosts for such pathogens, they may act as reservoir and spill-back hosts. Overlooking the close interconnection between wildlife, domestic animals and human health can hamper our ability to prevent and mitigate emerging diseases. Based on our findings, we propose that amphibians can serve as indicators of potentially pathogenic bacteria and anthropogenic disturbances in ecosystems. Therefore, monitoring amphibian-associated microbiomes can offer significant benefits beyond improving amphibian health. We strongly encourage future studies to specifically document the presence of potential pathogens to better understand and mitigate risks for wildlife, domestic animal and human populations.

Conclusions

In this study, we describe for the first time the skin microbiome and its relationship with Bd infection in Hm and Pp, two widely distributed amphibian species in Western Europe. Understanding the microbial communities in different species and regions, as well as the intrinsic and extrinsic factors that drive variation is the first step towards developing strategies to mitigate chytridiomycosis. We show that skin microbiome diversity and composition vary between our two frog species but not with Bd infection in asymptomatic hosts. Moreover, we begin to elucidate the role of Hm and Pp on chytridiomycosis dynamics by comparing their relative resistance and tolerance to infection, linking these differences to variations in microbiome diversity and composition. Given the protective role of skin microbiome in chytridiomycosis, the study of resistant and tolerant host species may be an effective approach to identifying key bacteria taxa for probiotic candidate selection and bioaugmentation approaches in succumbing species. Additionally, we highlight the utility of tracking amphibian microbiomes as indicators of ecosystem health and emerging public health threats. Further research should expand on the factors driving variations in amphibian skin microbiome, test the Bd-inhibitory function of different bacterial taxa in amphibian hosts and natural environments through transcriptomic approaches, and develop microbial therapies for species threatened by disease.

Data Availability

Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the primary accession code PRJNA1123624. Access link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1138540?reviewer=1buf0losg5r4rkaoj8a5dl78ir.

Full pipeline to perform the putative Bd-inhibitory bacteria analysis using 16S rRNA complete gene data sequenced through nanopore can be found at: https://github.com/MarietteViladomat/Amphibian-Skin-Microbiome-Bd-Inhibitory-taxa.

We have shared the rest of our data as Supplemental Information.

Supplementary Information

Table S1. Samples used for microbiome analysis. For *Hyla meridionalis* and *Pelophylax perezi* skin swabs were collected. and the presence of chytrid fungi DNA and skin microbiome were assessed. Infection loads are expressed as gene copy numbers. Environmental (water) microbiome was analyzed from filtered water samples. which were not tested for chytrid fungi presence. Accession numbers correspond to the sequence data deposited in National Center for Biotechnology Information.

ID	Species	Bd Status	Bd Load	Accession number
sample_02	Hyla meridionalis	Negative	0	SRR29928479
sample_04	Hyla meridionalis	Negative	0	SRR29928508
sample_09	Hyla meridionalis	Positive	208.297552	SRR29928486
sample_10	Hyla meridionalis	Positive	11.1741049	SRR29928513
sample_13	Hyla meridionalis	Negative	0	SRR29928512
sample_15	Hyla meridionalis	Negative	0	SRR29928501
sample_19	Hyla meridionalis	Negative	0	SRR29928490
sample_22	Hyla meridionalis	Negative	0	SRR29928477
sample_23	Hyla meridionalis	Negative	0	SRR29928476
sample_25	Hyla meridionalis	Negative	0	SRR29928475
sample_26	Hyla meridionalis	Negative	0	SRR29928474
sample_28	Hyla meridionalis	Negative	0	SRR29928473
sample_32	Hyla meridionalis	Negative	0	SRR29928511
sample_34	Pelophylax perezi	Negative	0	SRR29928510
sample_38	Pelophylax perezi	Positive	20.8998049	SRR29928509
sample_42	Pelophylax perezi	Positive	124.848497	SRR29928507
sample_43	Pelophylax perezi	Negative	0	SRR29928506
sample_47	Pelophylax perezi	Positive	12.8833937	SRR29928505
sample_55	Pelophylax perezi	Negative	0	SRR29928504
sample_60	Pelophylax perezi	Negative	0	SRR29928503
sample_65	Hyla meridionalis	Positive	4.18228161	SRR29928502
sample_66	Pelophylax perezi	Negative	0	SRR29928500
sample_68	Pelophylax perezi	Positive	4.55619614	SRR29928499
sample_69	Pelophylax perezi	Negative	0	SRR29928498
sample_71	Pelophylax perezi	Positive	39.2480569	SRR29928497
sample_73	Pelophylax perezi	Positive	5.56097949	SRR29928496
sample_76	Pelophylax perezi	Positive	6.8959684	SRR29928495
sample_79	Hyla meridionalis	Positive	81.1956108	SRR29928494
sample_81	Hyla meridionalis	Negative	0	SRR29928493
sample_82	Hyla meridionalis	Positive	35.3503368	SRR29928492

sample_83	Hyla meridionalis	Positive	265.716349	SRR29928491
sample_84	Hyla meridionalis	Positive	28.4055597	SRR29928489
sample_85	Hyla meridionalis	Positive	582.471371	SRR29928488
sample_88	Pelophylax perezi	Negative	0	SRR29928487
sample_92	Hyla meridionalis	Positive	10700.6204	SRR29928485
sample_93	Hyla meridionalis	Positive	463.844533	SRR29928484
sample_94	Hyla meridionalis	Positive	220.928616	SRR29928483
sample_95	Hyla meridionalis	Positive	3455.04654	SRR29928482
water_1	Water	NA	NA	SRR29928481
water_2	Water	NA	NA	SRR29928480
water_3	Water	NA	NA	SRR29928478

Table S2. List of potentially pathogenic bacteria used to search in our ASVs database. Bacteria are classified by their relevance as recognized (R) or opportunistic (O) and by their primary host as human (H) or animal (A). This list has been created based on the bacterial diseases listed by the European Centre for Disease Prevention and Control (ECDC. 2024). the bacterial priority pathogens list and bacteria of potential health concern by the World Health Organization (WHO) (Lightfoot. 2003; WHO. 2024) and the World Organization for Animal Health list of notifiable diseases (WOAH. 2024a. 2024b).

Bacteria	**Pathogenic relevance**	**Primary Host**	**Main species**	**List**
Acinetobacter baumannii	O	H, A		WHO, ECDC
Aeromonas spp.	R	H, A	*A. caviae*, *A. hydrophila*	WHO
Anaplasma spp.	R	H, A	*A. marginale*	WOAH
Babesia spp.	R	A	*B. bovis*, *B. bigemina*	WOAH
Bacillus anthracis	R	H, A		WHO, ECDC, WOAH
Bonamia exitiosa	R	A (only aquatic)		WOAH
Bonamia ostrae	R	A (only aquatic)		WOAH
Bordetella bronchiseptica	R (O for humans)	H, A		WOAH
Bordetella parapertussis	R	H		ECDC
Bordetella pertussis	R	H		ECDC
Borrelia spp.	R	H, A	*B. burgdorferi*	ECDC
Brucella spp.	R	H, A	*B. abortus*, *B. melitensis*, *B. ovis*, *B. suis*	WHO, ECDC, WOAH

Burkholderia mallei	R	H, A		WOAH
Burkholderia pseudomallei	R	H, A		WHO
Campylobacter fetus	R	A		WOAH
Campylobacter spp.	R	H, A	*C. coli, C. jejuni*	WHO, ECDC
Chlamydia abortus	R (O for humans)	H, A		WOAH
Chlamydia psittaci	R	H, A		WOAH
Chlamydia trachomatis	R	H		WHO, ECDC
Citrobacter spp.	O	H, A	*C. freundii, C. koseri*	WHO
Clostridioides difficile	R	H, A		ECDC
Clostridium botulinum	R	H, A		WHO, ECDC
Clostridium tetani	R	H, A		WHO, ECDC
Corynebacterium diphtheriae	R	H		ECDC
Corynebacterium ulcerans	R	H, A		ECDC
Coxiella burnetii	R	H, A		ECDC, WOAH
Entercoccus faecium	O	H		WHO
Enterobacter spp.	O	H	*E. cloacae, E. aerogenes*	WHO
Escherichia coli	R	H, A		WHO, ECDC
Francisella tularensis	R	H, A		WHO, ECDC, WOAH
Haemophilus influenzae	R	H		WHO, ECDC
Hepatobacter penaei	R	A (only aquatic)		WOAH
Klebsiella pneumoniae	R	H, A		WHO
Legionella pneumophila	R	H		WHO, ECDC
Leptospira interrogans	R	H, A		ECDC, WOAH
Listeria monocytogenes	R	H, A		WHO, ECDC
Marteilia refringens	R	A (only aquatic)		WOAH
Melisococcus plutonius	R	A (only bees)		WOAH
Morganella spp.	O	H	*M. morgani*	WHO
Mycobacterium paratuberculosis	R	H, A		WHO, WOAH

Mycobacterium bovis	R	H, A		WHO, ECDC, WOAH
Mycobacterium caprae	R	H, A		WOAH
Mycobacterium leprae	R	H		WHO
Mycobacterium tuberculosis	R	H, A		WHO, ECDC, WOAH
Mycobacterium ulcerans	o	H, A		WHO
Mycobacterium xenopi	R	H		WHO
Mycoplasma agalactiae	R	A		WOAH
Mycoplasma capricolum	R	A		WOAH
Mycoplasma gallisepticum	R	A		WOAH
Mycoplasma genitalium	R	H		WHO, ECDC
Mycoplasma mycoides	R	A		WOAH
Mycoplasma synoviae	R	A		WOAH
Neisseria gonorrhoeae	R	H		WHO
Neisseria meningitidis	R	H		WHO, ECDC
Paenibacillus larvae	R	A (only bees)		WOAH
Pasteurella multocida	R	H, A		WHO, WOAH
Perkinsus marinus	R	A (only aquatic)		WOAH
Perkinsus olseni	R	A (only aquatic)		WOAH
Proteus spp.	O	H, A	*P. mirabilis, P. vulgaris*	WHO
Pseudomonas aeruginosa	R	H, A		WHO
Rickettsia spp.	R (O some species)	H	*R. akari, R. bellii, R. canadensis, R. felis, R. massiliae, R. slovaca*	WHO
Salmonella abortusovis	R	A		WOAH
Salmonella bongori	R	H, A		WHO
Salmonella enterica	R	H, A		WHO, WOAH
Salmonella paratyphi	R	H, A		WHO, ECDC
Salmonella typhi	R	H		WHO, ECDC
Salmonella typhimurium	R	H, A		WOAH

Salmonella pullorum	R	A		WOAH
Serratia spp.	O	H, A	*S. fonticola, S. liquefaciens, S. marcescens*	WHO
Shigella spp.	R	H	*S. boydii, S. dysenteriae, S. flexneri, S. sonnei*	WHO
Staphylococcus aureus	R	H, A		WHO
Streptococcus agalactiae	O	H, A		WHO
Streptococcus pneumoniae	R	H		WHO, ECDC
Streptococcus spp. (others)	R, O	H, A	*S. equinus, S. gallinaceus, S. iniae, S. pyogenes, S. suis, S. uberis*	WHO
Taylorella equigenitalis	R	A		WOAH
Theileria spp.	R	A	*T. parva, T. annulata*	WOAH
Treponema pallidum	R	H, A		WHO, ECDC
Trichomonas foetus	R (O for humans)	H, A		WOAH
Trichomonas vaginalis	R	H		WHO
Vibrio vulnificus	R	H		WHO
Vibrio parahaemolyticus	R	H, A		WHO
Vibrio cholerae	R	H, A		WHO, ECDC
Xenohaliotis californiensis	R	A (only aquatic)		WOAH
Yersinia enterocolitica	R	H, A		WHO, ECDC, WOAH
Yersinia pestis	R	H, A		WHO, ECDC, WOAH
Yersinia pseudotuberculosis	R	H, A		WHO, ECDC, WOAH

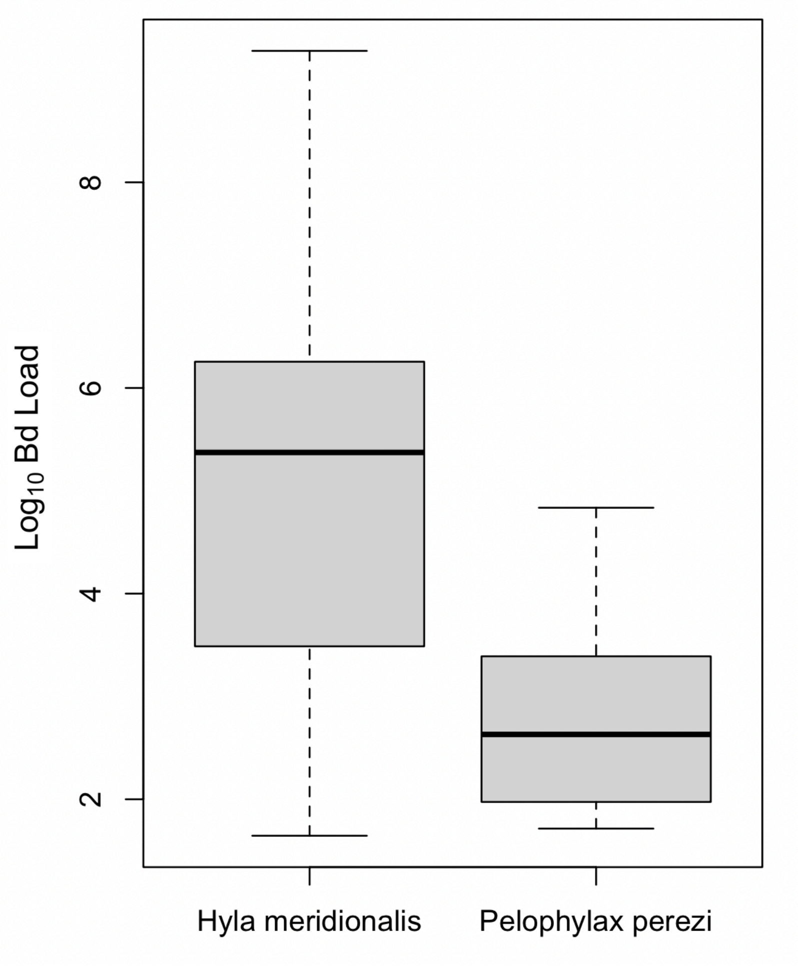


Figure S1. Boxplots showing log$_{10}$-transformed *Batrachochytrium dendrobatidis* (Bd) loads in *Hyla meridionalis* and *Pelophylax perezi*. Significant differences in Bd loads among amphibian species are observed (t=2.9976. df=16.837. p=0.0082).

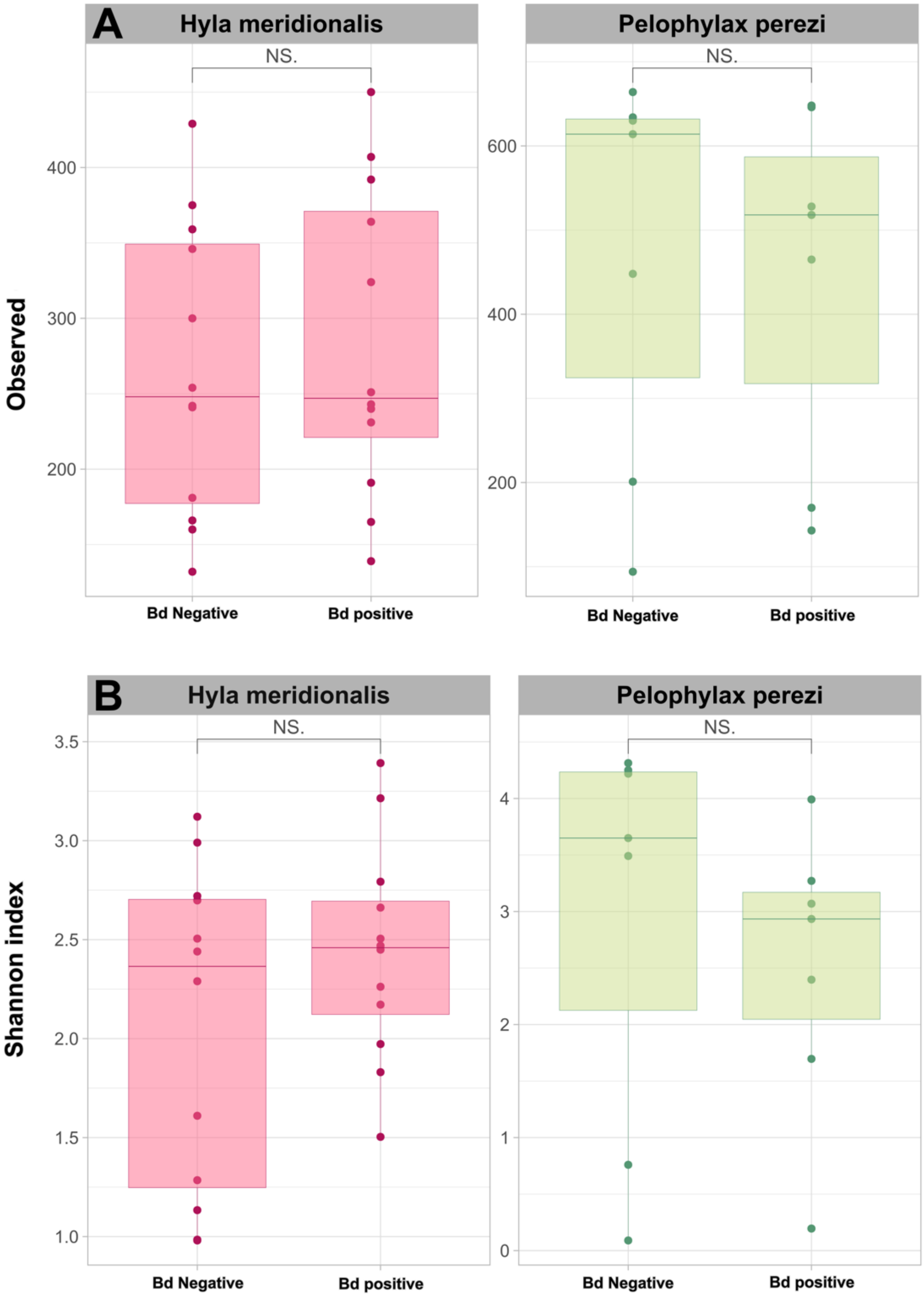


Figure S2. Bacterial richness (**A**) and Shannon index values (**B**) in *Batrachochytrium dendrobatidis* infected and non-infected *Hyla meridionalis* and *Pelophylax perezi*. NS = non-significant differences (p > 0.05).

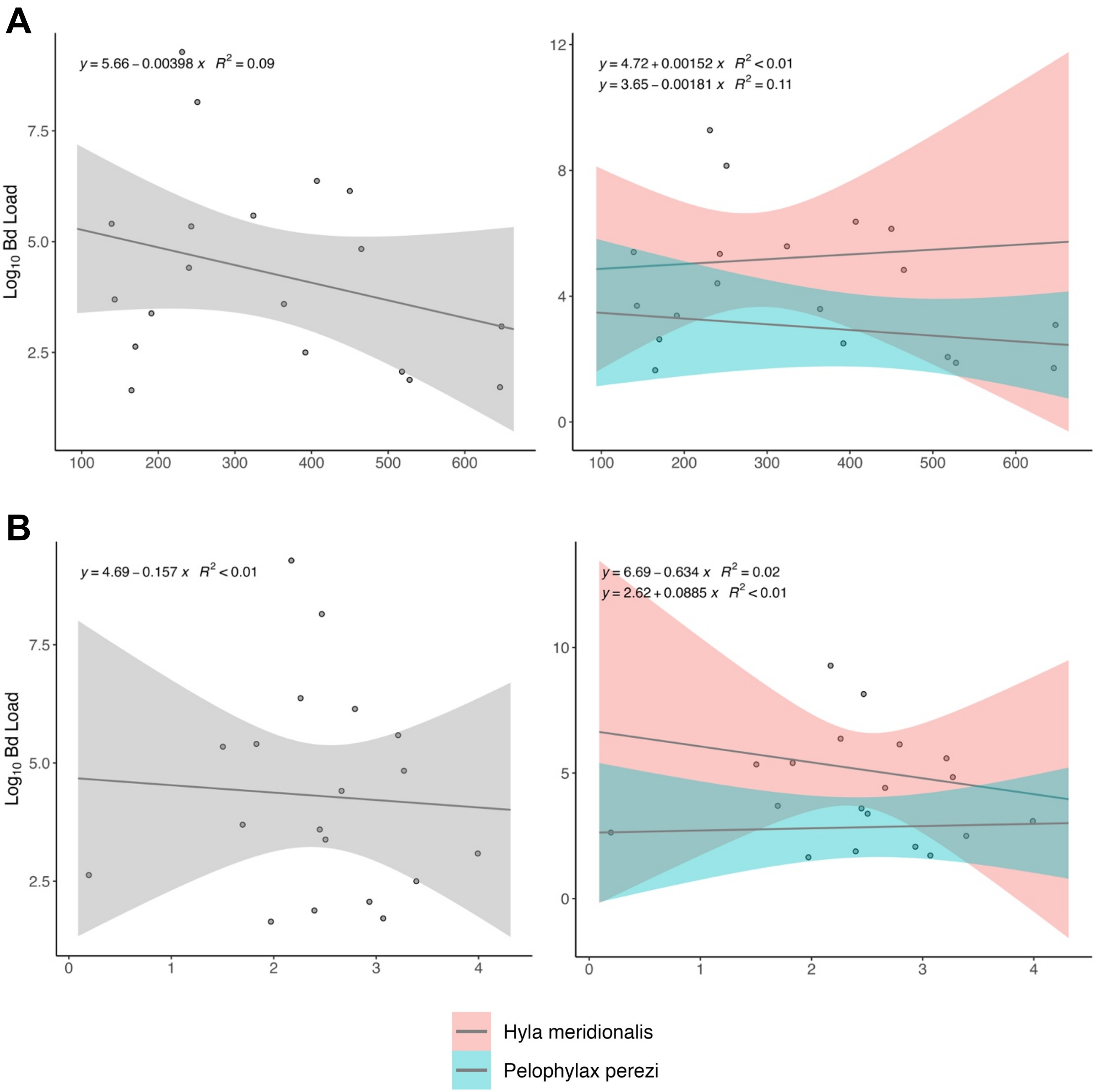


Figure S3. Linear regression between log10-transformed *Batrachochytrium dendrobatidis* (Bd) loads and observed operational taxonomic units (**A**) and Shannon index values (**B**). Regression is performed for both species combined (left graphs) and independently for each species (right graphs). Shaded areas show the 95% confidence intervals.

Figure S4. Relative abundance (top) and proportion (bottom) of putative *Batrachochytrium dendrobatidis* (Bd)-inhibitory bacteria in study samples. grouped by amphibian species. Bd status and water samples. Bd+ = *B. dendrobatidis* positive; Bd- = *B. dendrobatidis* negative.

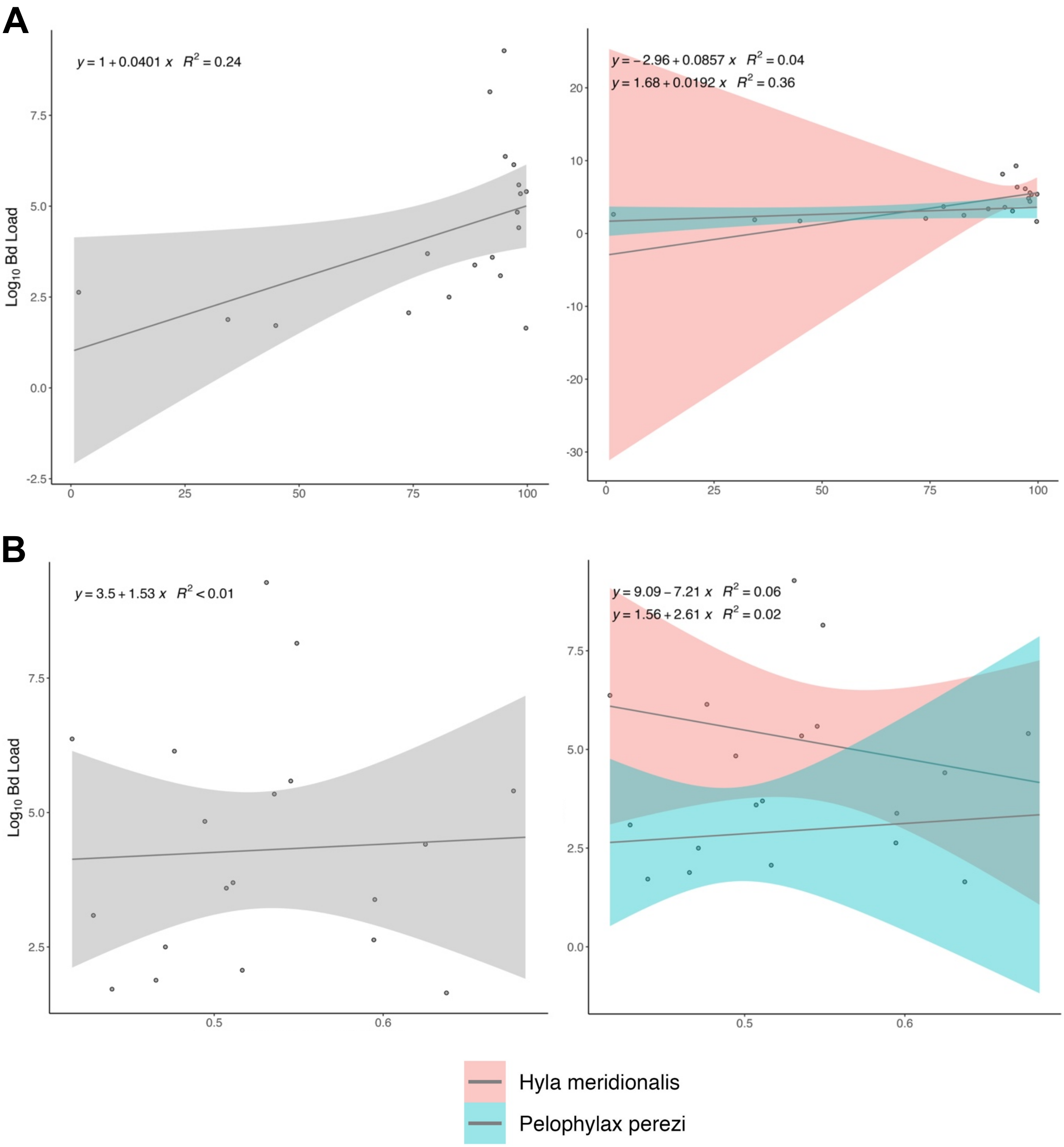


Figure S5. Linear regression between log10-transformed *Batrachochytrium dendrobatidis* (Bd) loads and putative Bd-inhibitory bacteria relative abundance (**A**) and proportion of taxa (**B**). Regression is performed for both species combined (left graphs) and independently for each species (right graphs). Shaded areas show the 95% confidence intervals.

Chapter 4

Coinfection of Chytrid Fungi in Urodeles during an Outbreak of Chytridiomycosis

Maria Puig Ribas
Oscar Cabezón
Roser Velarde
Josep Estruch
Emmanuel Serrano
Jaime Bosch
Barbora Thumsová
Albert Martínez-Silvestre

Journal of Wildlife Diseases (2022) 58(3): 658-663

Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal), has had an unprecedented impact on amphibian biodiversity. While Bd is globally widespread, Bsal is currently spreading, increasing the probability that both pathogens will co-occur in individual amphibians. Interactions among coinfecting parasites can have significant outcomes on disease dynamics and impact and, therefore, may have important consequences for amphibian conservation. Here we analyze the patterns of Bd-Bsal coinfections in two species of free-ranging urodeles during an outbreak of chytridiomycosis in Spain. Our goals were to assess (1) the probability of co-occurrence of both chytrid species, and (2) the correlation of pathogen loads in coinfected hosts. We detected coinfections in 81.58% of *Triturus marmoratus* (n=38) and in 18.75% of *Ichthyosaura alpestris* (n=16). Histopathological lesions of chytridiomycosis were observed only in *T. marmoratus*. Our results demonstrate a positive relationship between Bd and Bsal loads in *T. marmoratus* whereas the co-occurrence analysis showed a random association among pathogens in both urodele species. Overall, we show that Bd-Bsal coinfections intensify pathogen load in *T. marmoratus* and could, therefore, increase disease severity and have important consequences for the conservation of some amphibian species.

Introduction

Amphibian chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) fungi, is responsible for the greatest loss of biodiversity due to disease ever recorded (Fisher & Garner, 2020). Both pathogens likely originated in East Asia and have been introduced into naïve amphibian populations through human activities and international animal trade (Hanlon et al., 2018). Bd is now globally widespread, being able to infect over 1000 amphibian species (Fisher & Garner, 2020). Conversely, Bsal may be more host-specific, with infection and disease more frequently reported in urodele species while many European anurans appear tolerant and/or resistant to infection (Martel et al., 2014). Bsal has only recently been introduced and spreading in Western Europe, contributing to the near extirpation of *Salamandra salamandra* populations (Martel et al., 2013). In 2018, Bsal was also associated with a mortality event in *Triturus marmoratus* in Spain (Martel et al., 2020).

As Bsal continues to expand into areas where Bd is present, coinfections will be increasingly common in amphibian communities. Indeed, chytrid fungi coinfection has already been reported in *S. salamandra* from Germany (Lötters et al., 2018). However, the potential interactions among coinfecting chytrid fungi and its consequences for amphibian hosts are still largely unknown. Interactions among coinfecting parasites can be synergistic or antagonistic and can have important consequences on host susceptibility, transmission risk, infection duration and clinical outcome (Pedersen & Fenton, 2007). Synergism in chytrid fungi could result in increased prevalence and/or severity of disease, leading to further population declines and amphibian extinctions. On the other hand, antagonism between chytrid fungi could be protective for susceptible species and even aid the recovery of declining populations. Therefore, analyzing the patterns of coinfection is crucial to understanding disease dynamics and designing effective management strategies. Here, we analyze Bd-Bsal coinfections in two species of free-ranging urodeles at a focal area suffering an outbreak of chytridiomycosis. Our goals were to assess (1) the probability of co-occurrence of both chytrid species, and (2) the correlation of the pathogen loads of the two parasites in coinfected hosts.

Methods

In March 2018, Bsal was detected in a small reservoir in *Montnegre i el Corredor* Natural Park in Catalonia (NE Spain), associated with an outbreak of mortality in *T. marmoratus* (Martel et al., 2020). As part of the mitigation strategies, 38 native *T. marmoratus* and 16 introduced *Ichthyosaura alpestris* were culled soon after the detection of the index case (Martel et al., 2020; see online supplementary material at http://dx.doi.org/10.7589/JWD-D-21-00170. Toe-clip samples were collected and carcasses were fixed in 70% ethanol until histopathologic analysis. DNA was extracted from toe-clip samples using DNeasy Blood&Tissue Kit (Qiagen, Germany). Quantitative polymerase chain reaction (qPCR) assays for Bd (Boyle et al., 2004) and Bsal (Blooi et al., 2013) were run in duplicate for samples, positive and negative controls. Samples were considered positive when both duplicates revealed infection loads >0.1 zoospore genomic equivalents. Histopathological analysis was performed in seven well-preserved *T. marmoratus* and three *I. alpestris* to confirm the disease. Cross sections from each individual were obtained after soaking the carcass in a quick softener solution for 120 min. Histological examination of tissues was done using microscopic inspection of paraffin-embedded, 4-µm sections stained with hematoxylin and eosin. Due to overlap between Bd and Bsal infection patterns, no attempt to differentiate both chytrids was made. Prevalences with Wilson score confidence intervals (CI) of 95% were calculated using “epiR” package 2.0.19 (Stevenson et al., 2021). Pathogen prevalences and log-transformed pathogen loads were compared among species using Fisher’s exact tests and Student’s t-tests, respectively. We also explored whether Bd and Bsal were positive, negative or randomly associated using “co-occur” package 1.3 (Griffith et al., 2016). Finally, we assessed parasite interaction using a regression analysis of pathogen loads (zeros excluded). All statistical analyses were performed using R 4.0.3 (R Core Team, 2023).

Results

Prevalences of Bd and Bsal infections and coinfections were significatively higher in *T. marmoratus* than in *I. alpestris* ($p < 0.05$) (Table 1). Pathogen loads were not significantly different among urodele species for Bd ($t = -1.0455$, $df = 4.3849$, $p = 0.35$) but were significantly higher in *T. marmoratus* for Bsal ($t = -7.1336$, $df = 26.909$, $p < 0.05$) (Table 2). Our co-occurrence analysis showed a random association between Bd and Bsal infections in both newt species (p_lt = 1, p_gt = 1 for *T. marmoratus*, and p_lt = 0.82, p_gt = 0.63 for *I. alpestris*). In *T. marmoratus*, when coinfections occurred in a single host, pathogen loads were positively correlated ($n = 31$, $R^2 = 0.16$, $F(1, 29) = 5.36$, $p = 0.028$; Fig. 1). Correlation analysis was not performed in *I. alpestris* as only three individuals were coinfected. Chytridiomycosis was confirmed histopathologically in all seven *T. marmoratus* with multifocal areas of epithelial necrosis associated to the presence of sporangia (Fig. 2). Different degrees of inflammatory infiltrates associated with chytrid colonization in the skin were seen in six individuals. Interestingly, no inflammation was seen in the most severely affected individual (almost continued colonization of the skin), which also had the highest pathogen loads. The most intense inflammatory changes were seen in the newt with the lowest burden. No fungal colonization or dermal inflammation was detected in *I. alpestris*.

Table 1. Prevalence of *Batrachochytrium dendrobatidis* and *B. salamandrivorans* infection and coinfection in *Triturus marmoratus* and *Ichthyosaura alpestris*.

	Triturus marmoratus		*Ichthyosaura alpestris*		Fisher's exact test
	Positive / n	P ($CI_{95\%}$)	Positive / n	P ($CI_{95\%}$)	p-value
Batrachochytrium dendrobatidis	31/38	81.6 (66.6-90.8)	4/16	25 (10.2-49.5)	$1.338e^{-4}$
Batrachochytrium salamandrivorans	38/38	100 (90.8-100)	11/16	68.8 (44.4-85.8)	$1.381e^{-3}$
Coinfection	31/38	81.6 (66.6-90.8)	3/16	18.8 (6.6-43.0)	$2.305e^{-5}$

$CI_{95\%}$, 95% confidence intervals; n, number of tested individuals; P, prevalence of infection

Table 2. Summary of *Batrachochytrium dendrobatidis* and *B. salamandrivorans* loads (zoospore genomic equivalents) in infected *Triturus marmoratus* and *Ichthyosaura alpestris*.

	Triturus marmoratus		*Ichthyosaura alpestris*		Student's t-test*
	Median	Range	Median	Range	p-value
Batrachochytrium dendrobatidis load	10.2	1.1 - 1146.2	13.9	1.2 - 26.5	0.35
Batrachochytrium salamandrivorans load	273.6	5.7 - 7941.9	12.2	0.9 - 76.5	$1.159e^{-7}$

*Student's t-test were performed using log-transformed pathogen loads in order to conform to normality assumptions of the test.

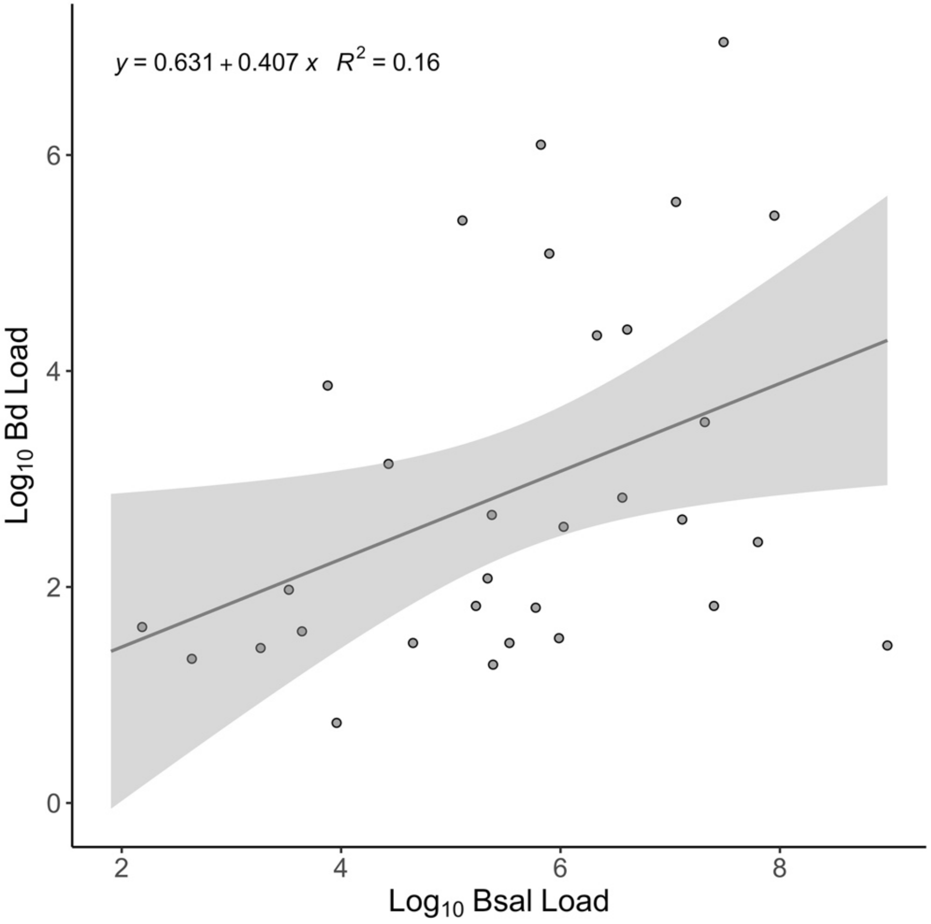


Figure 1. Linear regression between log₁₀-transformed *Batrachochytrium dendrobatidis* and *B. salamandrivorans* loads in *Triturus marmoratus*; shaded area shows the 95% confidence intervals. Bd/Bsal Load: number of genomic equivalents of zoospores.

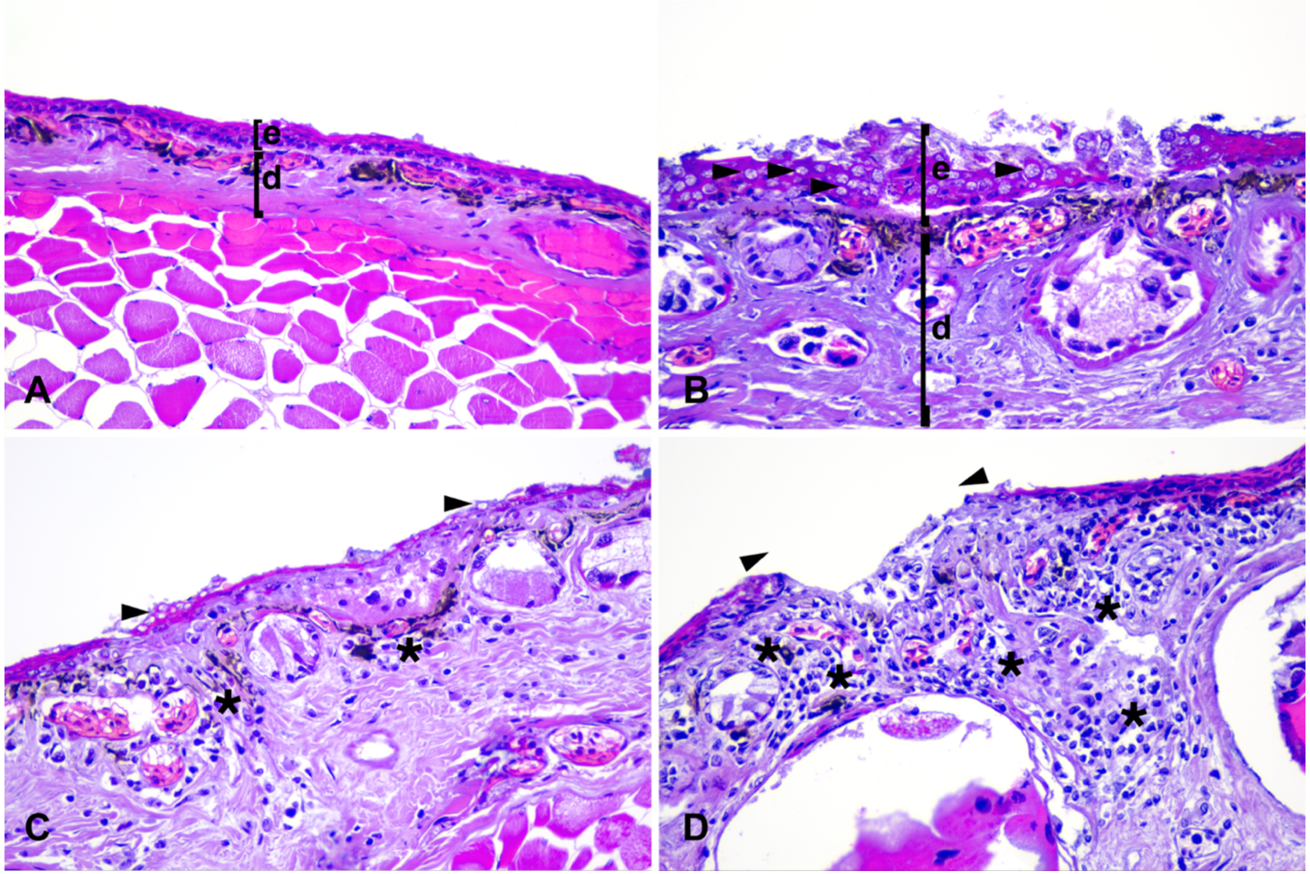


Figure 2. Microscopic lesions in the skin of *Triturus marmoratus* coinfected with *Batrachochytrium dendrobatidis* and *B. salamandrivorans*. Sections stained with hematoxylin and eosin stain and visualized at 200x magnification. (A) Non-affected area with intact epidermis (e) and compact dermal (d) tissue for comparison (Case AM051). (B) Extensive full thickness epithelial colonization by sporangia (arrowheads) with none to minimal inflammatory infiltrates and mild dermal edema (Case AM032). (C) Superficial invasion with sporangia (arrowhead), with epithelial cells jumbling and mild to moderate inflammatory infiltrates (asterisks), mainly mononuclear cells, and edema in the dermis (Case AM051). (D) Small ulcer (arrowheads) with very few sporangia at one of the margins, and a moderate mononuclear inflammatory infiltrate in the dermis (asterisks) (Case AM026).

Discussion

In this study, we analyzed the patterns of Bd-Bsal coinfections in free-ranging native *T. marmoratus* and introduced *I. alpestris* in Spain. To our knowledge, this is the first report of coinfection in these species and the second detection of natural Bd-Bsal coinfections globally (Lötters et al., 2018). Given the ongoing loss of biodiversity caused by Bd, its endemic occurrence in many areas and the emergence of Bsal, understanding and predicting the outcomes of coinfections is critical for amphibian conservation. This is of particular concern in urodeles, since many European species are highly susceptible to Bsal and coinfections may influence disease outcomes (Gilbert et al., 2020; Martel et al., 2014).

Our results demonstrate a positive relationship between Bd and Bsal loads in *T. marmoratus*, suggesting a synergistic interaction, where one parasite promotes the development of the other. Parasite synergism is often mediated by host immune system, through pathogen-induced immunosuppression, host tissue disruption and/or resource depletion (Pedersen & Fenton, 2007). In fact, there is evidence that Bd induces immune evasion, immunosuppression and immunopathology in some species (Grogan et al., 2018), and Bsal probably exhibits similar mechanisms (Farrer et al., 2017). A recent study described severely dysregulated immune responses in Bd-Bsal coinfected newts, showing that compounded immunopathology and immunosuppression could lead to more severe disease outcomes (C. A. McDonald et al., 2020). Nevertheless, few studies have evaluated the clinical course of coinfections and mixed results have been obtained thus far (Greener et al., 2020; Longo et al., 2019). In our study, most *T. marmoratus* had dermal inflammatory infiltrates consistent with an adaptive immune response (Grogan et al., 2018). Although it is not possible to determine the outcome of this inflammatory reaction (clearance or immunopathology) with only seven newts, the apparent effective induced defense response warrants further immunological studies in this species. In contrast, *T. marmoratus* experienced disease-induced mortality at the study site (Martel et al., 2020), suggesting that the existing immune response might not be entirely effective against chytrid fungi.

On the other hand, we showed that Bd and Bsal infections co-occur randomly in the species of urodeles studied. A random association between coinfecting chytrid fungi may be observed when constitutive defenses against first invasion remain unaltered. Despite both parasites depending on the same host resources/niche (i.e. epidermal cells), none of our analyses showed antagonistic interactions among chytrid fungi. These results suggest that resource limitation is not a constraint for fungal invasion and growth. Differences in chytrid fungi prevalence, load and pathology between *T. marmoratus* and *I. alpestris* probably reflect differences in species susceptibility as demonstrated experimentally (Fernández-Beaskoetxea et al., 2016; Martel et al., 2014). The lower pathogen prevalences, reduced Bsal loads and lack of histological lesions in *I. alpestris* are indicative of increased resistance and tolerance to chytrid fungi infection. Indeed, this species is considered a potential reservoir for Bsal in European amphibian communities (Gilbert et al., 2020).

Conclusion

We show that co-occurring Bd and Bsal elicit synergistic effects, intensifying pathogen load, in at least one urodele species. Chytrid fungi coinfections could, therefore, increase disease severity and have important consequences for host survival and conservation in susceptible amphibian species. A limitation of our study is the analysis of natural infections since it is not possible to determine the timing and sequence of coinfections. Experimental infections under controlled conditions are needed in order to reinforce our conclusions. Further research on parasite interactions, immune responses and clinical outcomes of chytrid fungi coinfections will be crucial to predict and prevent future biodiversity losses.

General discussion

He estat callat per dues raons: per pensar en la resposta i per demostrar que quan un pensa, la gent el mira malament. Per què es tan mal vist pensar? No és més censurable la gent que no reflexiona les coses?

— Merlí, Els peripatètics

Biodiversity in Catalonia is experiencing sharp declines, mirroring trends observed across the globe. According to the 2020 report on the state of biodiversity in Catalonia (Brotons et al., 2020), the region has lost 25% of its species over the past two decades. This decline is particularly pronounced in freshwater ecosystems, which are home to many endangered and protected species, including amphibians. Despite this alarming situation, current assessments largely overlook a fundamental dimension of biodiversity: the health status of wild populations. As a result, our understanding of endemic and emerging diseases – and their potential contribution to species declines – remains limited. This is particularly concerning for amphibians, the most threatened vertebrates, with disease being a major driver of their population declines worldwide (IUCN, 2024). Effective conservation requires evidence-based, standardized methods for monitoring species not only in terms of presence and abundance but also in terms of health. Understanding the epidemiological dynamics of diseases is essential for accurately determining conservation status and implementing appropriate management strategies.

This thesis contributes a significant advancement in this context by addressing one of the key gaps in the biodiversity framework of Catalonia: the lack of health-based monitoring of amphibian populations. Across four empirical chapters, we contribute to methodological advancements, explore the interplay between host, pathogen, and environment, and highlight the complexity of infectious disease dynamics in natural systems. Chytridiomycosis, an emerging infectious disease caused by chytrid fungi and known for its devastating impacts on amphibian populations worldwide, forms the central disease system throughout this work. Through validated methodologies and extensive field testing, we provide the first epidemiological data on Bd in Catalonia, setting the stage for future monitoring and research. This work lays the groundwork for integrating disease surveillance into broader conservation efforts for protected and declining amphibian species in the region. Moreover, the approaches and findings developed here have broader applicability, informing similar efforts in other countries facing comparable biodiversity and wildlife health challenges.

Beyond the PITfalls: toward ethical and evidence-based tagging in amphibians

Reliable population monitoring is a cornerstone of conservation biology, especially for assessing species' conservation status and understanding the impact of emerging threats such as infectious diseases. Without robust data on population dynamics, making informed decisions about when or where to intervene for conservation or disease management purposes is nearly impossible. Moreover, disease impacts on wild populations can be subtle and cumulative (Valenzuela-Sánchez et al., 2017); thus, detecting changes in survival, recruitment, or abundance depends entirely on a strong baseline of demographic data. Capture-mark-recapture (CMR) methods are widely used in this context, but the accuracy of demographic estimates is tightly linked to the reliability of the marking method. If marks are lost or if they inadvertently affect individual survival or behavior, population estimates can become biased or misleading (T. L. McDonald et al., 2003; Nichols, 1992). Among these, Passive Integrated Transponder (PIT) tags have gained popularity in amphibian research due to their potential advantages over traditional marking methods, such as increased durability, individual specificity, and ease of data retrieval (Cooke et al., 2013; Gibbons & Andrews, 2004). However, the use of PIT tags is not without challenges, as it is considered a surgical procedure in veterinary medicine and may not be suitable for all situations. Unfortunately, PIT tagging has not been sufficiently validated under controlled conditions – particularly in urodeles – and assumptions about their neutrality or effectiveness often go unchallenged.

To ensure that PIT tags yield valid population data while minimizing harm, implantation techniques must be evidence-based, species-specific, and aligned with ethical standards that prioritize animal welfare. As demonstrated in **Chapter 1,** relatively few studies rigorously validate PIT tagging methods for target urodele species, and many fail to report key methodological details. This represents a concerning gap in amphibian conservation, which can lead to the inappropriate application of marking techniques, resulting in increased tag rejection, biased demographic estimates, and undue suffering for the animals. Our findings in **Chapter 1** also reveal high and unpredictable rates of PIT tag loss in urodeles, which could inflate population estimates and downplay species' threat levels, ultimately undermining conservation decision-making. Furthermore, the lack of methodological transparency limits the ability to draw reliable comparisons across studies and obscures important factors influencing tag retention and animal welfare, thereby hindering the development of robust, standardized guidelines (Lyon et al., 2019; Musselman et al., 2017). These results underscore the necessity of conducting species- and method-specific trials before using PIT tags in urodeles and other amphibian populations.

To address these issues, in **Chapter 1** we emphasize the need for standardized, transparent reporting and the inclusion of veterinary professionals in developing and applying marking protocols for amphibians. This multidisciplinary approach would help ensure both methodological rigor and adherence to animal welfare standards, including considerations such as tag size, implantation site, anesthesia use, and post-operative care. Our study contributes to this goal by providing a validated, field-friendly, and cost-effective PIT tagging method for *Salamandra salamandra* and *Pleurodeles waltl* that does not require anesthesia and results in high tag retention with minimal impact. However, we also found that this method is not suitable for *Calotriton asper*, highlighting that the effectiveness of PIT tags is highly species- and context-dependent. Importantly, our methodological approach offers a blueprint for future validations in other amphibian taxa.

The welfare dimension of wildlife research and monitoring is increasingly gaining prominence, and rightly so (C. Berg et al., 2020). Many traditional marking methods in amphibians, including toe clipping and branding, are no longer ethically justifiable given our evolving understanding of animal sentience and suffering. Nevertheless, even some contemporary alternatives such as PIT tags are not free from scrutiny, especially when used in taxa like amphibians, where assessing pain, stress, or long-term effects is particularly challenging due to the subtlety of behavioral cues and a lack of species-specific welfare guidelines (Cortés Pérez & Maldonado Reséndiz, 2023). In this light, emerging technologies such as artificial intelligence and machine learning offer transformative potential. Non-invasive individual recognition based on natural body patterns – a method that eliminates physical marking entirely – can be a powerful tool, especially for small-bodied species or larval stages where traditional marking is either infeasible or ethically problematic (Dalibard et al., 2021; Takaya et al., 2023). These innovations can enhance welfare, reduce handling time, and improve public acceptance of monitoring activities, thereby aligning conservation goals more closely with ethical standards.

Amphibians are currently the most endangered group of vertebrates worldwide, yet population monitoring and health surveillance schemes remain alarmingly rare, even in regions where most species are legally protected. In the European Union, biodiversity directives call for conservation actions and recommend monitoring, but the absence of binding legislation mandating such programs has resulted in a lack of coordinated, long-term efforts. This gap is especially problematic in the face of emerging threats such as chytridiomycosis, a disease listed as reportable by the World Organization for Animal Health (WOAH). Without reliable individual marking techniques and systematic CMR studies, we are unable to assess population dynamics or disease impacts—challenges we encountered in Catalonia, where insufficient monitoring infrastructure prevented us from conducting longitudinal studies or investigating chytridiomycosis impacts in the following chapters of this thesis. We argue that establishing standardized, welfare-conscious marking methods – such as species-validated PIT tagging – is a critical first step toward enabling such studies. We encourage researchers and policy-makers to recognize this potential and prioritize amphibian monitoring as both a conservation and environmental health strategy. In **Chapter 1**, we make this case and provide a methodological foundation to support the development and implementation of standardized monitoring approaches in future research.

Host identity matters: variation in host responses drives chytridiomycosis dynamics

Host-specific traits play a fundamental role in shaping chytridiomycosis dynamics. Amphibian species differ markedly in their susceptibility, resistance, and tolerance to Bd, influencing both individual outcomes and broader epidemiological patterns (James et al., 2015). Our findings across **Chapters 2, 3, and 4** reinforce the concept that variation in host responses to infection may lead them to play different roles in the transmission and persistence of Bd. In endemic scenarios – such as the one we described in Catalonia – it becomes particularly important to identify both highly susceptible and potential reservoir hosts, in order to effectively target surveillance and management strategies. Even under endemic conditions, chytridiomycosis can cause mortality in susceptible hosts, suppress population densities, and reduce long-term population viability through increased vulnerability to other threats or stochastic events (Phillott et al., 2013). On the other hand, pathogen-tolerant hosts that sustain high levels of infection but exhibit low mortality may act as reservoirs, maintaining pathogen transmission in multi-host communities.

In **Chapter 2**, which provides the most comprehensive and comparative dataset across species, we observed pronounced interhost differences in Bd infection prevalence, intensity and mortality. *Alytes obstetricans* larvae showed the highest infection prevalence and high infection intensity without apparent mortality. This is consistent with their suggested role as tolerant reservoirs in other disease systems (Clare et al., 2016). However, Bd prevalence in post-metamorphic *A. obstetricans* was substantially lower, with the few infected individuals exhibiting high loads and, in some cases, mortality, suggesting increased susceptibility and decreased tolerance at the metamorphic stage. We also observed that *Hyla meridionalis* and *Pelophylax perezi* displayed distinct patterns of resistance and tolerance: *H. meridionalis* exhibited high prevalence and infection loads in the absence of clinical disease, suggesting tolerance, while *P. perezi* showed lower loads, indicating possible resistance mechanisms. These findings were supported in **Chapter 3**, where we further explored these two species and their skin microbiomes under asymptomatic Bd infection. Low Bd infection rates in other anuran and urodele species may indicate that some are relatively resistant and unlikely to function as effective reservoirs. In the case of urodeles, these observations also align with broader evidence suggesting that they are generally more resistant to *Bd* infection and disease (Scheele, Pasmans, et al., 2019; Van Rooij et al., 2015). Conversely, urodeles are the only amphibian taxa known to be susceptible to Bsal chytridiomycosis (Martel et al., 2014). In **Chapter 4**, we presented new evidence that *Triturus marmoratus* is susceptible to Bsal-induced disease and mortality and demonstrated that host responses to Bsal also vary markedly across species. However, limited sample sizes in some cases and the lack of longitudinal studies to assess individual infection dynamics call for caution when interpreting our findings and warrant further research.

Multiple factors likely drive this variation in host responses, including differences in innate and acquired immune responses, as well as behavioral and life-history traits (Brannelly et al., 2021; Grogan et al., 2018). In **Chapter 3**, we explored the potential role of skin microbiome as part of amphibians' constitutive immune defenses in such responses. Our microbiome analysis revealed significant differences in bacterial richness and composition between *H. meridionalis* and *P. perezi*, highlighting species-specific microbial communities that may reflect underlying ecological or physiological differences. Interestingly, *P. perezi* exhibited greater bacterial richness and lower Bd loads, potentially pointing toward a microbiome-mediated resistance, as previously proposed (Grogan et al., 2023). In contrast, while *H. meridionalis* carried higher Bd loads, it also harbored a higher relative abundance of putative Bd-inhibitory bacteria, suggesting a more complex relationship between microbiome composition and disease tolerance. Notably, we did not observe significant differences in microbiome diversity or structure between Bd-positive and Bd-negative individuals within species, possibly due to small sample sizes, asymptomatic infections, or environmental uniformity. Despite these limitations, our findings lay important groundwork for future studies tracking microbiome shifts as potential early indicators of susceptibility or disease risk. We emphasize that microbiome monitoring holds promise as an early-warning tool, particularly when integrated into long-term disease surveillance frameworks.

While amphibian species richness is often thought to play a crucial role in regulating disease dynamics, our modeling approach in **Chapter 2** shows that it does not significantly influence Bd epidemiology in Catalonia. Several authors have explored the concept of a dilution effect, where higher host diversity reduces disease risk, in the amphibian-Bd system. This effect is thought to occur because more diverse communities may limit the transmission of generalist pathogens by increasing the likelihood of encounters between susceptible and resistant hosts (Keesing et al., 2006). Laboratory studies generally support the occurrence of the dilution effect in Bd systems (Searle, Biga, et al., 2011; Venesky, Liu, et al., 2014), but field studies have produced mixed results, likely due to the variation in host composition, habitat complexity, and species-specific interactions (Becker & Zamudio, 2011; Liu et al., 2013). Our findings add to the growing body of evidence suggesting that the presence and identity of specific species – rather than overall species richness – play a more decisive role in shaping disease outcomes in complex natural systems. Thus, the presence of a particular species in a community can either suppress or amplify pathogen transmission depending on its susceptibility and ecological interactions. Understanding community-wide transmission dynamics and species-specific contributions is therefore essential for predicting disease outcomes in multi-host systems like amphibian chytridiomycosis.

Context is key: the role of environment and pathogen interactions

Batrachochytrium dendrobatidis is a globally distributed pathogen, with confirmed presence in at least 94 countries and across a wide range of environmental contexts (K. A. Murray et al., 2011; Olson et al., 2021; Ron, 2005). Its occurrence spans diverse climates and habitat types, from tropical rainforests to temperate highlands and Mediterranean systems. This ecological flexibility enhances its potential for spread and persistence, especially in biodiverse and climatically variable regions such as Catalonia. In line with this, in **Chapter 2**, we documented a broad geographic distribution of Bd across Catalonia, with prevalence not significantly associated with specific bioregions or habitat classifications.

Among environmental variables, temperature has consistently been identified as the primary driver of Bd dynamics (Alvarado-Rybak et al., 2021; K. A. Murray et al., 2011; Olson et al., 2021). Our findings support this pattern, with maximum summer temperature emerging as one of the strongest predictors of Bd prevalence in Catalonia. Interestingly, while high temperatures are generally considered inhibitory to Bd, we found a positive association, likely due to thermal buffering by microhabitats and behavioral adaptations of hosts that allow the pathogen to persist even in macroclimatically suboptimal conditions (Rohr & Raffel, 2010; Ron, 2005). Although altitude and precipitation have often been proposed as relevant predictors of Bd distribution (Alvarado-Rybak et al., 2021; Bacigalupe et al., 2019; Olson et al., 2021; Puschendorf et al., 2009; Walker et al., 2010), neither variable was retained in our final models. These results reinforce the idea that local thermal conditions are the most biologically meaningful factor influencing Bd occurrence in our system. This finding is particularly relevant in the context of climate change, which is expected to expand the availability of thermally suitable microhabitats and thus facilitate the persistence and spread of Bd (Venesky, Raffel, et al., 2014; Xie et al., 2016).

In addition to climatic factors, our results demonstrate that anthropogenic disturbances significantly predict Bd occurrence. Human-altered landscapes may facilitate the introduction and spread of Bd, whether through increased movement of amphibians and fomites (Fisher & Garner, 2020; Liu et al., 2013; O'Hanlon et al., 2018) or through habitat degradation that compromises host immunity (Jiménez & Sommer, 2017; Rollins-Smith et al., 2011). Habitat fragmentation and contaminants such as pesticides have been shown to alter the amphibian skin microbiome, a key barrier to infection, potentially increasing host vulnerability to chytrid fungi (Becker et al., 2017; Jiménez et al., 2020; McCoy & Peralta, 2018). These findings highlight how environmental conditions – both natural and human-driven – interact with host traits and pathogen biology to shape infection dynamics and disease outcomes.

Batrachochytrium salamandrivorans dynamics appear to be shaped by similar ecological drivers as Bd, with temperature and anthropogenic impacts emerging as key predictors in the few models developed to date (Beukema et al., 2021; Katz & Zellmer, 2018). However, given Bsal's narrower and cooler thermal tolerance range (Martel et al., 2013), its environmental suitability is expected to be higher in temperate regions, and its response to climate change may diverge considerably from that of Bd. In **Chapter 2**, we did not detect Bsal in Catalonia, preventing a direct evaluation of its environmental associations. Further research in Bsal-endemic areas will be essential to refine ecological models, improve our understanding of its disease dynamics, and inform targeted mitigation and conservation strategies.

Taken together, these insights suggest that environmental management represents a viable strategy to reduce Bd prevalence and disease risk.

Mitigation of climate change, restriction of human access and activity in high-risk areas, and preservation of environmental refugia that disrupt pathogen life cycles or enhance amphibian resilience represent promising conservation interventions (Garner et al., 2016; Scheele, Foster, et al., 2019). By targeting both abiotic and anthropogenic risk factors, such measures may reduce the burden of chytridiomycosis on vulnerable amphibian populations.

However, single-pathogen perspectives alone are insufficient to capture the full complexity of disease dynamics in free-ranging populations. An integrated approach must also account for interactions among multiple coinfecting pathogens. Coinfections can substantially alter infection dynamics and clinical outcomes, raising conservation concerns. This is particularly relevant for chytridiomycosis, as Bd and Bsal are increasingly co-circulating in European amphibian populations following Bsal's recent emergence. Yet despite this growing risk, very few studies have explored the effects of coinfection. To date, only two field studies have documented natural Bd-Bsal coinfections (Lötters et al., 2018; this thesis), and experimental studies remain scarce, lacking European species under realistic ecological conditions (C. A. McDonald et al., 2020). This highlights a critical gap in our understanding of pathogen interactions in amphibian communities.

In **Chapter 4**, we present one of the first documented cases of natural Bd-Bsal coinfection in Europe, and the first in Iberian urodeles. We observed a high prevalence of coinfections in two urodele species, one endemic and one introduced in Catalonia. Our results demonstrated a synergistic interaction between the two chytrid fungi that likely contributed to increased infection intensity and disease-induced mortality. Mechanisms such as pathogen-mediated immunosuppression, tissue disruption, or reduced efficiency of immune responses due to compounded pathogen pressures may explain these outcomes. Similar interactions have been observed under experimental conditions, where coinfected individuals showed more severe clinical outcomes and dysregulated immune responses compared to single infections (Longo et al., 2019; C. A. McDonald et al., 2020)

The implications for amphibian conservation are substantial. Synergistic interactions may accelerate disease progression, increase transmission potential, and reduce the effectiveness of natural or adaptive host defenses. As our study also showed, even in the presence of immune activation (e.g., inflammatory infiltrates), coinfections can lead to lethal outcomes, suggesting that coinfections can overwhelm host resilience. Therefore, there is an urgent need for controlled experimental infection studies to evaluate how Bd-Bsal coinfections influence pathogen load dynamics, disease progression, host survival, and immune responses. Additionally, understanding infection sequence, timing, and dose will be critical to designing effective conservation strategies and forecasting disease outcomes under realistic ecological scenarios.

Future perspectives

This thesis advances our understanding of amphibian disease ecology by highlighting the multifactorial nature of chytridiomycosis and the importance of integrating host traits, microbial defenses, environmental conditions, and pathogen interactions. Across its four empirical chapters, the work addresses crucial gaps, from validating field methods to investigating microbiome-pathogen dynamics and revealing the potentially compounding effects of co-infections. By situating these findings within the regional context of Catalonia, yet linking them to broader global trends, the thesis not only offers actionable insights for local conservation but also contributes to the wider field of wildlife health and emerging infectious disease ecology. To strengthen future research and conservation action, we offer the following recommendations:

- **Adopt ethical and standardized marking methodologies**: rigorous validation of marking techniques is essential, and such studies are still needed for many amphibian species, especially for those that are threatened. Methods must balance data reliability with animal welfare.
- **Invest in long-term, health-based monitoring:** robust, long-term population data are essential for accurately assessing conservation status and detecting the impacts of emerging threats such as disease. Equally important is the development of systematic health

surveillance schemes, which remain rare for most wildlife, including amphibians. Without baseline demographic and health data, it is difficult to evaluate population trends, understand species' vulnerability, or implement effective management strategies.

- **Prioritize microbiome-informed approaches**: given the role of the skin microbiome in host defense, future research should explore its causal relationship with chytridiomycosis resistance and tolerance, and evaluate its potential for conservation interventions, such as probiotic applications. Microbiome monitoring also holds promise as an early-warning tool for identifying shifts in host susceptibility or disease risk, particularly when integrated into long-term health surveillance frameworks.
- **Incorporate a multispecies, multi-pathogen perspective**: effective disease management must account for the ecological roles and susceptibility of individual host species, including their potential to act as reservoirs or amplifiers. Community-level approaches should incorporate host-pathogen interactions and the presence of co-infecting pathogens to better predict disease dynamics and inform targeted interventions.
- **Expand experimental coinfection studies**: controlled studies examining the effects of Bd-Bsal coinfection on infection load, immune response, disease progression, and host survival are urgently needed.
- **Implement proactive conservation strategies**: management efforts should target both abiotic (e.g., temperature regulation, climate refugia) and anthropogenic (e.g., habitat protection, movement restrictions) drivers to reduce disease risk in sensitive habitats.

In conclusion, this thesis highlights the urgency of adopting integrative, ethically grounded, and ecologically informed approaches to amphibian conservation. As amphibians continue to face unprecedented threats from habitat loss, climate change, and emerging diseases, only a holistic understanding of host–pathogen–environment interactions will allow us to develop effective and sustainable responses.

Conclusions

> No m’estranya que sigui més bona, la gent aquí dalt, més autèntica, més humana, si respiren cada dia aquest aire. I beuen aigua d’aquest riu. I contemplen cada dia la bellesa que fa mal a l’ànima d’aquestes muntanyes.
>
> — Irene Solà, Canto jo i la muntanya balla

1. The reliability of passive integrated transponder tags for marking urodeles is highly dependent on the species and implanting method, and it should be tested in controlled studies and reported appropriately before its use in field research.

2. *Batrachochytrium dendrobatidis* infection is broadly distributed and endemic in amphibian communities across Catalonia, while *Batrachochytrium salamandrivorans* is presumably absent.

3. At least seven native amphibian species in Catalonia are susceptible to *Batrachochytrium dendrobatidis* infection. However, only *Hyla meridionalis* and *Alytes obstetricans* larvae exhibit characteristics of potential reservoir hosts.

4. *Batrachochytrium dendrobatidis* occurrence in Catalonia is positively associated with maximum summer temperatures and anthropogenic disturbance.

5. The skin microbiome of amphibians does not necessarily shift in response to *Batrachochytrium dendrobatidis* infection in asymptomatic hosts but may contribute to species-specific mechanisms of tolerance and resistance.

6. Coinfection with *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* is synergic, increasing pathogen load in *Triturus marmoratus* and potentially other urodeles.

Acknowledgments
Agraïments
Agradecimientos

> Un fa el que pot i els amics fan la resta.
>
> — Andreu Buenafuente

Com escalar una muntanya, aquesta tesi m’ha posat a prova sense treva: amb trams exposats, passos insegurs i més d’una caiguda. Hi ha llargs que puges sola, i d’altres on algú t’assegura, et passa la corda o simplement et diu “tu pots” quan més ho necessites. He tingut la sort de formar part d’una cordada que no només m’ha acompanyat, sinó que m’ha posat les xapes quan calia, m’ha ajudat a tornar a enfilar-me, i ha fet que el camí fos més lleuger, amb complicitats, riures i bons moments. Aquest és el meu (insuficient) agraïment a tots els qui, d’una manera o altra, m’heu ajudat a no perdre l’agarre, a seguir pujant i, finalment, a fer cim.

A Óscar. ¿Qué te puedo decir? Si hay alguien a quien dedicarle esta tesis, sin duda eres tú. Gracias por tu pasión por nuestra profesión y por tu increíble capacidad de arrastrarnos a todos contigo. Entusiasta, emprendedor y líder nato – y también un gran liante – rebosas empatía, y por eso todos te seguimos sin dudar. Me has enseñado que las mejores ideas nacen del buen ambiente, de las risas, de una cerveza y una servilleta; y me has recordado día a día que lo más importante es ser felices con lo que hacemos y con quienes somos. ¡Ojalá el club de los positivos siga activo muchos años más!

A l’Ignasi, veu de la saviesa, la paciència i el bon rotllo. Gràcies per acompanyar-me en aquells primers mostrejos caòtics, on sovint acabàvem enmig d’un barranc sense sortida. Ets el nostre pilar: sempre capaç de calmar les aigües i, de passada, afegir-hi un toc d’humor sarcàstic.

A l’Albert, per aportar la teva experiència en el món dels amfibis i contagiar la passió que sents per aquesta feina. Gràcies per compartir el teu coneixement i per tot el suport que m’has donat.

Al Rafa, per la teva energia i el teu bon cor. Gràcies per totes les hores que has dedicat a compartir amb mi la teva experiència i el teu saber fer.

Al Marcel, perquè em vas donar l’impuls i la confiança que em van donar força per seguir el camí de la veterinària. Gràcies per ser el meu pare de professió.

Al Xavi, per tot el que hem compartit durant tants anys – la vida, la feina, la passió per la natura i innumerables aventures. Gràcies per haver estat al meu costat en tantes etapes d'aquest viatge.

A tota la gent que ha ajudat a construir WildCoM i amb qui he compartit dies intensos de feina, rialles, peripècies, confidències, calçotades, cerveses...Internes i interns – Irene, Carme, Josep, Pol, Lucía, Nacho, Nuri, Marc, Axelle, Angie, Rita, Ane, Paula, Charlotte, Lina, Simon, Sara, Carmen, Carol, Laura, Mireia, Sofia, Alessandra, Uxía, Irene, Alèxia, Cora i Neus – sense vosaltres aquesta tesi no tindria ni mostres ni, sobretot, tant bons records.

Carlotta, María y Mariette, gracias por acompañarme durante las últimas etapas de este camino, me hace muy feliz trabajar al lado de mujeres tan fuertes, brillantes y generosas.

Laura, honey, thanks for being the best housemate I could've imagined. I really miss our late-night chats over a glass of wine. Ti voglio un mondo di bene!

Lourdes, gràcies pels atacs de riure descontrolats, per retroalimentar les meves bogeries i per l'atenció al detall i la sensibilitat que poses en tot el que fas.

Andrea, gracias por tu buenrollismo contagioso, pero aún más por dejarme disfrutar de tu lado más agudo, divertido y deliciosamente sarcástico. Eres luz.

Laura, gràcies per posar banda sonora a la meva vida, per animar-me sempre i per l'energia desbordant que encomanes. Per totes les aventures que hem compartit, i les que encara ens esperen. Què hauria fet el Xip sense el seu Xop?

Johan, com trobo a faltar lliscar la cadira cap a la teva taula per comentar la jugada, tramar algun un pla o, simplement, xafardejar-te la pantalla. Treballar amb tu és tan fàcil que no ens cal ni parlar per entendre'ns. El millor company i amic que es pot tenir: m'has acceptat amb totes les meves bogeries i defectes, i m'has donat serenor quan més la necessitava. Ets la definició de bona persona, i per això t'estimo com un germà. I Aina, gràcies per fer-li sempre costat i ser com ets.

A Paula, por ser mi apoyo, interna, consejera, amiga, alma gemela y guía espiritual. Me faltan palabras para expresar la admiración que siento por ti: por tu fuerza, tu perseverancia, tu ingenio y esa inteligencia brillante que te permite llegar a entender lo más profundo de las personas. Te quiero siempre a mi lado.

Als sefitos, per ser la meva porta al món de la fauna salvatge i, a més, els millors veïns de passadís que es poden tenir. Roser, por demostrar cómo se puede amar una profesión y ejercerla siempre desde el rigor i la humildad.

A la gent del CReSA, investigadors i tècnics, per sempre estar disposats a compartir el vostre temps i coneixements amb generositat i paciència.

Al poble de Sancris i a la seva gent, per donar-me un entorn on créixer amb llibertat, arrelada a la natura i amb els peus a terra. Sempre sereu la meva tribu.

A la meva família – àvies, avis, tietes, tiets i un bon eixam de cosins – per creure sempre en mi i per apuntar-vos sense dubtar a qualsevol idea de bombero que se m'acudeix, com anar a "caçar" gripaus a les 12 de la nit.

Als Sumas i als Faros, per ser les nostres famílies escollides.

Al Segarra, per aparèixer quan tot just començava la pujada més dura i fer-la molt més lleugera. Per fer-me riure quan més ho necessitava, per tornar-me la il·lusió i per fer-me venir tantes ganes de totes les aventures que encara ens queden per viure junts. T'estimo!

Al meu germà Jordi, perquè sóc qui sóc gràcies a les mil trapelleries compartides, Zipi i Zape ens haurien demanat consell. Gràcies per ser-hi sempre i per ensenyar-me que el temps és relatiu i que l'esforç es pot dosificar...molt!

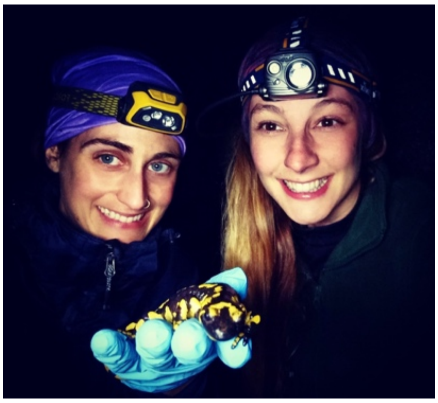
Al papa, pel teu suport incondicional en tot el que he fet, encara que no entenguessis ni una paraula del que t'explicava. M'alegro moltíssim haver heretat part del teu humor i del teu "desparpajo", i per l'equipàs que fèiem, com a bons hiperactius. Vas marxar massa aviat, però sé que estàs orgullosíssim de qui sóc, estiguis on estiguis.

A la mama, per despertar-me una curiositat insaciable i per educar-me en la sensibilitat i l'admiració cap a la natura. Sense tu no hi hauria tesi, perquè m'has recolzat sempre en tot el que fet i, fins i tot, m'has fet de "becària d'emergència" en molts mostrejos. Ningú més que tu demostra el que és la fortalesa i la dedicació. Ets una autèntica heroïna!

A la Kangoo, la meva estimada Putxineta, per portar-me durant deu anys a totes les aventures i mostrejos, fins i tot pujant per pistes d'esquí a 2.000 metres d'altitud.

Al Curry per ser l'alegria de la casa i recordar-me sempre allò més essencial de la vida. Per moltes més nits acurrucat als meus peus!


Marmot




Los PEORES INTERNOS ...
TE PIENSO Y TE ECOLOCALIZO
mascarilla o masbaratilla no sé... piénsalo
Paparras
¡TODO PARA mañaaana!
amplexus patronus
WildCoM Best Vibes 22 23

Funding and Collaborators

The author was supported by the FI-DGR 2021 predoctoral scholarship from the Government of Catalonia. The studies of this thesis were partially funded by the Barcelona Zoo Foundation, the Alt Pirineu Natural Park, and the generous contributors to our crowdfunding campaign, with special thanks to Oryx, Giacomini and Driving Events.

The professional commitment of the Government of Catalonia and the directors and personnel of the Catalan Natural Parks was fundamental for the successful development of this thesis. In particular, the author would like to acknowledge Ricard Casanovas Urgell, Marc Garriga Lujan, Elisenda Montserrat Freixa, Jordi Garcia Petit, Elena Julià Alsina, Montse Raurell Solà, Albert Vila Bonfill, Anna Planella Bosch, Sergio Romero de Tejada, Maria Pifarré i Olivé, Enric Capalleres, Emili Bassols i Isamat, Nati Franch, Helena Fanlo, Vanessa Cadenas Valdivieso and Diego Martínez Martínez. We also acknowledge the collaborative work of Salvador Carranza (Institut de Biologia Evolutiva, CSIC-UPF), Jordi Baucells, Alex López (Universitat de Girona), Octavi Borruel Trench (Ajuntament de Barcelona), Sergi García and Guillem Pascual Pijoan (Galanthus), Cinta Golorons (GEPEC) and Oriol Baena Crespo (Societat Catalana d'Herpetologia).

The help with field and laboratory work provided by the WildCoM team, visiting scholars and veterinary students was invaluable to the completion of this thesis. We also recognize the laboratory assistance by members of IRTA-CRESA Animal Health Research Centre. Special thanks to the Catalonian Amphibian and Reptile Rescue Center (CRARC) and its director, Joaquim Soler Massana, for their essential collaboration and dedication to amphibian and reptile conservation.

Finally, the author is deeply grateful to Dr. Andrew Cunningham, Louise Gibson and the Institute of Zoology (Zoological Society of London) for the opportunity to undertake a PhD placement under their support and hospitality.

References

Tolstoi, d’una tal Ana Karenina.

— Plats Bruts, Tinc una fan

Abarca, J. G., Vargas, G., Zuniga, I., Whitfield, S. M., Woodhams, D. C., Kerby, J., McKenzie, V. J., Murillo-Cruz, C., & Pinto-Tomás, A. A. (2018). Assessment of bacterial communities associated with the skin of Costa Rican amphibians at la Selva biological station. *Frontiers in Microbiology*, *9*(SEP). https://doi.org/10.3389/fmicb.2018.02001

Acevedo-Whitehouse, K., & Duffus, A. L. J. (2009). Effects of environmental change on wildlife health. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1534), 3429–3438.

Adams, M. J., Chelgren, N. D., Reinitz, D., Cole, R. A., Rachowicz, L. J., Galvan, S., Mccreary, B., Pearl, C. A., Bailey, L. L., Bettaso, J., Bull, E. L., & Leu, M. (2010). Using occupancy models to understand the distribution of an amphibian pathogen, *Batrachochytrium dendrobatidis*. In *Ecological Applications* (Vol. 20, Issue 1).

Alford, R. A., & Richards, S. J. (1999). Global Amphibian Declines: A Problem in Aplied Ecology. *Annu. Rev. Ecol. Syst.*, *30*, 133–65.

Allain, S. J. R., & Duffus, A. L. J. (2019). Emerging infectious disease threats to European herpetofauna. *Herpetological Journal*, *29*, 189–206. https://doi.org/10.33256/hj29.4.189206

Allison, S. D., & Martiny, J. B. H. (2008). Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences*, *105*(supplement_1), 11512–11519. https://doi.org/10.1073/pnas.0801925105

Alonso, A., Bosch, J., & Boyero, L. (2024). Loss of amphibian species alters periphyton communities in montane ponds. *Hydrobiologia*, *851*(16), 3977–3990. https://doi.org/10.1007/s10750-024-05551-0

Alvarado-Rybak, M., Lepe-Lopez, M., Peñafiel-Ricaurte, A., Valenzuela-Sánchez, A., Valdivia, C., Mardones, F. O., Bacigalupe, L. D., Puschendorf, R., Cunningham, A. A., & Azat, C. (2021). Bioclimatic and anthropogenic variables shape the occurrence of Batrachochytrium dendrobatidis over a large latitudinal gradient. *Scientific Reports*, *11*(1). https://doi.org/10.1038/s41598-021-96535-w

Amato, K. R., Kuthyar, S., Ekanayake-Weber, M., Salmi, R., Snyder-Mackler, N., Wijayathunga, L., Vandercone, R., & Lu, A. (2020). Gut microbiome, diet, and conservation of endangered langurs in Sri Lanka. *Biotropica*, *52*(5), 981–990.

Amstrup, S. C., McDonald, T., & Manly, B. F. J. (2010). *Handbook of Capture-Recapture Analysis*.

Arnason, A. N., & Mills, K. H. (1981). Bias and Loss of Precision Due to Tag Loss in Jolly–Seber Estimates for Mark–Recapture Experiments. *Canadian Journal of Fisheries and Aquatic Sciences*, *38*(9), 1077–1095. https://doi.org/10.1139/f81-148

Arntzen, J. W., Goudie, I. B. J., Halley, J., Jehle, R., & others. (2004). Cost comparison of marking techniques in long-term population studies: PIT-tags versus pattern maps. *Amphibia-Reptilia*, *25*(3), 305–315.

Astudillo-García, C., Hermans, S. M., Stevenson, B., Buckley, H. L., & Lear, G. (2019). Microbial assemblages and bioindicators as proxies for ecosystem health status: potential and limitations. *Applied Microbiology and Biotechnology*, *103*(16), 6407–6421. https://doi.org/10.1007/s00253-019-09963-0

Aylagas, E., Borja, Á., Tangherlini, M., Dell'Anno, A., Corinaldesi, C., Michell, C. T., Irigoien, X., Danovaro, R., & Rodríguez-Ezpeleta, N. (2017). A bacterial community-based index to assess the ecological status of estuarine and coastal environments. *Marine Pollution Bulletin*, *114*(2), 679–688. https://doi.org/https://doi.org/10.1016/j.marpolbul.2016.10.050

Bacigalupe, L. D., Soto-Azat, C., García-Vera, C., Barría-Oyarzo, I., & Rezende, E. L. (2017). Effects of amphibian phylogeny, climate and human impact on the occurrence of the amphibian-killing chytrid fungus. *Global Change Biology*, *23*(9), 3543–3553.

Bacigalupe, L. D., Vásquez, I. A., Estay, S. A., Valenzuela-Sánchez, A., Alvarado-Rybak, M., Peñafiel-Ricaurte, A., Cunningham, A. A., & Soto-Azat, C. (2019). The amphibian-killing fungus in a biodiversity hotspot: identifying and validating high-risk areas and refugia. *Ecosphere*, *10*(5). https://doi.org/10.1002/ecs2.2724

Bailey, L. L. (2004). Evaluating elastomer marking and photo identification methods for terrestrial salamanders: marking effects and observer bias. *Herpetological Review*, *35*(1), 38.

Baitchman, E., & Stetter, M. (2014). Amphibians. In G. West, D. Heard, & N. Caulkett (Eds.), *Zoo Animal and Wildlife Immobilization and Anesthesia, 2nd Edition* (pp. 303–311). Wiley-Blackwell. https://doi.org/doi:10.1002/9781118792919.ch17

Baker, R. E., Mahmud, A. S., Miller, I. F., Rajeev, M., Rasambainarivo, F., Rice, B. L., Takahashi, S., Tatem, A. J., Wagner, C. E., Wang, L.-F., Wesolowski, A., & Metcalf, C. J. E. (2022). Infectious disease in an era of global change. *Nature Reviews Microbiology*, *20*(4), 193–205. https://doi.org/10.1038/s41579-021-00639-z

Baláž, V., Vojar, J., Civiš, P., Šandera, M., & Rozínek, R. (2014). Chytridiomycosis risk among Central European amphibians based on surveillance data. *Diseases of Aquatic Organisms*, *112*(1), 1–8. https://doi.org/10.3354/dao02799

Ballare, K. M., Pope, N. S., Castilla, A. R., Cusser, S., Metz, R. P., & Jha, S. (2019). Utilizing field collected insects for next generation sequencing: Effects of sampling, storage, and DNA extraction methods. *Ecology and Evolution*, *9*(24), 13690–13705.

Bargalló, F., Martínez–Silvestre, A., & Fernandez, D. (2016). Detección de *Batrachochytrium dendrobatidis* en anfibios asintomáticos en Cataluña. *Journal of Herpetology*, *44*, 320–326.

Barton, K. (2024). MuMIn: Multi-Model Inference. In *R package version 1.48.4. https://CRAN.R-project.org/package=MuMIn*. https://doi.org/10.32614/CRAN.package.MuMIn

Bates, K. A., Sommer, U., Hopkins, K. P., Shelton, J. M. G., Wierzbicki, C., Sergeant, C., Tapley, B., Michaels, C. J., Schmeller, D. S., Loyau, A., Bosch, J., Viant, M. R., Harrison, X. A., Garner, T. W. J., & Fisher, M. C. (2022). Microbiome function predicts amphibian chytridiomycosis disease dynamics. *Microbiome*, *10*(1). https://doi.org/10.1186/s40168-021-01215-6

Becker, C. G., Longo, A. V, Haddad, C. F. B., & Zamudio, K. R. (2017). Land cover and forest connectivity alter the interactions among host, pathogen and skin microbiome. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1861), 20170582.

Becker, C. G., Rodriguez, D., Longo, A. V, Toledo, L. F., Lambertini, C., Leite, D. S., Haddad, C. F. B., & Zamudio, K. R. (2016). Deforestation, host community structure, and amphibian disease risk. *Basic and Applied Ecology*, *17*(1), 72–80.

Becker, C. G., & Zamudio, K. R. (2011). Tropical amphibian populations experience higher disease risk in natural habitats. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(24), 9893–9898. https://doi.org/10.1073/pnas.1014497108

Beebee, T. J. C., & Griffiths, R. A. (2005). The amphibian decline crisis: A watershed for conservation biology? *Biological Conservation*, *125*(3), 271–285. https://doi.org/10.1016/j.biocon.2005.04.009

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., Minbiole, K. P. C., House, L. L., Jensen, R. V., Becker, M. H., Walke, J. B., Medina, D., Ibáñez, R., & Harris, R. N. (2015). Panamanian frog species host unique skin bacterial communities. *Frontiers in Microbiology*, *6*(OCT), 1–21. https://doi.org/10.3389/fmicb.2015.01171

Berg, C., Lerner, H., Butterworth, A., & Walzer, C. (2020). Editorial: Wildlife Welfare. In *Frontiers in Veterinary Science* (Vol. 7). Frontiers Media S.A. https://doi.org/10.3389/fvets.2020.576095

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., … Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome*, *8*(1), 1–22. https://doi.org/10.1186/s40168-020-00905-x

Berger, L., Hyatt, A. D., Speare, R., & Longcore, J. E. (2005). Life cycle stages of the amphibian chytrid Batrachochytrium dendrobatidis. *Diseases of Aquatic Organisms*, *68*(1), 51–63. https://doi.org/10.3354/dao068051

Berger, L., Roberts, A. A., Voyles, J., Longcore, J. E., Murray, K. A., & Skerratt, L. F. (2016). History and recent progress on chytridiomycosis in amphibians. *Fungal Ecology*, *19*, 89–99. https://doi.org/10.1016/j.funeco.2015.09.007

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G., & Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*, *95*(15), 9031–9036. https://doi.org/10.1073/pnas.95.15.9031

Berger, L., Speare, R., Hines, H. B., Marantelli, G., Hyatt, A. D., McDonald, K. R., Skerratt, L. F., Olsen, V., Clarke, J. M., & Gillespie, G. (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal*, *82*(7), 434–439.

Bernardo-Cravo, A. P., Schmeller, D. S., Chatzinotas, A., Vredenburg, V. T., & Loyau, A. (2020). Environmental Factors and Host Microbiomes Shape Host–Pathogen Dynamics. In *Trends in Parasitology* (Vol. 36, Issue 7, pp. 616–633). Elsevier Ltd. https://doi.org/10.1016/j.pt.2020.04.010

Beukema, W., Erens, J., Schulz, V., Stegen, G., Spitzen-van der Sluijs, A., Stark, T., Laudelout, A., Kinet, T., Kirschey, T., & Poulain, M. (2021). Landscape epidemiology of Batrachochytrium salamandrivorans: reconciling data limitations and conservation urgency. *Ecological Applications*, *31*(5), e02342.

Blaustein, A. R., Urbina, J., Snyder, P. W., Reynolds, E., Dang, T., Hoverman, J. T., Han, B., Olson, D. H., Searle, C., & Hambalek, N. M. (2018). Effects of Emerging

Infectious Diseases on Amphibians : A Review of Experimental Studies. *Diversity*, *10*(81), 3–49. https://doi.org/10.3390/d10030081

Bletz, M. C., Archer, H., Harris, R. N., McKenzie, V. J., Rabemananjara, F. C. E., Rakotoarison, A., & Vences, M. (2017). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Frontiers in Microbiology*, *8*, 1530.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., & Harris, R. N. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters*, *16*(6), 807–820. https://doi.org/10.1111/ele.12099

Blooi, M., Pasmans, F., Longcore, J. E., Spitzen-Van Der Sluijs, A., Vercammen, F., & Martel, A. (2013). Duplex real-Time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. *Journal of Clinical Microbiology*, *51*(12), 4173–4177. https://doi.org/10.1128/JCM.02313-13

Bornbusch, S. L., & Drea, C. M. (2021). Antibiotic resistance genes in lemur gut and soil microbiota along a gradient of anthropogenic disturbance. *Frontiers in Ecology and Evolution*, *9*, 704070.

Bosch, J., & Martínez-Solano, I. (2006). Cytrid fungus infection related to unusual mortalities of *Salamandra* salamandra and *Bufo bufo* in the Peñalara Natural Park, Spain. *Oryx*, *40*(1), 84–89. https://doi.org/10.1017/S0030605306000093

Bosch, J., Martínez-Solano, I., & García-París, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation*, *97*, 331–337.

Bosch, J., Sanchez-Tomé, E., Fernández-Loras, A., Oliver, J. A., Fisher, M. C., & Garner, T. W. J. (2015). Successful elimination of a lethal wildlife infectious disease in nature. *Biology Letters*, *11*(11), 20150874.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T., & Hyatt, A. D. (2004). *Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay*. *60*, 141–148.

Brannelly, L. A., Hunter, D. A., Skerratt, L. F., Scheele, B. C., Lenger, D., Mcfadden, M. S., Harlow, P. S., & Berger, L. (2016). Chytrid infection and post-release fitness in the reintroduction of an endangered alpine tree frog. *Animal Conservation*, *19*(2), 153–162. https://doi.org/10.1111/acv.12230

Brannelly, L. A., McCallum, H. I., Grogan, L. F., Briggs, C. J., Ribas, M. P., Hollanders, M., Sasso, T., Familiar López, M., Newell, D. A., & Kilpatrick, A. M. (2021). Mechanisms underlying host persistence following amphibian disease emergence determine appropriate management strategies. In *Ecology Letters* (Vol. 24, Issue 1, pp. 130–148). Blackwell Publishing Ltd. https://doi.org/10.1111/ele.13621

Brannelly, L. A., Richards-Zawacki, C. L., & Pessier, A. P. (2012). Clinical trials with itraconazole as a treatment for chytrid fungal infections in amphibians. *Diseases of Aquatic Organisms*, *101*(2), 95–104. https://doi.org/10.3354/dao02521

Briggs, C. J., Knapp, R. A., & Vredenburg, V. T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National*

Academy of Sciences, *107*(21), 9695–9700. https://doi.org/10.1073/pnas.0912886107

Brooks, J. P., Edwards, D. J., Harwich, M. D., Rivera, M. C., Fettweis, J. M., Serrano, M. G., Reris, R. A., Sheth, N. U., Huang, B., Girerd, P., & others. (2015). The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiology*, *15*(1), 1–14.

Brooks, M. E., Kristensen, K., Benthem, K. J. , van, Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Mächler, M., & Bolker, B. M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, *9*(2), 378. https://doi.org/10.32614/RJ-2017-066

Brotons, L., Pou, N., Herrando, S., Bota, G., Villero, D., Garrabou, J., Ordóñez, J. L., Anton, M., Gual, G., Recoder, L., Alcaraz, J., Pla, M., Sainz de la Maza, P., Pont, S., & Pino, J. (2020). *Estat de la Natura a Catalunya 2020. Departament de Territori i Sostenibilitat. Generalitat de Catalunya. Barcelona.*

Brown, L. J. (1997). An evaluation of some marking and trapping techniques currently used in the study of anuran population dynamics. *Journal of Herpetology*, 410–419.

Brown, R. S., Cooke, S. J., Anderson, W. G., & McKinley, R. S. (1999). Evidence to challenge the “2% rule” for biotelemetry. *North American Journal of Fisheries Management*, *19*(3), 867–871.

Burrowes, P. A., Martes, M. C., Torres-Ríos, M., & Longo, A. V. (2017). Arboreality predicts *Batrachochytrium dendrobatidis* infection level in tropical direct-developing frogs. *Journal of Natural History*, *51*(11–12), 643–656. https://doi.org/10.1080/00222933.2017.1297504

Camper, J. D., & Dixon, J. R. (1988). *Evaluation of a microchip marking system for amphibians and reptiles*. Texas Parks \& Wildlife Department.

Cashins, S. D., Grogan, L. F., McFadden, M., Hunter, D., Harlow, P. S., Berger, L., & Skerratt, L. F. (2013). Prior Infection Does Not Improve Survival against the Amphibian Disease Chytridiomycosis. *PLoS ONE*, *8*(2), 1–7. https://doi.org/10.1371/journal.pone.0056747

CBD. (2020). *Secretariat of the Convention on Biological Diversity (2020) Global Biodiversity Outlook 5. Montreal.* www.emdashdesign.ca

Ceballos, G., Ehrlich, P. R., & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(30), E6089–E6096. https://doi.org/10.1073/pnas.1704949114

Ceballos, G., Ehrlich, P. R., & Raven, P. H. (2020). Vertebrates on the brink as indicators of biological annihilation and the sixth mass extinction. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(24), 13596–13602. https://doi.org/10.1073/pnas.1922686117

Cheng, H., Sun, Y., Yang, Q., Deng, M., Yu, Z., Zhu, G., Qu, J., Liu, L., Yang, L., & Xia, Y. (2022). A rapid bacterial pathogen and antimicrobial resistance diagnosis workflow using Oxford nanopore adaptive sequencing method. *Briefings in Bioinformatics*.

Christy, M. (1996). The efficacy of using Passive Integrated Transponder (PIT) tags without anaesthetic in free-living frogs. *Australian Zoologist*, *30*(2), 139–142.

Clare, F. C. (2014). *Quantifying the impact of infection by* Batrachochytrium dendrobatidis *on montane populations of* Alytes obstetricans *(Doctoral dissertation, Imperial College London).*

Clare, F. C., Halder, J. B., Daniel, O., Bielby, J., Semenov, M. A., Jombart, T., Loyau, A., Schmeller, D. S., Cunningham, A. A., Rowcliffe, M., Garner, T. W. J., Bosch, J., & Fisher, M. C. (2016). Climate forcing of an emerging pathogenic fungus across a montane multi-host community. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1709). https://doi.org/10.1098/rstb.2015.0454

Collins, J. P., & Storfer, A. (2003). Global amphibian declines: Sorting the hypotheses. *Diversity and Distributions*, *9*(2), 89–98. https://doi.org/10.1046/j.1472-4642.2003.00012.x

Cooke, S. J., Midwood, J. D., Thiem, J. D., Klimley, P., Lucas, M. C., Thorstad, E. B., Eiler, J., Holbrook, C., & Ebner, B. C. (2013). Tracking animals in freshwater with electronic tags: past, present and future. *Animal Biotelemetry*, *1*(1), 1–19.

Cooke, S. J., Woodley, C. M., Brad Eppard, M., Brown, R. S., & Nielsen, J. L. (2011). Advancing the surgical implantation of electronic tags in fish: a gap analysis and research agenda based on a review of trends in intracoelomic tagging effects studies. *Reviews in Fish Biology and Fisheries*, *21*(1), 127–151.

Cortés Pérez, E., & Maldonado Reséndiz, R. I. (2023). Welfare Indices in Anurans under Human Care. In *Journal of Zoological and Botanical Gardens* (Vol. 4, Issue 4, pp. 613–622). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/jzbg4030043

Costanza, R., de Groot, R., Sutton, P. C., van der Ploeg, S., Anderson, S., Kubiszewski, I., Farber, S., & Turner, R. K. (2014). Changes in the global value of ecosystem services. *Global Environmental Change*, *26*, 152–158. https://doi.org/10.1016/j.gloenvcha.2014.04.002

Cowie, R. H., Bouchet, P., & Fontaine, B. (2022). The Sixth Mass Extinction: fact, fiction or speculation? *Biological Reviews*, *97*(2), 640–663. https://doi.org/10.1111/brv.12816

Cunningham, A. A., Daszak, P., & Wood, J. L. N. (2017). One health, emerging infectious diseases and wildlife: Two decades of progress? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1725). https://doi.org/10.1098/rstb.2016.0167

Cunningham, M. M., Tran, L., McKee, C. G., Polo, R. O., Newman, T., Lansing, L., Griffiths, J. S., Bilodeau, G. J., Rott, M., & Guarna, M. M. (2022). Honey bees as biomonitors of environmental contaminants, pathogens, and climate change. *Ecological Indicators*, *134*, 108457.

Dalibard, M., Buisson, L., Calvez, O., Nguyen-hong, M., Trochet, A., & Laffaille, P. (2021). Can ventral pattern be used for individual recognition of the vulnerable Pyrenean brook newt (*Calotriton asper*)? *Herpetological Journal*, *31*, 99–110.

Danilevsky, A., Polsky, A. L., & Shomron, N. (2022). Adaptive sequencing using nanopores and deep learning of mitochondrial DNA. *Briefings in Bioinformatics*, *23*(4).

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science*, *287*(5452), 443–449. https://doi.org/10.1126/science.287.5452.443

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2003). Infectious disease and amphibian population declines. *Diversity and Distributions*, *9*(2), 141–150. https://doi.org/10.1046/j.1472-4642.2003.00016.x

De Castro, F., & Bolker, B. (2005). Mechanisms of disease-induced extinction. *Ecology Letters*, *8*(1), 117–126.

De León, L. F., Silva, B., Avilés-Rodríguez, K. J., & Buitrago-Rosas, D. (2023). Harnessing the omics revolution to address the global biodiversity crisis. *Current Opinion in Biotechnology*, *80*, 102901. https://doi.org/10.1016/j.copbio.2023.102901

Dell'Anno, A., Mei, M. L., Ianni, C., & Danovaro, R. (2003). Impact of bioavailable heavy metals on bacterial activities in coastal marine sediments. *World Journal of Microbiology and Biotechnology*, *19*(1), 93–100.

Departament Acció Climàtica, A. i A. R., & Generalitat de Catalunya. (2023). *Protected and threatened species of fauna of Catalonia*. Https://Mediambient.Gencat.Cat/ca/05_ambits_dactuacio/Patrimoni_natural/Fauna-Autoctona-Protegida/Gestio-Especies-Protegides-Amenacades/.

Díaz-Vallejo, E. J., Seeley, M., Smith, A. P., & Marín-Spiotta, E. (2021). A meta-analysis of tropical land-use change effects on the soil microbiome: Emerging patterns and knowledge gaps. *Biotropica*, *53*(3), 738–752.

Doddington, B. J., Bosch, J., Oliver, J. A., Grassly, N. C., Garcia, G., Schmidt, B. R., Garner, T. W. J., & Fisher, M. C. (2013). Context-dependent amphibian host population response to an invading pathogen. *Ecology*, *94*(8), 1795–1804. https://doi.org/10.1890/12-1270.1

Doody, J. S., Roe, J., Mayes, P., & Ishiyama, L. (2009). Telemetry tagging methods for some freshwater reptiles. *Marine and Freshwater Research*, *60*(4), 293–298.

DuRant, S. E., & Hopkins, W. A. (2008). Amphibian predation on larval mosquitoes. *Canadian Journal of Zoology*, *86*(10), 1159–1164.

Ebner, B. C. (2009). Preface. Tagging for telemetry of freshwater fauna. *Marine and Freshwater Research*, *60*(4), 281–283.

ECDC. (2024). *European Centre for Disease Prevention and Control (ECDC). Retrieved from https://www.ecdc.europa.eu/en*.

Elinson, R. P., & del Pino, E. M. (2012). Developmental diversity of amphibians. *Wiley Interdisciplinary Reviews: Developmental Biology*, *1*(3), 345–369. https://doi.org/10.1002/wdev.23

Estrada, A., Hughey, M. C., Medina, D., Rebollar, E. A., Walke, J. B., Harris, R. N., & Belden, L. K. (2019). Skin bacterial communities of neotropical treefrogs vary with local environmental conditions at the time of sampling. *PeerJ*, *7*, e7044.

Evans, T., Olson, S., Watson, J., Gruetzmacher, K., Pruvot, M., Jupiter, S., Wang, S., Clements, T., & Jung, K. (2020). Links between ecological integrity, emerging infectious diseases originating from wildlife, and other aspects of human health-an overview of the literature. *Wildlife Conservation Society*, *4*, e303.

Farrer, R. A., Martel, A., Verbrugghe, E., Abouelleil, A., Ducatelle, R., Longcore, J. E., James, T. Y., Pasmans, F., Fisher, M. C., & Cuomo, C. A. (2017). Genomic innovations linked to infection strategies across emerging pathogenic chytrid fungi. *Nature Communications*, *8*, 1–11. https://doi.org/10.1038/ncomms14742

Faust, K., Lahti, L., Gonze, D., de Vos, W. M., & Raes, J. (2015). Metagenomics meets time series analysis: Unraveling microbial community dynamics. *Current Opinion in Microbiology*, *25*(May), 56–66. https://doi.org/10.1016/j.mib.2015.04.004

Fernández-Beaskoetxea, S., Bosch, J., & Bielby, J. (2016). Infection and transmission heterogeneity of a multi-host pathogen (*Batrachochytrium dendrobatidis*) within an amphibian community. *Diseases of Aquatic Organisms*, *118*(1), 11–20. https://doi.org/10.3354/dao02963

Ferner, J. W. (2010). Measuring and marking post-metamorphic amphibians. *Amphibian Ecology and Conservation: A Handbook of Techniques*, 123–141.

Fisher, M. C., & Garner, T. W. J. (2020). Chytrid fungi and global amphibian declines. *Nature Reviews Microbiology*, *18*(6), 332–343. https://doi.org/10.1038/s41579-020-0335-x

Fisher, M. C., Garner, T. W. J., & Walker, S. F. (2009). Global emergence of <i>Batrachochytrium dendrobatidis</iZ and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology*, *63*(1), 291–310.

Flechas, S. V., Paz, A., Crawford, A. J., Sarmiento, C., Acevedo, A. A., Arboleda, A., Bolívar-García, W., Echeverry-Sandoval, C. L., Franco, R., Mojica, C., Muñoz, A., Palacios-Rodríguez, P., Posso-Terranova, A. M., Quintero-Marín, P., Rueda-Solano, L. A., Castro-Herrera, F., & Amézquita, A. (2017). Current and predicted distribution of the pathogenic fungus *Batrachochytrium dendrobatidis* in Colombia, a hotspot of amphibian biodiversity. *Biotropica*, *49*(5), 685–694. https://doi.org/10.1111/btp.12457

Frost, D. R. (2024). *Amphibian Species of the World: an Online Reference. Version 6.2 (accessed 01 June 2024). Electronic Database accessible at https://amphibiansoftheworld.amnh.org/index.php. American Museum of Natural History, New York, USA.* https://doi.org/doi.org/10.5531/db.vz.0001

Galbraith, E., & Convertino, M. (2021). The Eco-Evo Mandala: Simplifying Bacterioplankton Complexity into Ecohealth Signatures. *Entropy*, *23*(11), 1471.

García-Salmerón, A., Maluquer-Margalef, J., Rivera, X., & Pujol-Buxó, E. (2022). *Els amfibis dels Països Catalans*. Brau edicions.

Gargaglioni, L. H., & Milsom, W. K. (2007). Control of breathing in anuran amphibians. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *147*(3), 665–684. https://doi.org/10.1016/j.cbpa.2006.06.040

Garner, T. W. J., Schmidt, B. R., Martel, A., Pasmans, F., Muths, E., Cunningham, A. A., Weldon, C., Fisher, M. C., & Bosch, J. (2016). Mitigating amphibian chytridiomycoses in nature. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1709), 20160207.

Gascon, C. (2007). *Amphibian conservation action plan: proceedings IUCN/SSC Amphibian Conservation Summit 2005*. IUCn.

Geiger, C. C., Bregnard, C., Maluenda, E., Voordouw, M. J., & Schmidt, B. R. (2017). Antifungal treatment of wild amphibian populations caused a transient reduction in the prevalence of the fungal pathogen, Batrachochytrium dendrobatidis. *Scientific Reports*, *7*(1), 1–12. https://doi.org/10.1038/s41598-017-05798-9

Gibbons, W. J., & Andrews, K. M. (2004). PIT Tagging: Simple Technology at Its Best. *BioScience*, *54*(5), 447–454. https://doi.org/10.1641/0006-3568(2004)054[0447:PTSTAI]2.0.CO;2

Gilbert, M. J., Spitzen-van der Sluijs, A. M., Canessa, S., Bosch, J., Cunningham, A. A., Grasselli, E., Laudelout, A., Lötters, S., Miaud, C., Salvidio, S., Veith, M., Martel, A., & Pasmans, F. (2020). *Mitigating Batrachochytrium salamandrivorans in Europe. Batrachochytrium salamandrivorans Action Plan for European urodeles. Nijmegen, the Netherlands*.

Glasl, B., Bourne, D. G., Frade, P. R., Thomas, T., Schaffelke, B., & Webster, N. S. (2019). Microbial indicators of environmental perturbations in coral reef ecosystems. *Microbiome*, *7*(1), 94. https://doi.org/10.1186/s40168-019-0705-7

Goodwin, K. B., Hutchinson, J. D., & Gompert, Z. (2022). Spatiotemporal and ontogenetic variation, microbial selection, and predicted Bd-inhibitory function in the skin-associated microbiome of a Rocky Mountain amphibian. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.1020329

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, *16*(3), 183–190.

Graham, N. A. J., Wilson, S. K., Jennings, S., Polunin, N. V. C., Bijoux, J. P., & Robinson, J. (2006). Dynamic fragility of oceanic coral reef ecosystems. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(22), 8425–8429. https://doi.org/10.1073/pnas.0600693103

Grant, E. H. C., Miller, D. A. W., Schmidt, B. R., Adams, M. J., Amburgey, S. M., Chambert, T., Cruickshank, S. S., Fisher, R. N., Green, D. M., Hossack, B. R., Johnson, P. T. J., Joseph, M. B., Rittenhouse, T. A. G., Ryan, M. E., Waddle, J. H., Walls, S. C., Bailey, L. L., Fellers, G. M., Gorman, T. A., ... Muths, E. (2016). Quantitative evidence for the effects of multiple drivers on continental-scale amphibian declines. *Scientific Reports*, *6*(May), 1–9. https://doi.org/10.1038/srep25625

Gray, J. S., Dautel, H., Estrada-Peña, A., Kahl, O., & Lindgren, E. (2009). Effects of Climate Change on Ticks and Tick-Borne Diseases in Europe. *Interdisciplinary Perspectives on Infectious Diseases*, *2009*, 1–12. https://doi.org/10.1155/2009/593232

Greener, M. S., Verbrugghe, E., Kelly, M., Blooi, M., Beukema, W., Canessa, S., Carranza, S., Croubels, S., De Troyer, N., Fernandez-Giberteau, D., Goethals, P., Lens, L., Li, Z., Stegen, G., Strubbe, D., van Leeuwenberg, R., Van Praet, S., Vila-Escale, M., Vervaeke, M., ... Martel, A. (2020). Presence of low virulence chytrid fungi could protect European amphibians from more deadly strains. *Nature Communications*, *11*(1), 1–11. https://doi.org/10.1038/s41467-020-19241-7

Griffith, D. M., Veech, J. A., & Marsh, C. J. (2016). cooccur: Probabilistic Species Co-Occurrence Analysis in R. *Journal of Statistical Software*, *69*(1), 1–17. https://doi.org/10.18637/jss.v069.c02

Grogan, L. F., Mangan, M. J., & McCallum, H. I. (2023). Amphibian infection tolerance to chytridiomycosis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *378*(1882). https://doi.org/10.1098/rstb.2022.0133

Grogan, L. F., Robert, J., Berger, L., Skerratt, L. F., Scheele, B. C., Castley, J. G., Newell, D. A., & Mccallum, H. I. (2018). Review of the Amphibian Immune Response to Chytridiomycosis, and Future Directions. *Frontiers in Immunology*, *9:2536*. https://doi.org/10.3389/fimmu.2018.02536

Gweon, H. S., Shaw, L. P., Swann, J., De Maio, N., AbuOun, M., Niehus, R., Hubbard, A., Bowes, M. J., Bailey, M. J., Peto, T. E. A., & others. (2019). The impact of sequencing depth on the inferred taxonomic composition and AMR gene content of metagenomic samples. *Environmental Microbiome*, *14*(1), 1–15.

Halpern, B. S., Longo, C., Hardy, D., McLeod, K. L., Samhouri, J. F., Katona, S. K., Kleisner, K., Lester, S. E., Oleary, J., Ranelletti, M., Rosenberg, A. A., Scarborough, C., Selig, E. R., Best, B. D., Brumbaugh, D. R., Chapin, F. S., Crowder, L. B., Daly, K. L., Doney, S. C., … Zeller, D. (2012). An index to assess the health and benefits of the global ocean. *Nature*, *488*(7413), 615–620. https://doi.org/10.1038/nature11397

Hamer, A. J., & McDonnell, M. J. (2008). Amphibian ecology and conservation in the urbanising world: a review. *Biological Conservation*, *141*(10), 2432–2449.

Hanlon, S. M., Lynch, K. J., Kerby, J., & Parris, M. J. (2015). Batrachochytrium dendrobatidis exposure effects on foraging efficiencies and body size in anuran tadpoles. *Diseases of Aquatic Organisms*, *112*(3), 237–242. https://doi.org/10.3354/dao02810

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V. T., & Minbiole, K. P. C. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME Journal*, *3*(7), 818–824. https://doi.org/10.1038/ismej.2009.27

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., & Alford, R. A. (2009). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Diseases of Aquatic Organisms*, *83*(1), 11–16. https://doi.org/10.3354/dao02004

Hartig, F. (2024). DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. In *R package version 0.4.7. https://CRAN.R-project.org/package=DHARMa*. https://doi.org/10.32614/CRAN.package.DHARMa

Heard, G. W., Scroggie, M. P., Ramsey, D. S. L., Clemann, N., Hodgson, J. A., & Thomas, C. D. (2018). Can Habitat Management Mitigate Disease Impacts on Threatened Amphibians? *Conservation Letters*, *11*(2), 1–10. https://doi.org/10.1111/conl.12375

Heemeyer, J. L., Homyack, J. A., & Haas, C. A. (2007). Retention and readability of visible implant elastomer marks in eastern red-backed salamanders (Plethodon cinereus). *Herpetological Review*, *38*(4), 425.

Hocking, D. J., & Babbitt, K. J. (2014). Amphibian Contributions to Ecosystem Services. *Herpetological Conservation and Biology*, *9*(1), 1–17.

Hollanders, M., Grogan, L. F., McCallum, H. I., & Newell, D. A. (2024). High chytrid prevalence and infection intensities in tadpoles of *Mixophyes fleayi*. *Wildlife Research*, *51*(10).

Hudson, M. A., Young, R. P., Lopez, J., Martin, L., Fenton, C., McCrea, R., Griffiths, R. A., Adams, S. L., Gray, G., Garcia, G., & Cunningham, A. A. (2016). In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis epidemic. *Biological Conservation*, *195*, 37–45. https://doi.org/10.1016/j.biocon.2015.12.041

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F., & Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of

Batrachochytrium dendrobatidis. *Diseases of Aquatic Organisms*, *73*(3), 175–192. https://doi.org/10.3354/dao073175

IPBES. (2019). *Global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. E. S. Brondizio, J. Settele, S. Díaz, and H. T. Ngo (editors). IPBES secretariat, Bonn, Germany. 1148 pages. https://doi.org/10.5281/zenodo.3831673*.

IUCN. (2021). *The IUCN Red List of Threatened Species. Version 2021-3. https://www.iucnredlist.org. Accessed on 5 March 2022*.

IUCN. (2024). *The IUCN Red List of Threatened Species. Version 2023-1. https://www.iucnredlist.org. Accessed on 06 June 2024*.

IUCN SSC Amphibian Specialist Group. (2022). *Calotriton arnoldi. The IUCN Red List of Threatened Species 2022: E.T136131A89696462. Https://Dx.Doi.Org/10.2305/IUCN.UK.2022-1.RLTS.T136131A89696462.En. Accessed on 02 September 2024*.

IUCN-CEM. (2022). *The IUCN Red List of Ecosystems. Version 2022-1. <http://iucnrle.org>. Accessed on 12 of July 2024.r*.

IUCN/SSC. (2013). Guidelines for Reintroductions and Other Conservation Translocations IUCN. In *Ecologial Applications*. Gland, Switzerland: IUCN Species Survival Commission, viiii + 57 pp.

James, T. Y., Toledo, L. F., Rödder, D., da Silva Leite, D., Belasen, A. M., Betancourt-Román, C. M., Jenkinson, T. S., Soto-Azat, C., Lambertini, C., Longo, A. V., Ruggeri, J., Collins, J. P., Burrowes, P. A., Lips, K. R., Zamudio, K. R., & Longcore, J. E. (2015). Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: Lessons from the first 15 years of amphibian chytridiomycosis research. *Ecology and Evolution*, *5*(18), 4079–4097. https://doi.org/10.1002/ece3.1672

Jani, A. J., & Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(47), E5049–E5058. https://doi.org/10.1073/pnas.1412752111

Jani, A. J., Knapp, R. A., & Briggs, C. J. (2017). Epidemic and endemic pathogen dynamics correspond to distinct host population microbiomes at a landscape scale. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1857). https://doi.org/10.1098/rspb.2017.0944

Jansson, J. K., & Hofmockel, K. S. (2020). Soil microbiomes and climate change. *Nature Reviews Microbiology*, *18*(1), 35–46. https://doi.org/10.1038/s41579-019-0265-7

Jepsen, N., Schreck, C., Clements, S., & Thorstad, E. B. (2005). A brief discussion on the 2% tag/bodymass rule of thumb. *Aquatic Telemetry: Advances and Applications*, 255–259.

Jiménez, R. R., Alvarado, G., Estrella, J., & Sommer, S. (2019). Moving Beyond the Host: Unraveling the Skin Microbiome of Endangered Costa Rican Amphibians. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02060

Jiménez, R. R., Alvarado, G., Sandoval, J., & Sommer, S. (2020). Habitat disturbance influences the skin microbiome of a rediscovered neotropical-montane frog. *BMC Microbiology*, *20*(1). https://doi.org/10.1186/s12866-020-01979-1

Jiménez, R. R., & Sommer, S. (2017). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. In *Biodiversity and Conservation* (Vol. 26, Issue 4, pp. 763–786). Springer Netherlands. https://doi.org/10.1007/s10531-016-1272-x

Jin, L., Gerson, J. R., Rocca, J. D., Bernhardt, E. S., & Simonin, M. (2022). Alkaline mine drainage drives stream sediment microbial community structure and function. *Science of The Total Environment*, *805*, 150189. https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.150189

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, *451*(7181), 990–993. https://doi.org/10.1038/nature06536

Jovel, J., Patterson, J., Wang, W., Hotte, N., O'Keefe, S., Mitchel, T., Perry, T., Kao, D., Mason, A. L., Madsen, K. L., & others. (2016). Characterization of the gut microbiome using 16S or shotgun metagenomics. *Frontiers in Microbiology*, *7*, 459.

Kai, S., Matsuo, Y., Nakagawa, S., Kryukov, K., Matsukawa, S., Tanaka, H., Iwai, T., Imanishi, T., & Hirota, K. (2019). Rapid bacterial identification by direct PCR amplification of 16S rRNA genes using the MinION™ nanopore sequencer. *FEBS Open Bio*, *9*(3), 548–557.

Kakumanu, M. L., Reeves, A. M., Anderson, T. D., Rodrigues, R. R., & Williams, M. A. (2016). Honey bee gut microbiome is altered by in-hive pesticide exposures. *Frontiers in Microbiology*, *7*, 1255.

Kastellorizios, M., Tipnis, N., & Burgess, D. J. (2015). Foreign body reaction to subcutaneous implants. *Immune Responses to Biosurfaces*, 93–108.

Katz, T. S., & Zellmer, A. J. (2018). Comparison of model selection technique performance in predicting the spread of newly invasive species: a case study with Batrachochytrium salamandrivorans. *Biological Invasions*, *20*, 2107–2119.

Kearney, M. R., Isaac, A. P., & Porter, W. P. (2014). microclim: Global estimates of hourly microclimate based on long-term monthly climate averages. *Scientific Data*, *1*(1), 140006. https://doi.org/10.1038/sdata.2014.6

Keesing, F., Holt, R. D., & Ostfeld, R. S. (2006). Effects of species diversity on disease risk. *Ecology Letters*, *9*(4), 485–498.

Keesing, F., & Ostfeld, R. S. (2012). An ecosystem service of biodiversity: the protection of human health against infectious disease. In A. Aguirre, R. S. Ostfeld, & P. Daszak (Eds.), *Directions in Conservation Medicine: applied cases of ecological health.* (pp. 56–66). New Oxford University Press.

Khot, V., Strous, M., & Hawley, A. K. (2020). Computational approaches in viral ecology. *Computational and Structural Biotechnology Journal*, *18*, 1605–1612.

Konowalik, A., Najbar, A., Konowalik, K., Dylewski, Ł., Frydlewicz, M., Kisiel, P., Starzecka, A., Zaleśna, A., & Kolenda, K. (2020). Amphibians in an urban environment: a case study from a central European city (Wrocław, Poland). *Urban Ecosystems*, *23*(2), 235–243.

Kruger, A. (2020). Frog Skin Microbiota Vary With Host Species and Environment but Not Chytrid Infection. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01330

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., & McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology*, *23*(6), 1238–1250. https://doi.org/10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R., & McKenzie, V. J. (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1839), 20161553. https://doi.org/10.1098/rspb.2016.1553

Kühnel, S., Reinhard, S., & Kupfer, A. (2010). Evolutionary reproductive morphology of amphibians: an overview. *Bonn Zoological Bulletin*, *57*(2), 119–126.

Kuzmina, L. Y., Gilvanova, E. A., Galimzyanova, N. F., Arkhipova, T. N., Ryabova, A. S., Aktuganov, G. E., Sidorova, L. V, Kudoyarova, G. R., & Melent'ev, A. I. (2022). Characterization of the novel plant growth-stimulating strain *Advenella kashmirensis* IB-K1 and evaluation of its efficiency in saline soil. *Microbiology*, *91*(2), 173–183.

Larsen, E. H. (2021). Dual skin functions in amphibian osmoregulation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *253*, 110869. https://doi.org/https://doi.org/10.1016/j.cbpa.2020.110869

Latorre-Pérez, A., Gimeno-Valero, H., Tanner, K., Pascual, J., Vilanova, C., & Porcar, M. (2021). A round trip to the desert: in situ nanopore sequencing informs targeted bioprospecting. *Frontiers in Microbiology*, *12*, 768240.

Laudet, V. (2011). The origins and evolution of vertebrate metamorphosis. *Current Biology*, *21*(18), R726–R737. https://doi.org/10.1016/j.cub.2011.07.030

Lawler, O. K., Allan, H. L., Baxter, P. W. J., Castagnino, R., Tor, M. C., Dann, L. E., Hungerford, J., Karmacharya, D., Lloyd, T. J., & López-Jara, M. J. (2021). The COVID-19 pandemic is intricately linked to biodiversity loss and ecosystem health. *The Lancet Planetary Health*, *5*(11), e840–e850.

Lawson, L. A., Atkinson, C. L., & Jackson, C. R. (2022). The gut bacterial microbiome of the Threeridge mussel, Amblema plicata, varies between rivers but shows a consistent core community. *Freshwater Biology*.

Lear, G., Kingsbury, J. M., Franchini, S., Gambarini, V., Maday, S. D. M., Wallbank, J. A., Weaver, L., & Pantos, O. (2021). Plastics and the microbiome: impacts and solutions. *Environmental Microbiome*, *16*(1), 2. https://doi.org/10.1186/s40793-020-00371-w

Li, H. (2021). New strategies to improve minimap2 alignment accuracy. *Bioinformatics*, *37*(23), 4572–4574. https://doi.org/10.1093/bioinformatics/btab705

Life Tritó Montseny. (n.d.). *Life Tritó Montseny website*. Https://Lifetritomontseny.Eu.

Lightfoot, N. (2003). Bacteria of potential health concern. *World Health Organization (WHO) Heterotrophic Plate Counts and Drinking-Water Safety. IWA Publishing, London*, 61–79.

Lips, K. R. (2016). Overview of chytrid emergence and impacts on amphibians. In *Philosophical Transactions of the Royal Society B: Biological Sciences* (Vol. 371, Issue 1709). Royal Society of London. https://doi.org/10.1098/rstb.2015.0465

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P., & Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National*

Academy of Sciences, *103*(9), 3165–3170. https://doi.org/10.1073/pnas.0506889103

Liu, X., Rohr, J. R., & Li, Y. (2013). Climate, vegetation, introduced hosts and trade shape a global wildlife pandemic. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1753). https://doi.org/10.1098/rspb.2012.2506

Lobato-Bailón, L., García-Ulloa, M., Santos, A., Guixé, D., Camprodon, J., Florensa-Rius, X., Molleda, R., Manzano, R., Ribas, M. P., Espunyes, J., & others. (2023). The fecal bacterial microbiome of the Kuhl's pipistrelle bat (Pipistrellus kuhlii) reflects landscape anthropogenic pressure. *Animal Microbiome*, *5*(1), 1–14.

Longcore, J. E., Pessier, A. P., & Nichols, D. K. (1999). Batrachochytrium Dendrobatidis gen. et sp. nov., a Chytrid Pathogenic to Amphibians. *Mycologia*, *91*(2), 219. https://doi.org/10.2307/3761366

Longo, A. V., Fleischer, R. C., & Lips, K. R. (2019). Double trouble: co-infections of chytrid fungi will severely impact widely distributed newts. *Biological Invasions*, *21*(6), 2233–2245. https://doi.org/10.1007/s10530-019-01973-3

Longo, A. V, Savage, A. E., Hewson, I., & Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *Royal Society Open Science*, *2*(7), 140377.

Longo, A. V., & Zamudio, K. R. (2017). Temperature variation, bacterial diversity and fungal infection dynamics in the amphibian skin. *Molecular Ecology*, *26*(18), 4787–4797. https://doi.org/10.1111/mec.14220

Lötters, S., Wagner, N., Kerres, A., Vences, M., Steinfartz, S., Sabino-Pinto, J., Seufer, L., Preissler, K., Schulz, V., & Veith, M. (2018). First report of host co-infection of parasitic amphibian chytrid fungi. *Salamandra*, *54*(4), 287–290.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220–230.

Lu, Y., Zhou, G., Ewald, J., Pang, Z., Shiri, T., & Xia, J. (2023). MicrobiomeAnalyst 2.0: comprehensive statistical, functional and integrative analysis of microbiome data. *Nucleic Acids Research*, *51*(W1), W310–W318. https://doi.org/10.1093/nar/gkad407

Lyon, J. P., Hale, R., Kitchingman, A., O'Connor, J., Sharley, J., & Tonkin, Z. (2019). Effects of tag type, morphological location and tagger experience on tag retention rates in freshwater fishes. *Marine and Freshwater Research*, *70*(6), 891–895.

Mace, G. M., Masundire, H., & Baillie, J. E. M. (2005). *Biodiversity in Ecosystems and Human Well-Being: Current State and Trends Vol. 1, Ch. 4 (Millennium Ecosystem Assessment Series, Island Press)*.

Malacrinò, A., Sadowski, V. A., Martin, T. K., De Oliveira, N. C., Brackett, I. J., Feller, J. D., Harris, K. J., Heredia, O. C., Vescio, R., & Bennett, A. E. (2020). Biological invasions alter environmental microbiomes: A meta-analysis. *PLoS ONE*, *15*(10 October), 1–12. https://doi.org/10.1371/journal.pone.0240996

Malik, H., Ratovonamana, Y. R., Rakotondranary, S. J., Ganzhorn, J. U., Sommer, S., & others. (2022). Anthropogenic disturbance impacts gut microbiome homeostasis in a Malagasy primate. *Frontiers in Microbiology*, *13*.

Marantelli, G., Berger, L., Speare, R., & Keegan, L. (2004). Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conservation Biology*, *10*(3), 173–179.

Marijon, P., Chikhi, R., & Varré, J.-S. (2020). yacrd and fpa: upstream tools for long-read genome assembly. *Bioinformatics*, *36*(12), 3894–3896. https://doi.org/10.1093/bioinformatics/btaa262

Markert, B., & Wünschmann, S. (2011). Bioindicators and biomonitors: Use of organisms to observe the influence of chemicals on the environment. In *Organic Xenobiotics and Plants* (pp. 217–236). Springer.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., Farrer, R. A., Schmidt, B. R., Tobler, U., Goka, K., Lips, K. R., Muletz, C., Zamudio, K. R., Bosch, J., Lötters, S., Wombwell, E., Garner, T. W. J., Cunningham, A. A., Spitzen-Van Der Sluijs, A., … Pasmans, F. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*, *346*(6209), 630–631. https://doi.org/10.1126/science.1258268

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F., & Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*, *110*(38), 15325–15329. https://doi.org/10.1073/pnas.1307356110

Martel, A., Vila-Escale, M., Fernández-Giberteau, D., Martinez-Silvestre, A., Canessa, S., Van Praet, S., Pannon, P., Chiers, K., Ferran, A., Kelly, M., Picart, M., Piulats, D., Li, Z., Pagone, V., Pérez-Sorribes, L., Molina, C., Tarragó-Guarro, A., Velarde-Nieto, R., Carbonell, F., … Pasmans, F. (2020). Integral chain management of wildlife diseases. *Conservation Letters*, *13*(2), 1–6. https://doi.org/10.1111/conl.12707

Martin, S., Heavens, D., Lan, Y., Horsfield, S., Clark, M. D., & Leggett, R. M. (2022). Nanopore adaptive sampling: a tool for enrichment of low abundance species in metagenomic samples. *Genome Biology*, *23*(1), 1–27.

Martínez-Silvestre, A., Herpétologique, S., Shf, D. F., Calvez, O., Aubret, F., Mossoll-torres, M., Brien, D. O., & Ventura, M. (2020). Presence of the Fungus *Batrachochytrium dendrobatidis*, but not *Batrachochytrium salamandrivorans*, in Wild Pyrenean Brook Newts (*Calotriton asper*) in Spain and France. *Herpetological Review*, *51*(4), 738–743.

Martínez-Silvestre, A., Loras-Ortí, F., Garcia-Salmeron, A., Pujol-Buxó, E., Pérez-Novo, I., Maluquer-Margalef, J., Poch, S., Thumsová, B., & Bosch, J. (2023). Introduced Mediterranean painted frogs (*Discoglossus pictus*) are possible supershedders of the fungus *Batrachochytrium dendrobatidis* in Catalonia (NE Spain). *Amphibia-Reptilia*, *44*(2), 257–261.

Martinez-Silvestre, A., Velarde, R., Marschang, R. E., Pérez-Novo, I., Bisbal-Chinesta, J. F., Thumsová, B., & Bosch, J. (2021). First record of amphibian mortality associated with the fungus *Batrachochytrium dendrobatidis* in Catalonia (NE Spain). *Basic and Applied Herpetology*, *35*, 5–17. https://doi.org/10.11160/bah.210

McCallum, M. L. (2015). Vertebrate biodiversity losses point to a sixth mass extinction. *Biodiversity and Conservation*, *24*(10), 2497–2519. https://doi.org/10.1007/s10531-015-0940-6

McCarthy, M. A., Weller, W. F., & Parris, K. M. (2009). Effects of toe clipping on survival, recapture, and return rates of Jefferson Salamanders (Ambystoma jeffersonianum) in Ontario, Canada. *Journal of Herpetology*, *43*(3), 394–401.

McCoy, K. A., & Peralta, A. L. (2018). Pesticides could alter amphibian skin microbiomes and the effects of *Batrachochytrium dendrobatidis*. *Frontiers in Microbiology*, *9*, 748.

McDonald, C. A., Longo, A. V., Lips, K. R., & Zamudio, K. R. (2020). Incapacitating effects of fungal coinfection in a novel pathogen system. *Molecular Ecology*, *29*(17), 3173–3186. https://doi.org/10.1111/mec.15452

McDonald, T. L., Amstrup, S. C., & Manly, B. F. J. (2003). Tag loss can bias Jolly-Seber capture-recapture estimates. *Wildlife Society Bulletin*, *31*(3), 814–822. http://www.jstor.org/stable/3784604

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., & Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME Journal*, *6*(3), 588–596. https://doi.org/10.1038/ismej.2011.129

McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., Halstead, N. T., Lentz, G., Tenouri, N., Young, S., Civitello, D. J., Ortega, N., Fites, J. S., Reinert, L. K., Rollins-Smith, L. A., Raffel, T. R., & Rohr, J. R. (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature*, *511*(7508), 224–227. https://doi.org/10.1038/nature13491

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Mettee Zarecki, S. L., Bennett, S. D., Hall, J., Yaeger, J., Lujan, K., MPH, R., Adams-Cameron, M., Winpisinger Quinn, K., Brenden, R., Biggerstaff, G., Hill, V. R., Sholtes, K., Garrett, N. M., Lafon, P. C., Barton Behravesh, C., & Sodha, S. V. (2013). US Outbreak of Human *Salmonella* Infections Associated With Aquatic Frogs, 2008–2011. *Pediatrics*, *131*(4), 724–731. https://doi.org/10.1542/peds.2012-2031

Millennium Ecosystem Assessment. (2005). Ecosystems and Human Well-being: Biodiversity Synthesis. World Resources Institute, Washington, DC. In *World Resources Institute, Washington, DC.*

Mitchell, M. A. (2011). Zoonotic Diseases Associated with Reptiles and Amphibians: An Update. *Veterinary Clinics of North America: Exotic Animal Practice*, *14*(3), 439–456. https://doi.org/10.1016/j.cvex.2011.05.005

Montori, A., San Sebastián, O., Franch, M., Pujol-Buxó, E., Llorente, G. A., Fernández-Loras, A., Richter-Boix, À., & Bosch, J. (2019). Observations on the intensity and prevalence of *Batrachochytridium dendrobatidis* in sympatric and allopatric *Epidalea calamita* (native) and *Discoglossus pictus* (invasive) populations. *Basic and Applied Herpetology*, *33*, 5–17. https://doi.org/10.11160/bah.137

Moon, L. M., Butler, M., & Campbell, L. G. (2022). Evaluation of Tagging Methods for Unique Identification of Individuals in Three Aquatic Eurycea Salamander Species. *Ichthyology & Herpetology*, *110*(1), 77–86. https://doi.org/10.1643/h2021042

Mugge, R. L., Salerno, J. L., & Hamdan, L. J. (2021). Microbial functional responses in marine biofilms exposed to deepwater horizon spill contaminants. *Frontiers in Microbiology*, *12*, 636054.

Muletz Wolz, C. R., Yarwood, S. A., Campbell Grant, E. H., Fleischer, R. C., & Lips, K. R. (2018). Effects of host species and environment on the skin microbiome of Plethodontid salamanders. *Journal of Animal Ecology*, *87*(2), 341–353. https://doi.org/10.1111/1365-2656.12726

Muletz-Wolz, C. R., Fleischer, R. C., & Lips, K. R. (2019). Fungal disease and temperature alter skin microbiome structure in an experimental salamander system. *Molecular Ecology*, *28*(11), 2917–2931.

Murray, K. A., Retallick, R. W. R., Puschendorf, R., Skerratt, L. F., Rosauer, D., McCallum, H. I., Berger, L., Speare, R., & VanDerWal, J. (2011). Assessing spatial patterns of disease risk to biodiversity: implications for the management of the amphibian pathogen, *Batrachochytrium dendrobatidis*. *Journal of Applied Ecology*, *48*(1), 163–173.

Murray, K. A., Skerratt, L. F., Speare, R., & McCallum, H. (2009). Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, batrachochytrium dendrobatidis. *Conservation Biology*, *23*(5), 1242–1252. https://doi.org/10.1111/j.1523-1739.2009.01211.x

Murray, M. H., Lankau, E. W., Kidd, A. D., Welch, C. N., Ellison, T., Adams, H. C., Lipp, E. K., & Hernandez, S. M. (2020). Gut microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a wading bird. *PLoS One*, *15*(3), e0220926.

Musselman, W. C., Worthington, T. A., Mouser, J., Williams, D. M., & Brewer, S. K. (2017). Passive integrated transponder tags: Review of studies on warmwater fishes with notes on additional species. *Journal of Fish and Wildlife Management*, *8*(2), 353–364. https://doi.org/10.3996/122016-JFWM-091

Mutnale, M. C., Reddy, G. S., & Vasudevan, K. (2021). Bacterial Community in the Skin Microbiome of Frogs in a Coldspot of Chytridiomycosis Infection. *Microbial Ecology*, *82*, 554–558. https://doi.org/10.1007/s00248-020-01669-5/Published

Myers, R. A., Baum, J. K., Shepherd, T. D., Powers, S. P., & Peterson, C. H. (2007). Cascading effects of the loss of apex predatory sharks from a coastal ocean. *Science*, *315*(5820), 1846–1850. https://doi.org/10.1126/science.1138657

Nascimento, F. X., Glick, B. R., & Rossi, M. J. (2021). Multiple plant hormone catabolism activities: an adaptation to a plant-associated lifestyle by *Achromobacter spp. Environmental Microbiology Reports*, *13*(4), 533–539.

Natarajan, A., Han, A., Zlitni, S., Brooks, E. F., Vance, S. E., Wolfe, M., Singh, U., Jagannathan, P., Pinsky, B. A., Boehm, A., & others. (2021). Standardized preservation, extraction and quantification techniques for detection of fecal SARS-CoV-2 RNA. *Nature Communications*, *12*(1), 1–12.

Nichols, J. D. (1992). Capture-recapture models: using marked animals to study population dynamics. *Bioscience*, *42*(2), 94–102.

Nieves, D. J., & Heininger, U. (2016). *Bordetella pertussis*. *Emerging Infections 10*, 311–339.

Nogales, B., Lanfranconi, M. P., Piña-Villalonga, J. M., & Bosch, R. (2011). Anthropogenic perturbations in marine microbial communities. *FEMS Microbiology Reviews*, *35*(2), 275–298. https://doi.org/10.1111/j.1574-6976.2010.00248.x

Obon, E., Carbonell, F., Valbuena-Ureña, E., Alonso, M., Larios, R., Fernández-Beaskoetxea, S., Fisher, M. C., & Bosch, J. (2013). Chytridiomycosis surveillance in the critically endangered Montseny brook newt, Calotriton arnoldi, northeastern Spain. *Herpetological Journal*, *23*(4), 237–240.

O'Hanlon, S., Rieux, A., Farrer, R. A., Rosa, G. M., Waldman, B., Bataille, A., Kosch, T. A., Murray, K. A., Brankovics, B., Fumagalli, M., Martin, M. D., Wales, N., Alvarado-rybak, M., Bates, K. A., Berger, L., Böll, S., Brookes, L., Clare, F., Courtois, E. A., … Fisher, M. C. (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, *360*, 621–627.

Oksanen, J., & Stevens, H. (2007). *The Vegan Package*. http://cran.r-project.org/,

Oliveira, B. F., São-pedro, V. A., Santos-barrera, G., Penone, C., & Costa, G. C. (2017). Data Descriptor : AmphiBIO , a global database for amphibian ecological traits. *Scientific Data*, *4*(170123). https://doi.org/10.1038/sdata.2017.123

Olson, D. H., Aanensen, D. M., Ronnenberg, K. L., Powell, C. I., Walker, S. F., Bielby, J., Garner, T. W. J., Weaver, G., & Fisher, M. C. (2013). Mapping the Global Emergence of Batrachochytrium dendrobatidis, the Amphibian Chytrid Fungus. *PLoS ONE*, *8*(2), e56802. https://doi.org/10.1371/journal.pone.0056802

Olson, D. H., Ronnenberg, K. L., Glidden, C. K., Christiansen, K. R., & Blaustein, A. R. (2021). Global Patterns of the Fungal Pathogen *Batrachochytrium dendrobatidis* Support Conservation Urgency. *Frontiers in Veterinary Science*, *8*. https://doi.org/10.3389/fvets.2021.685877

Pedersen, A. B., & Fenton, A. (2007). Emphasizing the ecology in parasite community ecology. *Trends in Ecology and Evolution*, *22*(3), 133–139. https://doi.org/10.1016/j.tree.2006.11.005

Phillott, A. D., Grogan, L. F., Cashins, S. D., Mcdonald, K. R., Berger, L., & Skerratt, L. F. (2013). Chytridiomycosis and seasonal mortality of tropical stream-associated frogs 15 years after introduction of batrachochytrium dendrobatidis. *Conservation Biology*, *27*(5), 1058–1068. https://doi.org/10.1111/cobi.12073

Pickering, C. M., & Byrne, J. (2014). The benefits of publishing systematic quantitative literature reviews for PhD candidates and other early career researchers. *Higher Education Research and Development*, *33*, 534–548.

Pickett, E. J., Stockwell, M. P., Bower, D. S., Pollard, C. J., Garnham, J. I., Clulow, J., & Mahony, M. J. (2014). Six-year demographic study reveals threat of stochastic extinction for remnant populations of a threatened amphibian. *Austral Ecology*, *39*(2), 244–253. https://doi.org/10.1111/aec.12080

Pimm, S. L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., Raven, P. H., Roberts, C. M., & Sexton, J. O. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science*, *344*(6187). https://doi.org/10.1126/science.1246752

Piotrowski, J. S., Annis, S. L., & Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. In *Mycologia* (Vol. 96, Issue 1). by The Mycological Society of America.

Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., McKenzie, V., Lawler, S. P., & Foley, J. E. (2017). Greater Species Richness of Bacterial Skin Symbionts Better Suppresses the Amphibian Fungal Pathogen *Batrachochytrium dendrobatidis*. *Microbial Ecology*, *74*(1), 217–226. https://doi.org/10.1007/s00248-016-0916-4

Pleguezuelos, J. M., Márquez, R., & Lizana, M. (2002). Atlas y libro rojo de los anfibios y reptiles de España. In *Dirección General de Consrvación de la Naturaleza-Asociación Herpetológica Española (2ª impresión)*. https://doi.org/B-32.0/2002

Poll, C. P. (2009). Wound Management in Amphibians: Etiology and Treatment of Cutaneous Lesions. *Journal of Exotic Pet Medicine*, *18*(1), 20–35. https://doi.org/https://doi.org/10.1053/j.jepm.2008.10.005

Pounds, J. A., Bustamante, M. R., Coloma, L. A., Consuegra, J. A., Fogden, M. P. L., Foster, P. N., La Marca, E., Masters, K. L., Merino-Viteri, A., Puschendorf, R., Ron, S. R., Sánchez-Azofeifa, G. A., Still, C. J., & Young, B. E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, *439*(7073), 161–167. https://doi.org/10.1038/nature04246

Pradel, R. (1996). Utilization of Capture-Mark-Recapture for the Study of Recruitment and Population Growth Rate. *Biometrics*, *52*(2), 703–709.

Premke, K., Wurzbacher, C., Felsmann, K., Fabian, J., Taube, R., Bodmer, P., Attermeyer, K., Nitzsche, K. N., Schroer, S., Koschorreck, M., Hübner, E., Mahmoudinejad, T. H., Kyba, C. C. M., Monaghan, M. T., & Hölker, F. (2022). Large-scale sampling of the freshwater microbiome suggests pollution-driven ecosystem changes. *Environmental Pollution*, *308*, 119627. https://doi.org/https://doi.org/10.1016/j.envpol.2022.119627

Preuss, J. F., Greenspan, S. E., Rossi, E. M., Lucas Gonsales, E. M., Neely, W. J., Valiati, V. H., Woodhams, D. C., Becker, C. G., & Tozetti, A. M. (2020). Widespread pig farming practice linked to shifts in skin microbiomes and disease in pond-breeding amphibians. *Environmental Science \& Technology*, *54*(18), 11301–11312.

Puschendorf, R., Carnaval, A. C., Vanderwal, J., Zumbado-Ulate, H., Chaves, G., Bolaños, F., & Alford, R. A. (2009). Distribution models for the amphibian chytrid Batrachochytrium dendrobatidis in Costa Rica: Proposing climatic refuges as a conservation tool. *Diversity and Distributions*, *15*(3), 401–408. https://doi.org/10.1111/j.1472-4642.2008.00548.x

Pyke, G. H. (2005). The use of PIT tags in capture-recapture studies of frogs: a field evaluation. *Herpetological Review*, *36*(3), 281–285.

R Core Team. (2023). *R: A Language and Environment for Statistical Computing. _R Foundation for Statistical Computing, _Vienna, _Austria. <https://www.R-project.org/>.*

R Core Team. (2024). *_R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.*

Råberg, L., Graham, A. L., & Read, A. F. (2009). Decomposing health: tolerance and resistance to parasites in animals. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1513), 37–49. https://doi.org/10.1098/rstb.2008.0184

Råberg, L., Sim, D., & Read, A. F. (2007). Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*, *318*(5851), 812–814. https://doi.org/10.1126/science.1148526

Rachowicz, L. J., & Vredenburg, V. T. (2004). Transmission of Batrachochytrium dendrobatidis within and between amphibian life stages. *Diseases of Aquatic Organisms*, *61*(1–2), 75–83. https://doi.org/10.3354/dao061075

Raffel, T. R., Rohr, J. R., Kiesecker, J. M., & Hudson, P. J. (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Functional Ecology*, 819–828.

Ramey, A. M. (2021). Antimicrobial resistance: Wildlife as indicators of anthropogenic environmental contamination across space and through time. *Current Biology*, *31*(20), R1385--R1387.

Ramírez-Barahona, S., González-Serrano, F., Martínez-Ugalde, E., Soto-Pozos, A., Parra-Olea, G., & Rebollar, E. (2023). *Host phylogeny and environment shape the diversity of the salamander skin microbiome*.

Rapport, D. J., Costanza, R., & McMichael, A. J. (1998). Assessing ecosystem health. *Trends in Ecology and Evolution*, *13*(10), 397–402. https://doi.org/10.1016/S0169-5347(98)01449-9

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., & Belden, L. K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *The ISME Journal*, *10*(7), 1682–1695.

Ribas, M. P., García-Ulloa, M., Espunyes, J., & Cabezón, O. (2023). Improving the assessment of ecosystem and wildlife health: microbiome as an early indicator. *Current Opinion in Biotechnology*, *81*, 102923. https://doi.org/10.1016/j.copbio.2023.102923

Richards-Zawacki, C. L. (2010). Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1681), 519–528.

Rillig, M. C., Ryo, M., Lehmann, A., Aguilar-Trigueros, C. A., Buchert, S., Wulf, A., Iwasaki, A., Roy, J., & Yang, G. (2019). The role of multiple global change factors in driving soil functions and microbial biodiversity. *Science*, *366*(6467), 886–890. https://doi.org/10.1126/science.aay2832

Rocca, J. D., Simonin, M., Blaszczak, J. R., Ernakovich, J. G., Gibbons, S. M., Midani, F. S., & Washburne, A. D. (2019). The Microbiome Stress Project: Toward a global meta-analysis of environmental stressors and their effects on microbial communities. *Frontiers in Microbiology*, *10*(JAN). https://doi.org/10.3389/fmicb.2018.03272

Rodriguez, D., Becker, C. G., Pupin, N. C., Haddad, C. F. B., & Zamudio, K. R. (2014). Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. *Molecular Ecology*, *23*(4), 774–787. https://doi.org/10.1111/mec.12615

Rohr, J. R., & Raffel, T. R. (2010). Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proceedings of the National Academy of Sciences*, *107*(18), 8269–8274. https://doi.org/10.1073/pnas.0912883107

Roitman, S., Joseph Pollock, F., & Medina, M. (2018). Coral microbiomes as bioindicators of reef health. In *Population Genomics: Marine Organisms* (pp. 39–57). Springer.

Roland, A. B., & O'Connell, L. A. (2015). Poison frogs as a model system for studying the neurobiology of parental care. *Current Opinion in Behavioral Sciences*, *6*, 76–81. https://doi.org/10.1016/j.cobeha.2015.10.002

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K., & Woodhams, D. C. (2011). Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integrative and Comparative Biology*, *51*(4), 552–562. https://doi.org/10.1093/icb/icr095

Rollins-Smith, L. A., Reinert, L. K., O'Leary, C. J., Houston, L. E., & Woodhams, D. C. (2005). Antimicrobial peptide defenses in amphibian skin. *Integrative and Comparative Biology*, *45*(1), 137–142. https://doi.org/10.1093/icb/45.1.137

Ron, S. R. (2005). Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the new world. *Biotropica*, *37*(2), 209–221. https://doi.org/10.1111/j.1744-7429.2005.00028.x

Root, T., Price, J., Hall, K., & Schneider, S. (2003). Fingerprints of global warming on wild animals and plants. *Nature*, *421*(6918), 57–60. https://doi.org/10.1038/nature01309.1.

Rosa, G. M., Anza, I., Moreira, P. L., Conde, J., Martins, F., Fisher, M. C., & Bosch, J. (2013). Evidence of chytrid-mediated population declines in common midwife toad in Serra da Estrela, Portugal. *Animal Conservation*, *16*(3), 306–315. https://doi.org/10.1111/j.1469-1795.2012.00602.x

Rosenblum, E. B., Voyles, J., Poorten, T. J., & Stajich, J. E. (2010). The Deadly Chytrid Fungus: A Story of an Emerging Pathogen. *PLoS Pathogens*, *6*(1), e1000550. https://doi.org/10.1371/journal.ppat.1000550

Rowley, J. J. L., & Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in nature. *Scientific Reports*, *3*(1), 1515.

Sabino-Pinto, J., Bletz, M., Hendrix, R., Perl, R. G. B., Martel, A., Pasmans, F., Lötters, S., Mutschmann, F., Schmeller, D. S., Schmidt, B. R., Veith, M., Wagner, N., Vences, M., & Steinfartz, S. (2015). First detection of the emerging fungal pathogen Batrachochytrium salamandrivorans in Germany. *Amphibia-Reptilia*, *36*(4), 411–416. https://doi.org/10.1163/15685381-00003008

Sannolo, M., Gatti, F., Mangiacotti, M., Scali, S., & Sacchi, R. (2016). Photo-identification in amphibian studies: a test of I3S Pattern. *Acta Herpetologica*, *11*(1), 63–68. https://doi.org/10.13128/Acta

Scheele, B. C., Foster, C. N., Hunter, D. A., Lindenmayer, D. B., Schmidt, B. R., & Heard, G. W. (2019). Living with the enemy: Facilitating amphibian coexistence with disease. *Biological Conservation*, *236*, 52–59. https://doi.org/10.1016/j.biocon.2019.05.032

Scheele, B. C., Hunter, D. A., Brannelly, L. A., Skerratt, L. F., & Driscoll, D. A. (2017). Reservoir-host amplification of disease impact in an endangered amphibian. *Conservation Biology*, *31*(3), 592–600. https://doi.org/10.1111/cobi.12830

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Martel, A., Beukema, W., Acevedo, A. A., Burrowes, P. A., Carvalho, T., Catenazzi, A., & others. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, *363*(6434), 1459–1463.

Schloegel, L. M., Picco, A. M., Kilpatrick, A. M., Davies, A. J., Hyatt, A. D., & Daszak, P. (2009). Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). *Biological Conservation*, *142*(7), 1420–1426. https://doi.org/10.1016/j.biocon.2009.02.007

Schloegel, L. M., Toledo, L. F., Longcore, J. E., Greenspan, S. E., Vieira, C. A., Lee, M., Zhao, S., Wangen, C., Ferreira, C. M., Hipolito, M., Davies, A. J., Cuomo, C. A., Daszak, P., & James, T. Y. (2012). Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology*, *21*(21), 5162–5177. https://doi.org/10.1111/j.1365-294X.2012.05710.x

Schmeller, D. S., Blooi, M., Martel, A., Garner, T. W. J., Fisher, M. C., Azemar, F., Clare, F. C., Leclerc, C., Jäger, L., Guevara-Nieto, M., Loyau, A., & Pasmans, F. (2014). Microscopic aquatic predators strongly affect infection dynamics of a globally emerged pathogen. *Current Biology*, *24*(2), 176–180. https://doi.org/10.1016/j.cub.2013.11.032

Schmeller, D. S., Cheng, T., Shelton, J., Lin, C. F., Chan-Alvarado, A., Bernardo-Cravo, A., Zoccarato, L., Ding, T. S., Lin, Y. P., Swei, A., Fisher, M. C., Vredenburg, V. T., & Loyau, A. (2022). Environment is associated with chytrid infection and skin microbiome richness on an amphibian rich island (Taiwan). *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-20547-3

Searle, C. L., Biga, L. M., Spatafora, J. W., & Blaustein, A. R. (2011). A dilution effect in the emerging amphibian pathogen Batrachochytrium dendrobatidis. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(39), 16322–16326. https://doi.org/10.1073/pnas.1108490108

Searle, C. L., Gervasi, S. S., Hua, J., Hammond, J. I., Relyea, R. A., Olson, D. H., & Blaustein, A. R. (2011). Differential Host Susceptibility to *Batrachochytrium dendrobatidis*, an Emerging Amphibian Pathogen. *Conservation Biology*, *25*(5), 965–974. https://doi.org/10.1111/j.1523-1739.2011.01708.x

Seelenfreund, E., Robinson, W. A., Amato, C. M., Tan, A.-C., Kim, J., & Robinson, S. E. (2014). Long term storage of dry versus frozen RNA for next generation molecular studies. *PloS One*, *9*(11), e111827.

Sehnal, L., Brammer-Robbins, E., Wormington, A. M., Blaha, L., Bisesi, J., Larkin, I., Martyniuk, C. J., Simonin, M., & Adamovsky, O. (2021). Microbiome Composition and Function in Aquatic Vertebrates: Small Organisms Making Big Impacts on Aquatic Animal Health. *Frontiers in Microbiology*, *12*(March). https://doi.org/10.3389/fmicb.2021.567408

Seitz, T. J., Schütte, U. M. E., & Drown, D. M. (2022). Unearthing Shifts in Microbial Communities Across a Soil Disturbance Gradient. *Frontiers in Microbiology*, *13*(May), 1–12. https://doi.org/10.3389/fmicb.2022.781051

Shi, L., Dossa, G. G. O., Paudel, E., Zang, H., Xu, J., & Harrison, R. D. (2019). Changes in fungal communities across a forest disturbance gradient. *Applied and Environmental Microbiology*, *85*(12), e00080--19.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., Hines, H. B., & Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, *4*(2), 125–134. https://doi.org/10.1007/s10393-007-0093-5

Smith, S. N., Watters, J. L., & Siler, C. D. (2023). Host ecology drives frog skin microbiome diversity across ecotone in South-Central North America. *Frontiers in Microbiomes*, *2*. https://doi.org/10.3389/frmbi.2023.1286985

Spitzen-Van Der Sluijs, A., Marte, A., Hallmann, C. A., Bosman, W., Garner, T. W. J., Van Rooij, P., Jooris, R., Haesebrouck, F., & Pasmans, F. (2014). Environmental Determinants of Recent Endemism of *Batrachochytrium dendrobatidis* Infections in Amphibian Assemblages in the Absence of Disease Outbreaks. *Conservation Biology*, *28*(5), 1302–1311. https://doi.org/10.1111/cobi.12281

Springborn, M. R., Weill, J. A., Lips, K. R., Ibáñez, R., & Ghosh, A. (2022). Amphibian collapses increased malaria incidence in Central America. *Environmental Research Letters*, *17*(10). https://doi.org/10.1088/1748-9326/ac8e1d

Stegen, G., Pasmans, F., Schmidt, B. R., Rouffaer, L. O., Van Praet, S., Schaub, M., Canessa, S., Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F., & Martel, A. (2017). Drivers of salamander extirpation mediated by Batrachochytrium salamandrivorans. *Nature*, *544*(7650), 353–356. https://doi.org/10.1038/nature22059

Stevenson, M., Sergeant, E., Nunes, T., Heuer, C., Marshall, J., Sanchez, J., Thornton, R., Reiczigel, J., Robison-Cox, J., Sebastiani, P., Solymos, P., Yoshida, K., Jones, G., Pirikahu, S., Firestone, S., Kyle, R., Popp, J., Jay, M., Reynard, C., … Szabo, A. (2021). *epiR: Tools for the Analysis of Epidemiological Data. R package version 2.0.38. https://CRAN.R-project.org/package=epiR*.

Stice, M. J., & Briggs, C. J. (2010). Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Journal of Wildlife Diseases*, *46*(1), 70–77.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, S. L. A., Fischman, D. L., & Waller, R. W. (2004). Status and Trends of Amphibian Declines and Extinctions Worldwide. *Science*, *306*(5702), 1783–1786.

Sugden, S., Sanderson, D., Ford, K., Stein, L. Y., & St. Clair, C. C. (2020). An altered microbiome in urban coyotes mediates relationships between anthropogenic diet and poor health. *Scientific Reports*, *10*(1), 22207.

Sunagawa, S., Coelho, L. P., Chaffron, S., Kultima, J. R., Labadie, K., Salazar, G., Djahanschiri, B., Zeller, G., Mende, D. R., Alberti, A., Cornejo-Castillo, F. M., Costea, P. I., Cruaud, C., d'Ovidio, F., Engelen, S., Ferrera, I., Gasol, J. M., Guidi, L., Hildebrand, F., … Velayoudon, D. (2015). Structure and function of the global ocean microbiome. *Science*, *348*(6237), 1261359. https://doi.org/10.1126/science.1261359

Takaya, K., Taguchi, Y., & Ise, T. (2023). Individual identification of endangered amphibians using deep learning and smartphone images: case study of the Japanese giant salamander (*Andrias japonicus*). *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-40814-1

Tattersall, G. J. (2007). Skin breathing in amphibians. *Endothelial Biomedicine*, 85–91. https://doi.org/10.1017/CBO9780511546198.010

Temperton, B., & Giovannoni, S. J. (2012). Metagenomics: microbial diversity through a scratched lens. *Current Opinion in Microbiology*, *15*(5), 605–612.

Therneau, T. M. (2022). *A Package for Survival Analysis in R*.

Thumsová, B., Alarcos, G., Ayres, C., Rosa, G. M., & Bosch, J. (2024). Relationship between two pathogens in an amphibian community that experienced mass mortalities. *Conservation Biology*, e14196.

Thumsová, B., González-Miras, E., Rubio, Á., Granados, I., Bates, K. A., & Bosch, J. (2024). Chemical disinfection as a simple and reliable method to control the amphibian chytrid fungus at breeding points of endangered amphibians. *Scientific Reports*, *14*(1). https://doi.org/10.1038/s41598-024-55946-1

Tobler, U., & Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE*, *5*(6), 1–8. https://doi.org/10.1371/journal.pone.0010927

Toledo, R. C., & Jared, C. (1995). Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology*, *111*(1), 1–29. https://doi.org/10.1016/0300-9629(95)98515-I

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The Human Microbiome Project. *Nature*, *449*(7164), 804–810. https://doi.org/10.1038/nature06244

UNEP. (2023). *CBD/COP/15/17. Report of the Conference of the Parties to the Convention of Biological Diversity on the Second Part of its Fifteenth Meeting. 19-20 October 2023, Montreal, Canada.*

Valenzuela-Sánchez, A., Schmidt, B. R., Uribe-Rivera, D. E., Costas, F., Cunningham, A. A., & Soto-Azat, C. (2017). Cryptic disease-induced mortality may cause host extinction in an apparently stable host-parasite system. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1863). https://doi.org/10.1098/rspb.2017.1176

Van Rooij, P., Martel, A., Haesebrouck, F., & Pasmans, F. (2015). Amphibian chytridiomycosis: A review with focus on fungus-host interactions. *Veterinary Research*, *46*(1), 1–22. https://doi.org/10.1186/s13567-015-0266-0

Venesky, M. D., Liu, X., Sauer, E. L., & Rohr, J. R. (2014). Linking manipulative experiments to field data to test the dilution effect. *Journal of Animal Ecology*, *83*(3), 557–565. https://doi.org/10.1111/1365-2656.12159

Venesky, M. D., Raffel, T. R., Mcmahon, T. A., & Rohr, J. R. (2014). Confronting inconsistencies in the amphibian-chytridiomycosis system: Implications for disease management. *Biological Reviews*, *89*(2), 477–483. https://doi.org/10.1111/brv.12064

Vitousek, P. M., Mooney, H. A., Lubchenco, J., & Melillo, J. M. (1997). Human Domination of Earth's Ecosystems. *Science*, *277*(5325), 494–499. https://doi.org/10.1126/science.277.5325.494

Vo, A.-T., & Jedlicka, J. A. (2014). Protocols for metagenomic DNA extraction and Illumina amplicon library preparation for faecal and swab samples. *Molecular Ecology Resources*, *14*(6), 1183–1197.

Voyles, J., Woodhams, D. C., Saenz, V., Byrne, A. Q., Perez, R., Rios-Sotelo, G., Ryan, M. J., Bletz, M. C., Sobell, F. A., McLetchie, S., Reinert, L., Rosenblum, E. B., Rollins-Smith, L. A., Ibáñez, R., Ray, J. M., Griffith, E. J., Ross, H., & Richards-Zawacki, C. L. (2018). Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. *Science*, *359*, 1517–1519. https://doi.org/10.1126/science.aao4806

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., & Dinudom, A. (2009). Pathogenesis of Chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, *326*, 582–586.

Vredenburg, V. T., Briggs, C. J., & Harris, R. N. (2011). Host-pathogen dynamics of amphibian chytridiomycosis: the role of the skin microbiome in healthy and disease. In *Fungal Dis. An Emerg. Threat to Human, Anim. Plant Heal. Work. Summ. (eds. Pray, L., Relman, D.A., Choffens, E.R. & Olsen, L.). National Academies Press, Washington, DC, p. 488*.

Waddle, A. W., Clulow, S., Aquilina, A., Sauer, E. L., Kaiser, S. W., Miller, C., Flegg, J. A., Campbell, P. T., Gallagher, H., & Dimovski, I. (2024). Hotspot shelters stimulate frog resistance to chytridiomycosis. *Nature*, 1–6.

Waddle, A. W., Rivera, R., Rice, H., Keenan, E. C., Rezaei, G., Levy, J. E., Vasquez, Y. S., Sai, M., Hill, J., & Zmuda, A. (2021). Amphibian resistance to chytridiomycosis increases following low-virulence chytrid fungal infection or drug-mediated clearance. *Journal of Applied Ecology*, *58*(10), 2053–2064.

Waddle, J. H., Rice, K. G., Mazzotti, F. J., & Percival, H. F. (2008). Modeling the effect of toe clipping on treefrog survival: beyond the return rate. *Journal of Herpetology*, *42*(3), 467–473.

Wagg, C., Bender, S. F., Widmer, F., & Van Der Heijden, M. G. A. (2014). Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proceedings of the National Academy of Sciences*, *111*(14), 5266–5270.

Wake, D. B., & Koo, M. S. (2018). Amphibians. *Current Biology*, *28*(21), R1237–R1241. https://doi.org/10.1016/j.cub.2018.09.028

Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *PNAS*, *105*(supplement_1), 11466-11473. www.pnas.orgcgidoi10.1073pnas.0801921105

Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., & Belden, L. K. (2017). Dominance-function relationships in the amphibian skin microbiome. *Environmental Microbiology*, *19*(8), 3387–3397. https://doi.org/10.1111/1462-2920.13850

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P. C., & Belden, L. K. (2015). Community structure and function of amphibian skin microbes: An experiment with bullfrogs exposed to a chytrid fungus. *PLoS ONE*, *10*(10). https://doi.org/10.1371/journal.pone.0139848

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D. A., Ginestet, C., Arthur, C. P., & Fisher, M. C. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters*, *13*(3), 372–382. https://doi.org/10.1111/j.1461-0248.2009.01434.x

Wang, Z., Han, M., Li, E., Liu, X., Wei, H., Yang, C., Lu, S., & Ning, K. (2020). Distribution of antibiotic resistance genes in an agriculturally disturbed lake in China: Their links with microbial communities, antibiotics, and water quality. *Journal of Hazardous Materials*, *393*, 122426.

Warne, R. W., LaBumbard, B., LaGrange, S., Vredenburg, V. T., & Catenazzi, A. (2016). Co-infection by chytrid fungus and ranaviruses in wild and harvested frogs in the tropical Andes. *PloS One*, *11*(1), e0145864.

Watson, S. E., Hauffe, H. C., Bull, M. J., Atwood, T. C., McKinney, M. A., Pindo, M., & Perkins, S. E. (2019). Global change-driven use of onshore habitat impacts polar bear faecal microbiota. *The ISME Journal*, *13*(12), 2916–2926. https://doi.org/10.1038/s41396-019-0480-2

Whitfield, S. M., Geerdes, E., Chacon, I., Rodriguez, E. B., Jimenez, R. R., Donnelly, M. A., & Kerby, J. L. (2013). Infection and co-infection by the amphibian chytrid fungus and ranavirus in wild Costa Rican frogs. *Diseases of Aquatic Organisms*, *104*(2), 173–178.

WHO. (2024). *WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. Geneva: World Health Organization; 2024. Licence: CC BY-NC-SA 3.0 IGO.*

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Williams, C. L., Caraballo-Rodríguez, A. M., Allaband, C., Zarrinpar, A., Knight, R., & Gauglitz, J. M. (2018). Wildlife-microbiome interactions and disease: exploring opportunities for disease mitigation across ecological scales. *Drug Discovery Today: Disease Models*, *28*, 105–115. https://doi.org/10.1016/j.ddmod.2019.08.012

Winter, J. D. (1996). Advances in underwater biotelemetry. In *Murphy BR, Willis DW (eds) Fisheries techniques, 2nd edn. American Fisheries Society, Bethesda, Maryland* (pp. 555–590).

WOAH. (2024a). *World Organization for Animal Health. Aquatic Animal Health Code, 26th Ed. OIE, Paris.*

WOAH. (2024b). *World Organization for Animal Health. Terrestrial Animal Health Code, 32nd Ed. OIE, Paris.*

Wobeser, G. A. (2007). Disease in Wild Animals : Investigation and Management. In *Management* (2nd Editio). Springer-Verlag Berlin Heidelberg, Germany.

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., Bell, S. C., Bletz, M., Daskin, J. H., Davis, L. R., Flechas, S. V., Lauer, A., Gonzalez, A., Harris, R. N., Holden, W. M., Hughey, M. C., Ibáñez, R., Knight, R., Kueneman, J., … McKenzie, V. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology*, *96*(2), 595–595. https://doi.org/10.1890/14-1837.1

Woodhams, D. C., Alford, R. A., Briggs, C. J., Johnson, M., & Rollins-Smith, L. A. (2008). Life-history trade-offs influence disease in changing climates: Strategies of an amphibian pathogen. *Ecology*, *89*(6), 1627–1639.

WWF. (2022). *Living Planet Report 2022 – Building a nature- positive society.* http://www.ncbi.nlm.nih.gov/pubmed/3067

Xie, G. Y., Olson, D. H., & Blaustein, A. R. (2016). Projecting the global distribution of the emerging amphibian fungal pathogen, *Batrachochytrium dendrobatidis*, based on IPCC climate futures. *PLoS ONE*, *11*(8). https://doi.org/10.1371/journal.pone.0160746

Young, A., & McNaught, C.-E. (2011). The physiology of wound healing. *Surgery (Oxford)*, *29*(10), 475–479.

Ziegler, M., Roik, A., Porter, A., Zubier, K., Mudarris, M. S., Ormond, R., & Voolstra, C. R. (2016). Coral microbial community dynamics in response to anthropogenic impacts near a major city in the central Red Sea. *Marine Pollution Bulletin*, *105*(2), 629–640. https://doi.org/https://doi.org/10.1016/j.marpolbul.2015.12.045

Zipkin, E. F., DiRenzo, G. V, Ray, J. M., Rossman, S., & Lips, K. R. (2020). Tropical snake diversity collapses after widespread amphibian loss. *Science*, *367*(6479), 814–816.