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Habitat split as a driver of disease in amphibians

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ABSTRACT

Anthropogenic habitat disturbance is fundamentally altering patterns of disease transmission and immunity across the vertebrate tree of life. Most studies linking anthropogenic habitat change and disease focus on habitat loss and fragmentation, but these processes often lead to a third process that is equally important: *habitat split*. Defined as spatial separation between the multiple classes of natural habitat that many vertebrate species require to complete their life cycles, habitat split has been linked to population declines in vertebrates, e.g. amphibians breeding in lowland aquatic habitats and overwintering in fragments of upland terrestrial vegetation. Here, we link habitat split to enhanced disease risk in amphibians (*i*) by reviewing the biotic and abiotic forces shaping elements of immunity and (*ii*) through a spatially oriented field study focused on tropical frogs. We propose a framework to investigate mechanisms by which habitat split influences disease risk in amphibians, focusing on three broad host factors linked to immunity: (*i*) composition of symbiotic microbial communities, (*ii*) immunogenetic variation, and (*iii*) stress hormone levels. Our review highlights the potential for habitat split to contribute to host-associated microbiome dysbiosis, reductions in immunogenetic repertoire, and chronic stress, that often facilitate pathogenic infections and disease in amphibians and other classes of vertebrates. We highlight that targeted habitat-restoration strategies aiming to connect multiple classes of natural habitats (e.g. terrestrial–freshwater, terrestrial–marine, marine–freshwater) could enhance priming of the vertebrate immune system through repeated low-load exposure to enzootic pathogens and reduced stress-induced immunosuppression.

Key words: landscape epidemiology, immune responses, *Batrachochytrium*, corridors, fragmentation, conservation.

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I. INTRODUCTION

Anthropogenic habitat disturbance contributes to immense variation in vertebrate immunity against infectious diseases. Habitat disturbance may have particularly strong effects on immunity and disease in vertebrate species that move among multiple habitats over daily, seasonal, or ontogenetic timescales, including migratory species in which a proportion of the population changes habitat cyclically or during their life history. Across scales ranging from cross-continental (e.g. birds and large mammals) to watershed-specific (e.g. amphibians and fish), movements through multiple classes of natural and non-natural habitats may impose unique physiological demands and challenges by different assemblages of predators and parasites, often requiring trade-offs between investment in immunity and the high energetic costs of movement through inhospitable environments (Phillips, 1986; Alerstam, 1993; Malmgren, 2002; Martin, Scheuerlein & Wikelski, 2003). Recovery from infection may have favoured the evolution of migratory behaviour in several classes of vertebrates (Todd, 2007; Daversa *et al.*, 2018*a*,*b*; Satterfield *et al.*, 2018; Shaw *et al.*, 2019; Shaw & Binning, 2020; Poulin & de Angeli Dutra, 2021), and thus understanding the relationship between habitat disturbance and immunity in species that require multiple habitats during their life cycle is critical for predicting disease susceptibility in wild populations under global change. However, the study of environmental variation in immune function is in its early stages and is commonly limited by spatial autocorrelation, low spatial replication (Albery *et al.*, 2019; Becker *et al.*, 2020), and a focus on habitat loss and spatial connectivity of a single habitat class. As a result, the specific mechanisms underlying the associations between habitat disturbance, immunity, and disease are largely unresolved.

One type of anthropogenic habitat disturbance that is particularly pertinent to vertebrates is fragmentation, the process of breaking apart a single continuous natural habitat (e.g. forest), typically resulting in a large number of small patches of natural habitat within a matrix of anthropogenic habitats (Fahrig, 2003; Ewers & Didham, 2006). Recent heated discussions around the independent and interactive effects of habitat loss *versus* fragmentation *per se* on biodiversity highlight the complexity of mechanisms operating under the umbrella of habitat fragmentation at multiple spatial scales (Fahrig, 2017; Fletcher *et al.*, 2018; Fahrig *et al.*, 2019). The fragmentation process could often lead to habitat split, defined as spatial separation between the multiple classes of natural habitat that many vertebrate species require to complete their life cycles (Becker *et al.*, 2007). Habitat split disrupts landscape complementation (Pope, Fahrig & Merriam, 2000) and poses several potential challenges to vagile species with complex life cycles, including restricted movement, overcrowding, changes in habitat quality of migratory routes, limited gene flow, and increased pathogen diversification in isolated habitat patches (Becker *et al.*, 2007; Zohdy, Schwartz & Oaks, 2019; Betts *et al.*, 2020). These factors, separately or combined, have the potential to cause profound shifts in pathogen pressure and the biotic and abiotic conditions that shape immune function and wildlife disease dynamics (Teitelbaum *et al.*, 2018; Poulin & de Angeli Dutra, 2021). Here we demonstrate that habitat split between natural terrestrial environments and aquatic breeding sites is linked to increased pathogen infection intensity in amphibians during the breeding season. We then propose a framework to investigate mechanisms by which anthropogenic habitat split influences disease risk, using three broad host factors linked to immunity: (*i*) composition of symbiotic microbial communities; (*ii*) immunogenetic variation; and (*iii*) physiological indices of chronic stress.

Hosts carry remarkably diverse assemblages of symbiotic bacteria and microeukaryotes that may act as a barrier to pathogenic infections or prime host immunity. These microbiota and their metabolic products, collectively known as the microbiome, constitute a rapidly evolving area of research in vertebrate immunity. Microbiome function can be influenced by environmental stressors (Kueneman *et al.*, 2019; Rocca *et al.*, 2019) including invading pathogens (Bletz *et al.*, 2018; Jani & Briggs, 2018; Weitzman *et al.*, 2019). When the microbiome is challenged with low pathogen

loads, competitive microbial interactions coupled with host responses may result in microbiomes enriched with anti-pathogen members. Upon recovery from a pathogenic infection, these enriched microbial communities are primed for a second exposure and are able to proliferate rapidly in response to new pathogen infection (Jin Song *et al.*, 2019). Under this adaptive microbiome hypothesis, microbiome enrichment can lead to adaptive shifts in the host microbiome with subsequent host recovery, and when transmitted through a population, microbial rescue may stabilize host population trajectories (Mueller *et al.*, 2019). By altering pathogen pressure, transmission pathways and microbiome recruitment, habitat split may thus interfere with these adaptive processes.

Immunogenetic variation, defined as population-level genetic variation that influences host immune responses, is linked to variable infectious disease outcomes in a wide range of vertebrate taxa (Acevedo-Whitehouse & Cunningham, 2006). A majority of research on host immunogenetic factors linked to pathogen infection and susceptibility focuses on genes of the major histocompatibility complex (MHC; Bernatchez & Landry, 2003; Radwan, Biedrzycka & Babik, 2010). MHC genes are the most diverse across the entire vertebrate genome (Klein, 1986) and the proteins they encode play central roles in generating acquired immune responses (Messaoudi *et al.*, 2002). In particular, MHC class II genetic variation is exceptionally high within populations due to balancing and negative frequency-dependent selection promoting ever-increasing numbers of alleles that have novel pathogen-recognition abilities (Edwards & Hedrick, 1966; Spurgin & Richardson, 2010; Eizaguirre *et al.*, 2012; Lenz, 2018). MHC polymorphisms are thus a particularly relevant metric of immunogenetic diversity to consider in contributing to variation in immunity across vertebrate populations across heterogeneous environments. Habitat split has the potential to impact population immunogenetic diversity through changes in microevolutionary and demographic processes including genetic drift, gene flow, and population reductions (Dixo *et al.*, 2009). While these effects will likely cause negative population impacts (e.g. lower immune gene diversity and less pathogen resistance in smaller populations; Savage *et al.*, 2018), it is also plausible that habitat split could reduce gene flow and better enable local immunogenetic adaptation (e.g. high frequency of the beneficial MHC allele Q in genetically isolated populations; Savage & Zamudio, 2016). Loss of spatial connectivity among multiple required habitats could also alter host immunity more generally, by exacerbating host contact rates with pathogens in environmental reservoirs (Adams, Weber & Johnson, 2020) or through host crowding in habitat refugia.

A third plausible mechanism underlying disease increases in wildlife moving between 'split' natural habitats is chronic stress-induced immunosuppression. Physiological stress in vertebrates is a state of threatened homeostasis that activates the hypothalamic–pituitary–adrenal/interrenal (HPA/I) axis and sympathetic nervous system. Among vertebrates, this highly conserved physiological response can be measured as behavioural and hormonal changes from baseline levels, for example, as prolonged elevated circulating glucocorticoid levels due to a loss in negative feedback function which, in acute stress conditions, reduces glucocorticoid levels to baseline (Sapolsky, Romero & Munck, 2000; Lopes *et al.*, 2021). Because chronic activation of the HPA axis can be immunosuppressive (Sapolsky *et al.*, 2000), correlations between habitat disturbance and wildlife disease are often interpreted as stress-induced susceptibility (Brearley *et al.*, 2013). The reactive scope model suggests that allostatic load may impact the ability of hosts to respond to acute stressors including pathogen exposure (Romero, Dickens & Cyr, 2009). Yet, demonstrating the causal links between an environmental change, physiological stress, and disease incidence can be challenging in wildlife disease systems (but see Hall *et al.*, 2020). In the context of habitat split, chronic stress-induced immunosuppression could be induced by any combination of limited resources, altered biotic and abiotic conditions and high-cost migrations among multiple classes of natural habitats during breeding or ontogenetic development. Though complex, the interplay between physiological and behavioural adaptations may provide insight into how some individuals or populations are more resilient to the combined stressors of habitat split and infectious disease.

Our review of disease dynamics in amphibians, the most threatened taxon among vertebrates (IUCN, 2021), serves as a case study for understanding how habitat split alters immunity in natural vertebrate populations threatened with pathogens. We review the vast body of literature on microbiome, immunogenetic, and stress-mediated mechanisms affecting amphibian immunity and link them to habitat split. While recent studies have examined the relationship between host immunity and population biogeography (Woodhams *et al.*, 2010; Becker *et al.*, 2020) or habitat fragmentation (Belasen *et al.*, 2022; Bordes *et al.*, 2015), explicit consideration of how habitat split can drive vertebrate disease outcomes by altering immune processes has not been addressed. We discuss and link amphibian-specific findings (red arrows in Fig. 1) and expand on some of the known and unresolved mechanisms driving vertebrate immune responses in other classes of vertebrates (all arrows in Fig. 1).

II. AMPHIBIANS AS IDEAL MODEL ORGANISMS

Amphibians are well-suited model organisms to test for effects of habitat split on disease. The most distinctive features of the amphibian life cycle for many species are annual landscape-scale migrations of adults between terrestrial and aquatic habitats for reproduction/overwintering, and dispersal of recently metamorphosed juveniles from aquatic to terrestrial habitats (Sinsch, 1988; Smith & Green, 2005; Popescu & Hunter Jr, 2011; Joly, 2019). Anthropogenic habitat loss frequently occurs on a scale that disconnects remnants of natural terrestrial habitat and aquatic

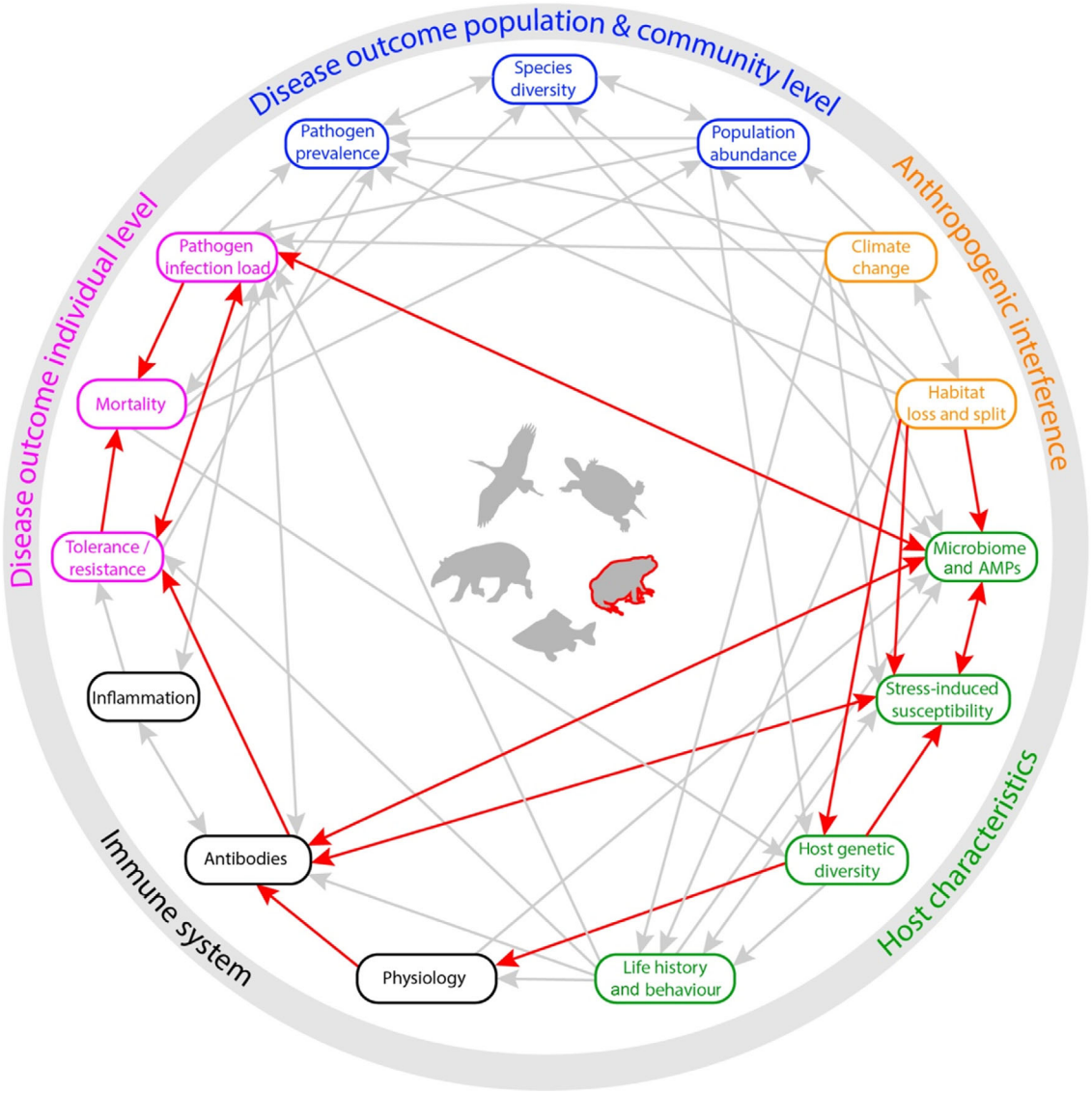

Fig. 1. Direct and indirect links between elements of anthropogenic interference, host characteristics, immune system, and disease outcomes at individual, population and community levels. Associations (arrows) reviewed in our focal amphibian system are highlighted in red. This study also examines some of the well-known and unresolved associations driving wildlife diseases in other vertebrates (grey and red arrows). AMP, antimicrobial peptide.

breeding/overwintering sites, leading to habitat split, and alters both taxonomic and genetic diversity of amphibian communities (Becker *et al.*, 2007; Dixo *et al.*, 2009). Pre- and post-breeding migrations through disturbed habitats have been linked to population declines in forest fragments (Harper, Rittenhouse & Semlitsch, 2008; Todd *et al.*, 2009; Becker *et al.*, 2010). At the landscape scale, habitat split has been identified as the leading cause of amphibian biodiversity decline across Brazil's Atlantic Forest, even after accounting for the independent and interactive effects of habitat loss and fragmentation (Becker *et al.*, 2007).

Habitat-specialist amphibians tend to avoid disturbed habitats and sharp edges (Popescu & Hunter, 2011; Jehle & Arntzen, 2000), although interpopulation variation in habitat preferences and migration behaviour has been reported (Constible, Gregory & Larsen, 2010). Habitat specialists thus tend to exhibit more limited dispersal abilities than habitat generalists; amphibians with more generalist life histories are often adapted to migrate through incredibly long distances (Smith & Green, 2005). However, long-distance migration after the breeding season has been associated with a high likelihood of lateral movement (Coster, Veysey-Powell & Babbitt, 2014), indicating that even habitat generalists could suffer from lack of orientation and impaired immune function due to the physiological stress of long-distance movement to locate high-quality terrestrial habitats disconnected from water bodies. These findings suggest that habitat split could be linked to reduced survival of both

habitat specialist and generalist species to a different degree. The proximate causes driving this process, including the potential role of diseases, require further investigation.

A compelling case study of habitat split exacerbating infectious disease risk in amphibians is chytridiomycosis, a skin disease caused by the waterborne fungus *Batrachochytrium dendrobatidis* (Bd) that has been linked to amphibian population declines and extinctions globally (Woodhams *et al.*, 2008; Lips, 2016; Scheele *et al.*, 2019). The infective life stage of Bd is a flagellated zoospore that can swim in water bodies (Longcore, Pessier & Nichols, 1999; Berger *et al.*, 2005; Raffel *et al.*, 2015), and attacks the moist keratinized skin of post-metamorphic amphibians and mouthparts of larvae (Longcore *et al.*, 1999; Berger *et al.*, 2005; Greenspan, Longcore & Calhoun, 2012). In the host, the fungus develops as a stationary zoosporangium, which eventually discharges a new generation of zoospores onto the skin, causing re-infection of the same individual or transmission to other amphibian hosts (Berger *et al.*, 2005; Briggs, Knapp & Vredenburg, 2010). Zoospores are also discharged into water bodies; thus, aquatic habitats can serve as environmental Bd reservoirs. Host responses to exposure can range from disease susceptibility (Voyles, Rosenblum & Berger, 2011), to learned pathogen avoidance behaviours, to adaptive immunity (McMahon *et al.*, 2014).

Chytridiomycosis disease dynamics involve complex interactions between the ecologies and evolutionary histories of host amphibian populations and the Bd lineages that infect them (Jenkinson *et al.*, 2018; O'Hanlon *et al.*, 2018; Scheele *et al.*, 2019; Basanta *et al.*, 2022). Perhaps even more important in driving disease outbreaks is how the environment mediates these host–pathogen interactions. In particular, the thermal mismatch hypothesis (Cohen *et al.*, 2017, 2019), related to the lag and seasonal acclimation hypotheses originally described by Raffel *et al.* (2006, 2015), posits that hosts are more vulnerable to parasite infection outside of host thermal optima because parasites can acclimate faster to shifts in temperature than can hosts. Bd can also gain a competitive edge over host immunity after temperature decreases (Raffel *et al.*, 2013, 2015; Greenspan *et al.*, 2017*b*), potentially affecting hibernation survival, and can decrease host critical thermal maxima, affecting the ability of hosts to clear Bd through behavioural thermoregulation (Woodhams, Alford & Marantelli, 2003; Greenspan *et al.*, 2017*a*). Habitat disturbances (e.g. landscape-scale deforestation) and natural disasters (e.g. canopy-thinning by cyclones) have substantial effects on Bd infections in tropical and temperate amphibians (Becker & Zamudio, 2011; Liu, Rohr & Li, 2013; Roznik *et al.*, 2015), likely through causing shifts in microclimates (Becker *et al.*, 2016, 2017). Moisture can also be an important mediating factor. For instance, decreased moisture can limit Bd infections (Brem & Lips, 2008; Bustamante, Livo & Carey, 2010; Raffel *et al.*, 2015) and faster drying conditions in disturbed habitats may predispose juvenile amphibians to disease (Kohli *et al.*, 2019). Landscapes affected by habitat split are shaped not only by variable microclimatic conditions but also by distinctive connectivity and spatial characteristics. Thus, the ecophysiology of disease under habitat split requires further study.

III. HABITAT SPLIT IS LINKED TO INCREASED PATHOGEN INFECTION INTENSITY DURING THE BREEDING SEASON

Habitat split between terrestrial and aquatic habitats is a widespread phenomenon in Neotropical landscapes, particularly in regions of intermediate topographic complexity (Viana, Tabanez & Batista, 1997). In our focal study region, Brazil's Atlantic Forest in São Paulo state, anthropogenic habitat disturbances, including agriculture, livestock farming, industry, and urban settlements, are concentrated in valleys. Forest fragments without water bodies are often left on steeper slopes and hilltops (Viana *et al.*, 1997; da Silva & Casteleti, 2003). The fragmentation process thus (*i*) produces a large proportion of small upland forest fragments without perennial water bodies (e.g. ponds, streams) and (*ii*) reduces the number of forest patches connected to perennial aquatic breeding sites. In such landscapes, several amphibian species are forced to migrate between upland forest fragments and aquatic habitats through open disturbed environments to complete their life cycle (Becker *et al.*, 2010; Fig. 2). Our previous findings also indicate that metamorphosing amphibians are similarly faced with dispersal through disturbed habitats (Becker *et al.*, 2010). Limited encounters with water bodies during non-breeding months should preclude host populations from frequent Bd exposures in water bodies, which could prevent development of anti-pathogen responses

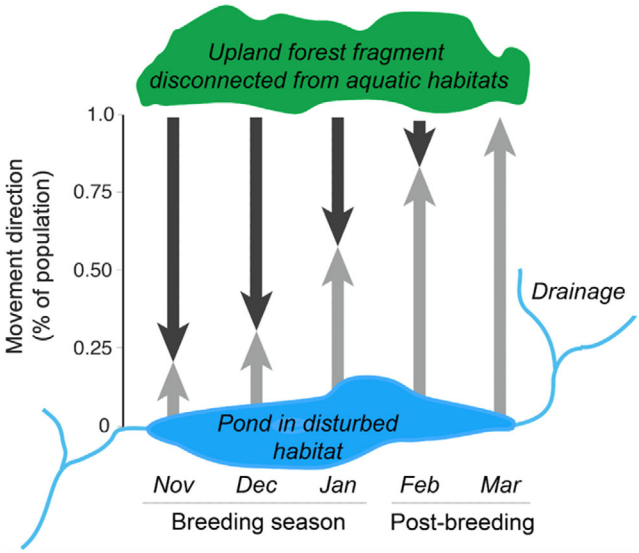

Fig. 2. Breeding migration of aquatic-breeding amphibians from upland 'dry' forest fragments to aquatic environments in disturbed habitats (black arrows). Post-breeding migration from aquatic habitats to forest fragments (grey arrows), including recently metamorphosed individuals. Average movement direction shifts significantly over time; redrawn from Becker *et al.* (2010).

(i.e. immunity). In continuous natural landscapes, however, amphibians may experience consistent encounters with water bodies year-round through random and directional foraging behaviour during breeding and non-breeding months. This could facilitate host tolerance to waterborne Bd (Ramsey *et al.*, 2010; McMahon *et al.*, 2014) by priming elements of the immune system (i.e. immunological memory, microbiome recruitment and natural selection, stress hormones) with repeated low-load exposures, facilitating temporally consistent anti-Bd immunity. Recent findings indicate that radio-tracked toads often lose infections towards the end of the breeding season, and remain Bd-free following post-breeding migrations (Daversa *et al.*, 2018*a*). This indicates alternative hypotheses: (1) changes in environmental conditions post-breeding are responsible for decreases in pathogen detectability, or (2) Bd exposure during breeding could indeed prime the amphibian immune system.

To test whether habitat split is linked to increased disease risk in amphibians, we surveyed for Bd in five forest-associated anuran species endemic to Brazil's Atlantic Forest (see online supporting information, Fig. S1). We sampled in 40 natural forest fragments and continuous forest sites within eight Atlantic Forest landscapes arranged in a latitudinal transect (Figs S2 and S3), including a focal landscape where we had previously quantified amphibian migrations through split and connected habitats (Becker *et al.*, 2010). Sampling took place at the onset of the breeding season of most local anurans during the Austral spring (breeding season) of 2018. The landscapes comprised a deforestation gradient ranging from pristine continuous forest to one of the most heavily fragmented regions of Brazil's Atlantic Forest. We quantified habitat split as the average minimum distance between the edge of a natural forest remnant and the nearest drainage network at north, south, east and west. Small focal forest fragments of comparable size often had varying levels of habitat split, which allowed us to tease apart the effects of habitat fragmentation from habitat split in our analysis. The following variables were considered as explanatory in our zero inflated negative binomial model selection [compared using Akaike's information criterion with small sample correction (AICc)] in which Bd infection load was treated as the response variable: habitat split, natural habitat cover, forest fragment size (excluded from final model, see below), host species, longitude, and Julian date. Detailed descriptions of spatial metrics, field sampling, and statistical analyses are provided in Appendix S1.

We found that habitat split was linked to increased Bd infection loads in amphibian hosts during the breeding season when accounting for other biotic and abiotic predictors (Table 1). Per cent natural habitat cover measured at the landscape scale was a strong positive predictor of Bd, likely due to optimal climatic conditions for Bd growth in continuous natural forests as suggested by previous studies (e.g. Becker & Zamudio, 2011). Our model also predicted a decrease in Bd infection loads towards the end of the breeding season, highlighting that acquired resistance after pathogen exposure during breeding aggregations could indeed evolve in our system, as described in a recent mesocosm study using amphibians from one of our highly disturbed landscapes (Becker *et al.*, 2016). Our model also highlighted that infection loads increased towards higher longitudes, as landscapes with higher levels of habitat split were located in the east. Although our model also indicated that Bd infection loads significantly varied among species (Table 1), suggesting host-specific differences in exposure and/or immune responses, both aquatic- and terrestrial-breeding species showed high average infection loads in split forest fragments. This may suggest that aquatic-breeding species could be carrying Bd to upland forest fragments after breeding in split aquatic environments, and spreading it to populations of territorial terrestrial-breeding species such as *Ischnocnema henselii*, but this hypothesis remains to be tested. Our ΔAICc model selection approach excluded forest fragment size as an explanatory variable (Fig. S4), underscoring the key role of processes that occur at wider spatial scales, which consider sets of fragments with their surrounding environment. In particular, the degree of landscape-scale natural habitat cover and habitat split were both significant explanatory variables for Bd infection intensity in our system, highlighting the need to identify and rigorously investigate potential mechanisms linking shifts in landscape configuration, spatial connectivity and elements of host immunity in wildlife.

Table 1. Zero inflated negative binomial (ZINB) regression including habitat split, natural habitat cover (landscape scale), longitude, Julian date and host species explaining Bd infection intensity in tropical amphibians.

Variable	d.f.	β	Wald χ^2	P
Habitat split	1	0.020	11.349	0.001
Natural habitat cover	1	0.090	17.600	<0.0001
Longitude	1	0.001	5.532	0.019
Julian date	1	−0.015	5.236	0.022
Host species	4	–	23.272	<0.0001

Estimation method: maximum likelihood; $N = 777$ observations; highest variance inflation factor (VIF) = 2.498.

IV. POTENTIAL MECHANISMS LINKING HABITAT SPLIT AND ELEMENTS OF AMPHIBIAN IMMUNE DEFENCES

(1) Skin microbiomes

Recent findings indicate that habitat loss and fragmentation cause shifts in community composition of the amphibian microbiome, such as decreases in skin bacterial diversity (Becker *et al.*, 2017) and microbiome dysbiosis (Jiménez *et al.*, 2020; Neely *et al.*, 2022) in disturbed environments. Using methods to quantify the function (i.e. anti-Bd defence capacity) of amphibian skin bacteria (Woodhams *et al.*, 2015; Rebollar *et al.*, 2016, 2019), several studies have demonstrated that exposure to water bodies and/or aquatic

Bd shapes microbiome composition in ways that influence the potential of the microbiome to inhibit Bd growth. For example, a recent study of anti-Bd function highlights that uninfected hosts captured in water bodies have a greater number and abundance of putative anti-Bd microbes than Bd-positive hosts (Fig. S5C). Among species, aquatic-breeding treefrogs had a significantly higher number of culturable Bd-inhibitory bacterial taxa compared to terrestrial-breeding frogs in Panama (Rebollar *et al.*, 2019). Although this hypothesis remains to be tested, shifts in host microbiome structure and function is a plausible mechanism to explain the observed disproportionate increase in infection levels during the breeding season in split environments. Here we propose several mechanisms underlying amphibian microbiome changes in the context of habitat split and how these changes affect Bd dynamics.

First, we propose that the restricted movement in split habitats may reduce the adaptive potential of the amphibian skin microbiome during non-breeding months, which could explain the higher Bd infection intensity in split habitats at the onset of the breeding season observed in our study system. Frequent exposures to Bd in aquatic habitats are known to increase host acquired resistance (McMahon *et al.*, 2014; Becker *et al.*, 2016), and studies have demonstrated that infection with Bd causes shifts in the amphibian skin microbiome often capable of inhibiting Bd (Loudon *et al.*, 2016; Bates *et al.*, 2018; Harrison *et al.*, 2019; Jin Song *et al.*, 2019). In terrestrial habitats split from water bodies, aquatic-breeding amphibians are likely to experience relatively few exposures to waterborne Bd during overwintering (Fig. 3A*i*) and pre-breeding seasons (Fig. 3A*ii*). This is expected to impede the consistent but mild pathogen exposures required to prime the host microbiome ahead of increases in pathogen pressure during the breeding season. Under these conditions, natural selection for microbiomes competitive against Bd may not operate, which could lead to increased Bd susceptibility upon exposure to aquatic habitats during the breeding season (Fig. 3A*iii*). Restricted host movement during non-breeding months as a result of habitat split may also overwhelm the host microbiome with continuous high-load pathogen exposure in crowded and isolated patchy populations (Brannelly *et al.*, 2015). Conversely, in connected terrestrial and aquatic habitats where host movement is unrestricted, frogs should experience multiple low-load pathogen exposures in or around water bodies during non-breeding months (Fig. 3B). This should thereby maintain low pathogen loads and increase overall health and immunity of amphibian hosts before subsequent periods of seasonal pathogen pressure (annual breeding migrations) and throughout the year. These hypotheses require empirical support.

Habitat split could also impact microbiome composition and anti-Bd function through exposure to both varied microclimates and environmentally sourced bacteria in the anthropogenic matrix habitats. Environmental conditions are known to drive shifts in host surface microbiomes (Woodhams *et al.*, 2020), including amphibian skin microbiomes (Kueneman *et al.*, 2019; Walke *et al.*, 2021). Microclimate conditions such as humidity and temperature that vary in split habitats predict microbiome composition within (Muletz-Wolz *et al.*, 2017) and among species (after controlling for phylogeny; e.g. Bletz *et al.*, 2017*a*). A variety of other factors impact the skin microbiome, including host development, ecological niche, habitat disturbance, and host assemblage (see Fig. S6 for microbiome drivers across biological scales). In connected terrestrial and aquatic habitats, hosts may encounter more diverse pools of microbial taxa in water bodies surrounded by natural land cover (Becker *et al.*, 2017). Furthermore, livestock waste in disturbed agricultural landscapes is linked to skin microbiome dysbiosis, increased dominance of Bd-facilitative bacteria and risk of chytridiomycosis in frogs traversing and breeding in agricultural ponds (Preuss *et al.*, 2020). This suggests that amphibians moving through landscapes with high levels of habitat split may also recruit a smaller pool of symbionts able to hamper Bd growth effectively because water bodies disconnected from natural habitats are more likely to suffer from agricultural runoff, and this may further reduce host adaptive potential to persist when they are challenged by disease.

Well-replicated mechanistic studies linking microbiomes and host–pathogen interactions across increasingly disturbed landscapes are needed to unravel the effects of anthropogenic habitat split and landscape connectivity on amphibian diseases. The Stress Gradient Hypothesis (Bertness & Callaway, 1994) proposes that primary space-holders buffer neighbours from potentially limiting stresses and has been expanded to include the potential for host–microbiome interactions to mediate host stressors (David, Thapa-Magar & Afkhami, 2018). Specifically, microbial effects are classified on a continuum ranging from microbiome mitigation, i.e. inducing microbial taxa with functions that mitigate harmful effects of stress on host performance, to exacerbation of the effects of stress on hosts. Infection may be a particular stress leading to microbiome mitigation. However, anthropogenic habitat split could interfere with this natural buffering process if infections are limited to high-intensity exposures during the breeding season that overwhelm the host immune system, rather than under non-split conditions when additional low-load exposures during non-breeding seasons may allow microbiome mitigation processes to operate more effectively.

(2) Immunogenetics

Genetic variation within key immune loci is associated with differential Bd infection and chytridiomycosis outcomes in diverse amphibian taxa. To date, immunogenetic studies of host Bd responses have primarily investigated genetic diversity in two categories of amphibian defence molecules: antimicrobial peptides (AMPs) and MHC class II proteins. Amphibians constitutively produce AMPs that are secreted onto the skin surface (Demori *et al.*, 2019), and over 1000 unique peptides and genes have been identified in amphibians (Varga, Bui-Marinos & Katzenback, 2019). Some of these AMPs contribute to resistance against Bd

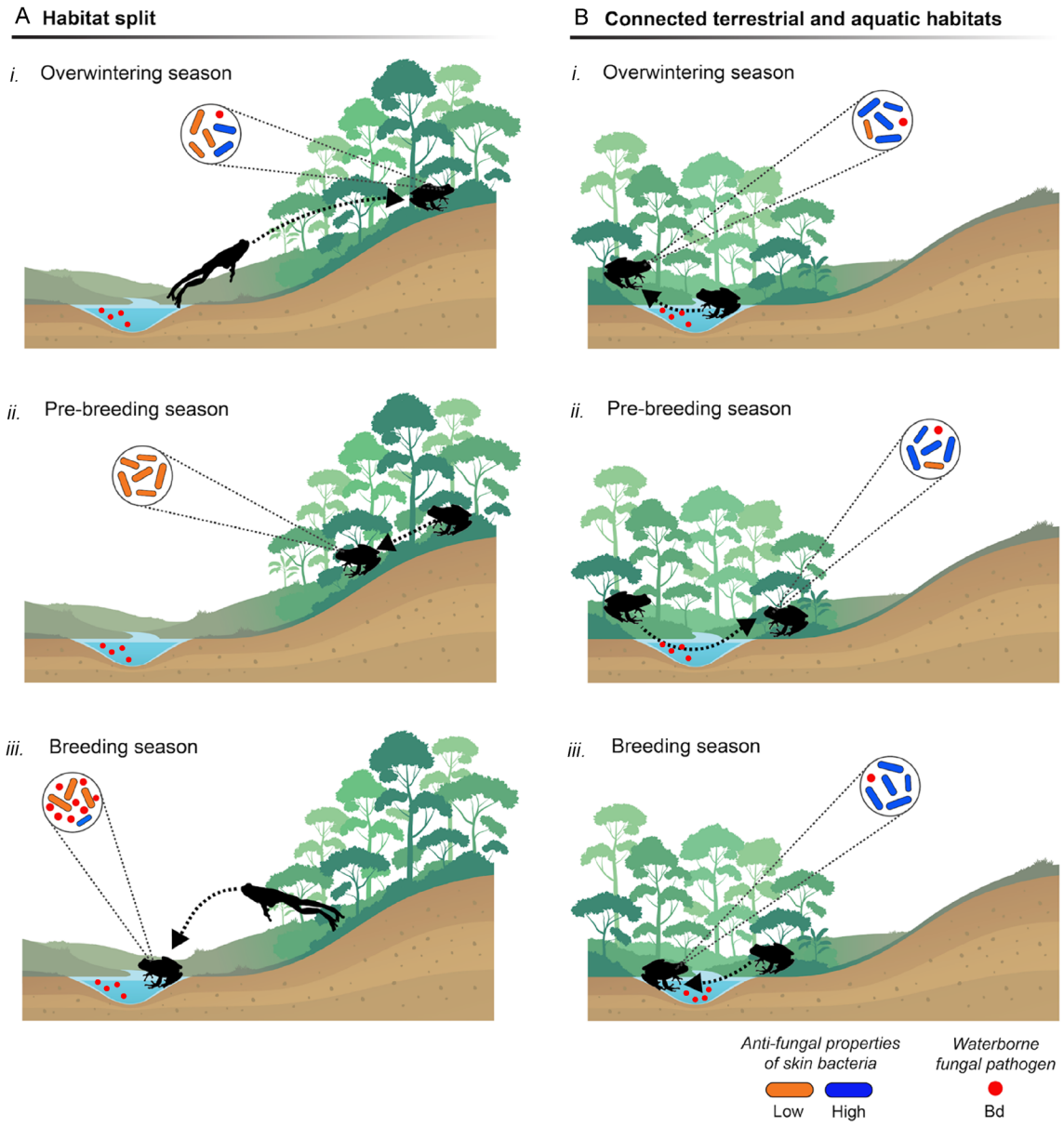

Fig. 3. Hypothetical skin microbiome assembly under contrasting scenarios of high and low spatial connectivity between terrestrial and aquatic habitats. (A) In forest fragments disconnected from riparian zones, lack of regular exposure to *Batrachochytrium dendrobatidis* (Bd) during overwintering (*i*) and pre-breeding seasons (*ii*) should select for a Bd-naive skin microbiome, likely increasing the risk of chytridiomycosis and die-offs at the onset of the breeding season (*iii*). (B) In forest fragments connected to riparian zones, however, forest-associated amphibians experience multiple exposures to Bd in water bodies during overwintering (*i*), pre-breeding (*ii*), and the breeding season (*iii*), which should select for skin microbiomes dominated by bacteria with antifungal properties. Arrows indicate movement of the same individual frog.

(Myers *et al.*, 2012; Pask, Cary & Rollins-Smith, 2013) and in certain cases the diversity of AMPs produced is associated with Bd resistance (Daum *et al.*, 2012) and selection for AMP activity against Bd is associated with population recovery to disease (Voyles *et al.*, 2018).

MHC class II genes are the other group of immune loci that have been extensively studied in the context of Bd. MHC class II pathogen surveillance receptors show allelic and gene expression associations with Bd survival across diverse host species (Savage & Zamudio, 2011, 2016; Bataille *et al.*, 2015; Fu & Waldman, 2017; Savage *et al.*, 2018). Notably, a recent transcriptome study of immune gene expression in lowland leopard frogs (*Lithobates yavapaiensis*) exposed to Bd found that susceptible frogs maintained elevated levels of induced immune gene expression, including MHC class II, whereas surviving frogs showed

reduced expression of these same loci, even relative to control animals (Savage *et al.*, 2020). We hypothesize that this pattern arises from effective regulation of acquired immunity in surviving frogs compared to poor immune system control in susceptible individuals (Benacerraf, 2006), likely connected to the MHC alleles present within an individual (Savage *et al.*, 2020). Several transcriptomic studies of chytridiomycosis have also identified elevated expression of other immune loci that may be important for disease outcomes (Ramsey *et al.*, 2010; Ellison *et al.*, 2014; Price *et al.*, 2015; Grogan *et al.*, 2018*a*), but genetic variation at these loci has not yet been explored in amphibians. Indeed, other variable immunogenetic loci may be important for mediating amphibian responses to infectious pathogens. For example, Toll-like receptors (TLRs) contribute to vertebrate innate immune defence by recognizing invading pathogens, and polymorphism of TLR genes is linked to disease outcomes in humans (Mukherjee, Huda & Sinha Babu, 2019), livestock (Novák, 2014), and natural vertebrate populations (Loots *et al.*, 2018).

An analogous mechanism to microbiome recruitment or natural selection might operate simultaneously modulating immune gene diversity and expression in landscapes varying in levels of habitat disturbance. In areas of high habitat split, limited encounters with water bodies during non-breeding months may preclude host populations from frequent pathogen exposures, which may limit expression (priming) of immune genes and pathogen tolerance during the breeding season. Thus, host species in these split habitats should lack an immunological memory of Bd due to reduced regular pathogen exposures during overwintering and pre-breeding seasons, which could plausibly cause immune dysregulation (McMahon *et al.*, 2014). Conversely, prior Bd exposure in hosts with high immunogenetic diversity should promote multiple primed immune genes, rapid increases in immune gene expression, and faster and more effective immune responses. This process is independent of habitat fragmentation *per se*; a small forest fragment connected to perennial water bodies should allow for the same continuous exposure to Bd and the priming of immune genes as large stretches of continuous and connected natural habitats.

Another important consideration is how immunogenetic diversity and differentiation may change within and among populations due to habitat split in a pathogen-riddled landscape. Conservation genetic theory (Frankham *et al.*, 2002) predicts that disease pressure and habitat fragmentation will each act to reduce effective population sizes, increase drift, and therefore cause a loss of overall genetic diversity and heterozygosity within amphibian populations (Allentoft & O'Brien, 2010). By contrast, balancing selection for resistance to multiple fluctuating pathogens can maintain immunogenetic diversity in small populations that have lost diversity at other loci (e.g. Aguilar *et al.*, 2004). While fragmentation will also isolate populations from each other and increase genetic differentiation at all loci, if the same pathogen is dominant within each population, that common selective pressure may serve to keep populations more genetically similar at immune loci by favouring the same pathogen-resistance alleles (e.g. Alves *et al.*, 2019; Hill *et al.*, 1991). Habitat split may exacerbate the overall genetic consequences of fragmentation, because populations will lose extra individuals during risky migrations in the breeding season, thereby suffering even stronger consequences of drift. Alternatively, habitat split combined with habitat loss could lead to increased genetic variation within populations due to greater gene flow if breeding migrations from multiple source populations are shifted from many to a single or few breeding locations. Because habitat split should reduce host immune priming to pathogens, split should exacerbate pathogen-linked mortalities, enhance drift, and accelerate the loss of genetic variation. The increase in disease mortalities due to habitat split should also increase the strength of selection for pathogen-resistance alleles, making it more likely that selection will be strong enough to cause immunogenetic adaptation to a dominant pathogen even in small populations suffering from the effects of drift.

While considerable empirical work is needed to test these population genetic predictions in the specific context of habitat split, several studies contrasting overall genetic diversity with MHC diversity in Bd-endemic populations shed some light on the consequences of disease and fragmentation for immune genes relative to other genetic loci. Amphibian communities in our focal landscapes with the highest levels of habitat split harbour lower neutral genetic diversity (Dixo *et al.*, 2009), lower MHC diversity (Belasen *et al.*, 2018), lower taxonomic diversity (Becker *et al.*, 2007), and experience relatively high disease susceptibility during the breeding season, consistent with the population genetic scenarios outlined here. In other regions of the world, populations with endemic Bd infections show higher genetic differentiation at neutral loci relative to MHC class II loci in bullfrogs (*Lithobates catesbeianus*; LaFond *et al.*, 2022), northern leopard frogs (*Lithobates pipiens*; Trujillo *et al.*, 2021), and lowland leopard frogs (*Lithobates yavapaiensis*; Savage & Zamudio 2016), suggesting that parallel selection for Bd resistance could be maintaining similar MHC allelic diversity among genetically isolated populations. Within populations, some amphibian studies find higher MHC diversity relative to neutral loci (Savage & Zamudio, 2016; Trujillo *et al.*, 2021), suggesting that pathogen selection maintains immunogenetic diversity despite drift, whereas other studies find the opposite pattern of reduced MHC diversity relative to neutral loci (Savage *et al.*, 2018; Kosch *et al.*, 2016), which may arise from directional selection for resistance to Bd. These contrasting findings highlight the complexity of selection on hypervariable immune genes (Spurgin & Richardson, 2010), and serve as a reminder that habitat split adds further complexity that requires carefully designed studies to tease apart.

(3) Chronic stress

Habitat split could also affect amphibian disease dynamics through stress-induced disease susceptibility, in which chronic stress suppresses pathogen defences. To understand the physiological challenges and consequences of habitat split, we use

the allostasis model (McEwen & Wingfield, 2003), a concept describing the physiological mechanisms that maintain homeostasis during environmental change (Fig. 4). In the allostasis model, the response to stressful conditions brought on by unpredictable environmental changes is termed the emergency life-history stage (Wingfield *et al.*, 1998). This strategy is thought to minimize energy loss through the tissue-specific metabolic actions of glucocorticoids that effectively shut down non-essential processes and release energy from storage for essential ones (Wingfield *et al.*, 1998). After the perturbation passes, a negative feedback loop of the HPA/I axis returns glucocorticoid levels to baseline (Sapolsky *et al.*, 2000). When elevated glucocorticoid levels are sustained for prolonged periods because feedback loops become dysregulated, sickness behaviours can become detrimental (Lopes *et al.*, 2021) and certain aspects of immune function can become suppressed (Dhabhar, 2009). Though the generality of this consequence of chronic stress is debated (see Boonstra, 2013), current studies provide some support for chronic stress as a potential mechanism for habitat split to exacerbate disease in amphibians.

Although evidence across taxa suggests a positive relationship between habitat disturbance and glucocorticoids [see reviews in Busch & Hayward (2009) and Baker, Gobush & Vynne (2013)], no study to date has demonstrated a causal link between glucocorticoids and reduced immunity in this context (Ellis, McWhorter & Maron, 2012; Messina *et al.*, 2018). Further, the role of glucocorticoids in Bd tolerance and resistance remains unclear [see reviews in Grogan *et al.* (2018*b*) and Hammond *et al.* (2020)]. Because the bioactivity of glucocorticoids is regulated through transport molecules (i.e. corticosteroid-binding globulins), receptor expression and signalling, and intracellular enzymes that can deactivate glucocorticoids prior to receptor binding (reviewed in Romero & Wingfield, 2015), the relationship between circulating glucocorticoid concentrations and immune responses is rarely straightforward. Therefore, we frame current research within the context of energy requirements (i.e. allostatic load) to maintain homeostasis, attain sufficient resources, and fight Bd infections to identify putative chronic stressors introduced by habitat split and their role in Bd dynamics.

First, habitat split between natural terrestrial and aquatic habitats is likely to increase energy expenditures due to greater risks of dehydration and longer breeding migration and juvenile dispersal distances (Fig. 4A). Recent forest clearing substantially shifts hydrothermal microclimate conditions (i.e. humidity, temperature, solar radiation, and wind speed; Ghuman & Lal, 1987), often creating hazardous landscapes between split natural habitats. Indeed, desiccation-prone amphibian species were more dispersal limited in a tropical landscape with xeric matrix types between forest patches (Watling & Braga, 2015). Amphibians have unique capacities to survive water loss, within limits (Greenberg & Palen, 2021), but these adaptations expend energy (i.e. increased heart rate and cardiac contractility), induce stress on the cardiovascular system (i.e. blood hyperosmolality, hypovolemia, and hyperviscosity), and increase production of reactive oxygen species (ROS; see review in Hillman *et al.*, 2008). In addition, corticosterone in part mediates the physiological response to dehydration stress (Uchiyama & Konno, 2006) and may also play a role

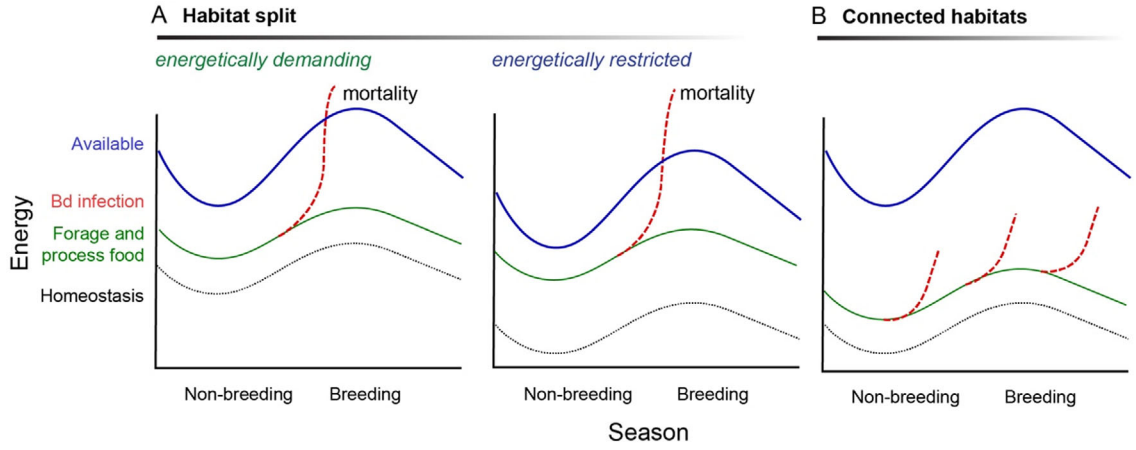

Fig. 4. Conceptual model of energy requirements in relation to available energy in the system (i.e. allostatic load, *sensu* McEwen & Wingfield, 2003) of amphibians in split (A) and connected (B) habitats during non-breeding and breeding seasons. Frogs migrating through split habitats face several stressors that can increase the energy required to maintain homeostasis (e.g. increased heart rate due to dehydration stress), thereby shifting the energy required to attain sufficient resources higher (energetically demanding). In addition, habitat split can decrease the available energy and increase the energy needed to obtain resources (e.g. find prey) in disturbed habitats (energetically restricted). In both cases, which are not mutually exclusive, the energy needed to fight severe *Batrachochytrium dendrobatidis* (Bd) infections in the breeding season might exceed what is available, causing allostatic overload and likely activating the hypothalamic–pituitary–adrenal/interrenal (HPA/I) axis. In connected natural landscapes, the energy required to maintain homeostasis and to find and process food should never exceed the energy available in the system. Even when additional energy is needed to fight Bd infections (red dashed line), which occur repeatedly through the seasons at low levels, this increase in required energy does not exceed the available energy.

in water-seeking behaviour (Madelaire *et al.*, 2020). Mounting an appropriate corticosterone response appears vital for survival and limiting water loss in two toad species invading arid habitats (Jessop *et al.*, 2013; Barsotti *et al.*, 2021). However, whether persistent exposure to dehydration stress affects immunity still remains largely unexplored. In one example, reduced water availability under cool temperatures or droughts caused rainforest frogs to clump together, and this change in behaviour increased mortality from chytridiomycosis (Longo, Burrowes & Joglar, 2010). Given that chytridiomycosis causes water and ion balance disruption (Voyles *et al.*, 2007), leads to substantial increases in water loss rate (Russo *et al.*, 2018), puts stress on cardiac function (Salla *et al.*, 2018), and can also stimulate an increase in corticosterone levels (Fig. S5A; Gabor, Fisher & Bosch, 2013; but see Hammond *et al.*, 2020), the potential for synergy between chytridiomycosis and additional dehydration stress imposed by migratory behaviour through disturbed matrix habitats is an important direction for future research in landscapes with high levels of habitat split (Moura-Campos *et al.*, 2021).

The longer migration distances of amphibians in split habitat landscapes (Cushman, 2006; Becker *et al.*, 2007, 2010) are likely to increase energy expenditures. When dispersing through disturbed habitats, amphibians may be faced with several barriers to movement (Nowakowski *et al.*, 2015). Spotted salamander, *Ambystoma maculatum*, males migrating across pavement to reach fragmented breeding ponds had elevated baseline corticosterone levels compared to those migrating through forest (Homan *et al.*, 2003). However, conflicting findings of lower baseline and stress-induced corticosterone levels in males migrating to and from disturbed (i.e. recently developed subdivisions) compared to undisturbed sites suggest that corticosterone responses may be more nuanced, but could be interpreted as evidence for chronic stress (Homan *et al.*, 2003). On the other hand, mole salamanders, *Ambystoma talpoideum*, maintained high growth rates and lipid storage despite exposure to forest clear cutting (Chazal & Niewiarowski, 1998). Because this tolerance appears related to the availability of burrows to escape desiccation stress (Rothermel & Luhring, 2005), responses to migration and dispersal distances are likely quite variable depending on burrow use and vagility. More accurate landscape resistance estimates are needed for many species to determine appropriate threshold distances and cover-class resistance values for migrating amphibians, as well as incorporating an ecophysiology framework to predict individual/population responses to hydrothermal changes induced by habitat split [e.g. mechanistic modelling such as NicheMapR from Kearney & Porter (2020)].

Second, disturbed matrix habitats may change food quality or availability for migrating species, reducing the available energy in the system (Fig. 4A). Habitat fragmentation and edges appear to drive drastic, unpredictable changes in food web dynamics and arthropod abundance (Debinski & Holt, 2000; Murphy *et al.*, 2016), suggesting decreases in food availability and/or quality for amphibians forced to use disturbed habitats while moving between terrestrial and aquatic habitats. In some cases, specific diet needs of some species may not be met (e.g. carotenoids; Dugas, Yeager & Richards-Zawacki, 2013). Because food deprivation can increase corticosterone levels (Crespi & Denver, 2005) to quantities that are potentially immunosuppressive in amphibians (Rollins-Smith 2001), nutritional stress is another potential mechanism for greater disease susceptibility in split habitats. Regardless of corticosterone levels, nutritional stress and a loss in body condition can lead to decreased infection resistance (e.g. Venesky *et al.*, 2012). In one study, Janin, Léna & Joly (2011) concluded that reduced food quality or increased energy expenditures likely explain lower body condition and increased baseline corticosterone responses in common toads (*Bufo bufo*) in recent habitat disturbance compared to preserved landscapes. On the other hand, some generalist species may benefit from reduced competition and appear to increase in abundance in disturbed environments (Schneider-Maunoury *et al.*, 2016). Overall, there appear to be many substantial gaps in our understanding of whether amphibians generally experience nutritional stress when migrating between split terrestrial and aquatic habitats.

Stress induced by more energetically demanding/costly breeding migrations and subsequent Bd exposure could also push individuals with the poorest energetic condition past a threshold where breeding-induced steroid levels become immunosuppressive (see review in Carr, 2011). In comparison, populations in connected or continuous natural habitats are expected to be more continuously exposed to low levels of Bd, thus we predict that the energy to fight infections rarely increases above what is attainable in the system without these additional expenditures (Fig. 4B). In summary, the study of amphibian physiological responses to habitat split has great potential to identify stressors and their role in Bd dynamics, and whether glucocorticoids are a reliable indicator of chronic stress for amphibian populations (Fig. 5). Note that careful experimental design is needed to consider interspecific differences in the perception of new environments as stressful, differences in habituation or adaptive capacity, and whether hormone levels are indicative of chronic stress [i.e. the direction/magnitude of change from baseline (Dickens & Romero, 2013); see field techniques in Romero & Wingfield (2015)]. We therefore recommend that future studies combine diverse metrics that causally link habitat split stressors, physiological responses, and immunity.

(4) Synergistic interactions among mechanisms of amphibian immunity under habitat split

Shifts in host microbiome composition, chronic stress, and immune gene diversity and expression are often tightly linked. Thus, these focal mechanisms could act synergistically to boost host immune capacity in continuous natural habitats and, conversely, act antagonistically to exacerbate pathogen dominance in the microbiome and disease in split natural environments. Continuous terrestrial and aquatic habitats should enable higher gene flow among populations, reducing

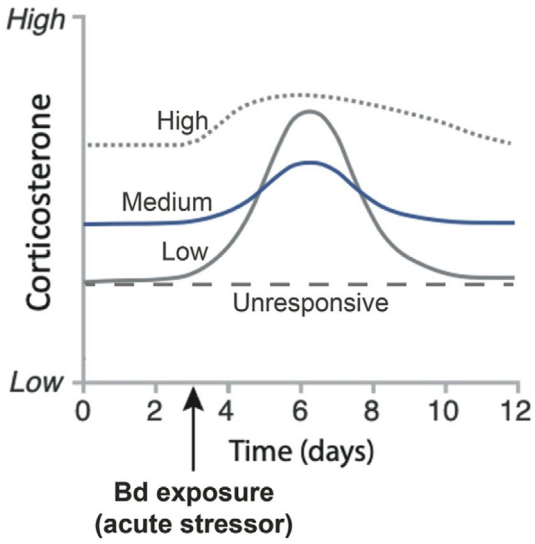

Fig. 5. Conceptual model and alternative hypotheses of corticosterone dynamics in amphibians under continuous habitat/low stress (grey line) and low habitat split/moderate stress (blue line), and high habitat split/high stress conditions (dotted line). Exposure to *Batrachochytrium dendrobatidis* (Bd) is indicated as an acute stressor on day 3. Under moderate chronic stress (blue line) there may be a higher baseline of corticosterone, a muted response to Bd and a relatively long time lag of recovery. Under low chronic stress conditions such as continuous habitat and no previous Bd infection (grey line) there may be a robust corticosterone response and recovery upon initial Bd exposure. Alternatively, chronic infection with Bd in continuous habitat/low stress conditions may produce higher baseline corticosterone or reduced responses to additional Bd exposures (approaching the blue line). There may be no corticosterone response to Bd exposure during breeding when a corticosterone increase may be costly to reproductive output (dashed line), and a measure of reproductive output then may be more indicative of stress level. These hypothesized responses require testing in a range of amphibian species to determine whether responses to Bd exposure can be generalized or whether responses depend on species-specific traits.

drift and allowing balancing selection to maintain a higher pool of MHC alleles (Belasen *et al.*, 2022) and facilitating microbiome rescue (see Becker *et al.*, 2017) across multiple host species, thereby maintaining low pathogen loads and increasing overall health and immunity of the amphibian community throughout the year. Habitat split-related reductions in immunogenetic diversity and changes in MHC class II genotype abundance and expression within populations could covary with microbiome structure and function, as observed in fragmented populations of Ozark hellbenders *Cryptobranchus alleganiensis bishopi* (Hernández-Gómez, Briggler & Williams, 2018); see also Grieves *et al.* (2021) for a similar correlation between pairwise MHC similarity and preen gland microbiota in birds. Torres-Sánchez & Longo (2022) found heterozygote advantage to recruitment of beneficial symbionts, providing defences that increase host fitness. Likewise, microbiome recruitment and natural selection and/or sustained glucocorticoid elevation during development in poor aquatic habitat within a disturbed landscape matrix could affect the development of juvenile skin defences (Hayes, 1995; Knutie *et al.*, 2017*b*). Migrating through disturbed habitats may not only reduce food quality during times of high energy expenditure, but also significantly alter gut microbiome composition with potential negative effects on energy assimilation (Fontaine, Novarro & Kohl, 2018). Synergistic studies combining landscape genetics and stress/thermal physiology are needed to identify genes and phenotypes associated with energetic costs of movement through split natural habitats and their potential to influence disease dynamics. For instance, mechanisms of genetic erosion driving a loss in MHC-II or AMP diversity (e.g. genetic drift, reduced gene flow, and small population sizes) could cause a loss of within-population variation in stress responsiveness, reducing the capacity for a series of host adaptive responses (reviewed in Bijlsma & Loeschcke, 2012).

V. HABITAT SPLIT *VERSUS* IMMUNE RESPONSES IN OTHER VERTEBRATES AND ACROSS SPATIAL SCALES

At the local or landscape scale, habitat disturbances leading to disruption of spatial connectivity among multiple required natural habitats (e.g. breeding, non-breeding, overwintering) can have detrimental effects on immunity in a wide variety of vertebrate taxa in addition to amphibians (Fig. 1; Dingle, 2014). For example, flying foxes (*Pteropus* spp.) that remain isolated in urban habitat fragments for abnormally long periods often lose herd immunity to Hendra virus, resulting in more severe disease outbreaks (Plowright *et al.*, 2011). Similarly, common fruit bats (*Artibeus jamaicensis*) in isolated forest fragments in Panama carry higher blood parasite burdens, leading to changes in immune parameters linked to reduced fitness (Cottontail, Wellinghausen & Kalko, 2009). Reduced fat stores due to limited or distant foraging common in habitat split landscapes also increase the risk of severe white-nose syndrome in infected hibernating brown bats *Myotis lucifugus* (Cheng *et al.*, 2019), highlighting that reduced spatial connectivity between foraging and overwintering habitats could lead to reduced host immune capacity. In a seabird system (threatened marbled murrelet, *Brachyramphus marmoratus*), ocean conditions (natural feeding habitat) and loss of coastal forested nesting habitat interacted to reduce bird populations (Betts *et al.*, 2020), a pattern that could be explained by shifts in host immune capacity against opportunistic pathogens in response to longer migration distances between the ocean and more inland nesting habitats (Vásquez-Carrillo *et al.*, 2014). In addition, roadway impediments to snake movement may increase physiological stress and reduce genetic diversity, factors expected to increase the severity of snake fungal disease in several species

(Clark *et al.*, 2010; McCoy, Lind & Farrell, 2017; Agugliaro *et al.*, 2020). Finally, a rapidly growing body of literature reports increases in antibiotic-resistant microbiota in a diverse array of hosts from human-dominated ecosystems (Power, Emery & Gillings, 2013; Dolejska, 2020). Together, these empirical data sets demonstrate the disease risks and immune system compromises that highly mobile and migratory species face as a result of habitat split.

In other cases, effects of habitat disturbance and split on immunity are less straightforward. For instance, higher abundances of anthropogenic food sources in disturbed landscapes were associated with increases in pathogen-inhibitory gut microbiota (Ingala *et al.*, 2019) and other improved immune defences against bacterial pathogens, but also higher stress in migrating vampire bats *Desmodus rotundus* (Becker *et al.*, 2018). Similarly, capybaras (*Hydrochoerus hydrochaeris*) in isolated and disturbed water bodies often forage through the surrounding cropland matrix and are exposed to high levels of biotic and abiotic stressors (Schivo *et al.*, 2015), but the associations between stress, immune investment, and infection varied among different types of parasite infections (Eberhardt *et al.*, 2013). In addition, supplemental food resources around urban and suburban forest fragments were found to improve innate immune defences but were also linked to reduced immune parameters such as higher pathogen infections in a variety of taxa (Wilcoxen *et al.*, 2015; Strandin, Babayan & Forbes, 2018).

At continental or other long-distance scales, migrations may increase host exposure (and adaptive responses) to myriad microparasites through large-scale environmental sampling and/or environmental tracking, but could also reduce exposure through parasite escape from harsh seasonality and contaminated habitats (Altizer *et al.*, 2006; Altizer, Bartel & Han, 2011; Daversa *et al.*, 2018*a*; Teitelbaum *et al.*, 2018; Poulin & de Angeli Dutra, 2021). Anthropogenic climate warming and habitat change affect large-scale and continental seasonal migration routes in several vertebrate groups such as salmonids, migratory birds, humpback whales, caribous (Altizer *et al.*, 2011; Dingle, 2014), with significant effects on immune responses through stress, immune gene expression, and exposure to environmental microbes, both pathogenic and beneficial. In migratory salmonids, different levels of habitat split between freshwater and marine environments can alter pathogen exposure (Lennox *et al.*, 2020), microbiome composition and function (Reid *et al.*, 2017), immune gene expression (Twardek *et al.*, 2019) and metabolic stress (Twardek *et al.*, 2019), all of which potentially influence immune responses at the individual level as well as herd immunity. Aquaculture may exacerbate the impacts of anthropogenic habitat split on acquired resistance to viruses by exposing or trapping salmon in a network of fish farms (pathogen hotspots) throughout long migratory routes (Lafferty *et al.*, 2015). Another example of habitat split at long-distance scales is the removal or degradation of stopover sites used by migratory birds, which is known to reduce migration success with downstream impacts on stress levels, immune gene repertoire and expression, microbiome homeostasis, and survival (Aharon-Rotman, Bauer & Klaassen, 2016; Xu *et al.*, 2019). Disruption to spatial connectivity among multiple classes of natural habitats should also alter host immune responses in migratory ungulates (Becker *et al.*, 2020) by impeding seasonal social behaviours necessary to maintain pools of symbiotic microorganisms linked to adaptive immunity (Fryxell, 1995; Guttal & Couzin, 2010).

In an era of global change, other anthropogenic disturbances could function analogously to habitat split to influence migration and immunity at large spatial scales. For instance, ocean warming may 'split' marine habitats by disrupting sea currents and salinity gradients at high enough levels to influence migration routes and therefore impact elements of host immunity in migratory fishes, sea turtles, birds, and sea mammals (Lennox *et al.*, 2016). As an example, skin microbiome composition in humpback whales differed between early *versus* late migration and feeding *versus* starving (Apprill *et al.*, 2014). A recent study suggests that the observed richer blow microbiota of whales at the beginning of fasting and annual migration is linked to changes in the respiratory microbiome during migration (Vendl *et al.*, 2020). Likewise, shifts in salinity can influence intestinal microbiome composition and immunity in yellow drum, *Nibea albiflora* (Tian *et al.*, 2020). These findings highlight how habitat split occurring at multiple spatial scales could alter the frequency and intensity of pathogen exposure, with important implications for the vertebrate immune system.

VI. CONCLUSIONS

(1) Habitat split is a widespread phenomenon with negative impacts on biodiversity. Our framework for investigating the impacts of habitat split on amphibian disease risk links accelerated global change stressors with host characteristics, components of the immune system, and disease outcomes at both population and community levels.

(2) Evidence indicates that restricted movement stemming from habitat split could impair the assembly and natural selection of a healthy microbiome, lead to a reduction of host immunogenetic diversity, and drive a sustained downregulation of immune genes, with downstream negative impacts on host immune function.

(3) Our review also highlights that habitat split likely leads to stress-induced disease susceptibility, in which chronic host stress suppresses pathogen defences. Specifically, habitat split is likely to increase host energy expenditures (and reduce energy intake) due to physiological constraints posed by costlier movement through non-natural environments.

(4) We are at the cusp of being able to predict host–pathogen dynamics in diverse systems by crossing spatial connectivity data with host physiological, genetic, microbiological and environmental parameters. Advancements in analytical tools such as machine learning and artificial intelligence will likely allow us to develop comprehensive predictive and forecasting

models, but we still lack a basic understanding of how these underpinning mechanisms shape host immune responses in disturbed and increasingly fragmented environments.

(5) Understanding mechanisms by which habitat split influences disease dynamics in amphibians and in other vertebrates will also allow us to develop effective habitat restoration strategies that will not only improve local wildlife management and ecosystem health, but could also mitigate the risk of emerging zoonotic diseases.

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IX. SUPPORTING INFORMATION

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

Fig. S1. The five amphibian species sampled in our study.
Fig. S2. Eight focal landscapes (red dots) of Atlantic Forest in the State of São Paulo, southeastern Brazil.

Fig. S3. Occurrence of five focal amphibian species in eight focal landscapes in the Atlantic Forest in the State of São Paulo, southeastern Brazil.
Appendix S1. Supplementary methods.
Fig. S4. Zero inflated negative binomial (ZINB) regression model selection.
Fig. S5. The acute-phase response to infection in amphibians involves several interrelated components.
Fig. S6. Drivers of amphibian microbiomes across multiple ecological scales.

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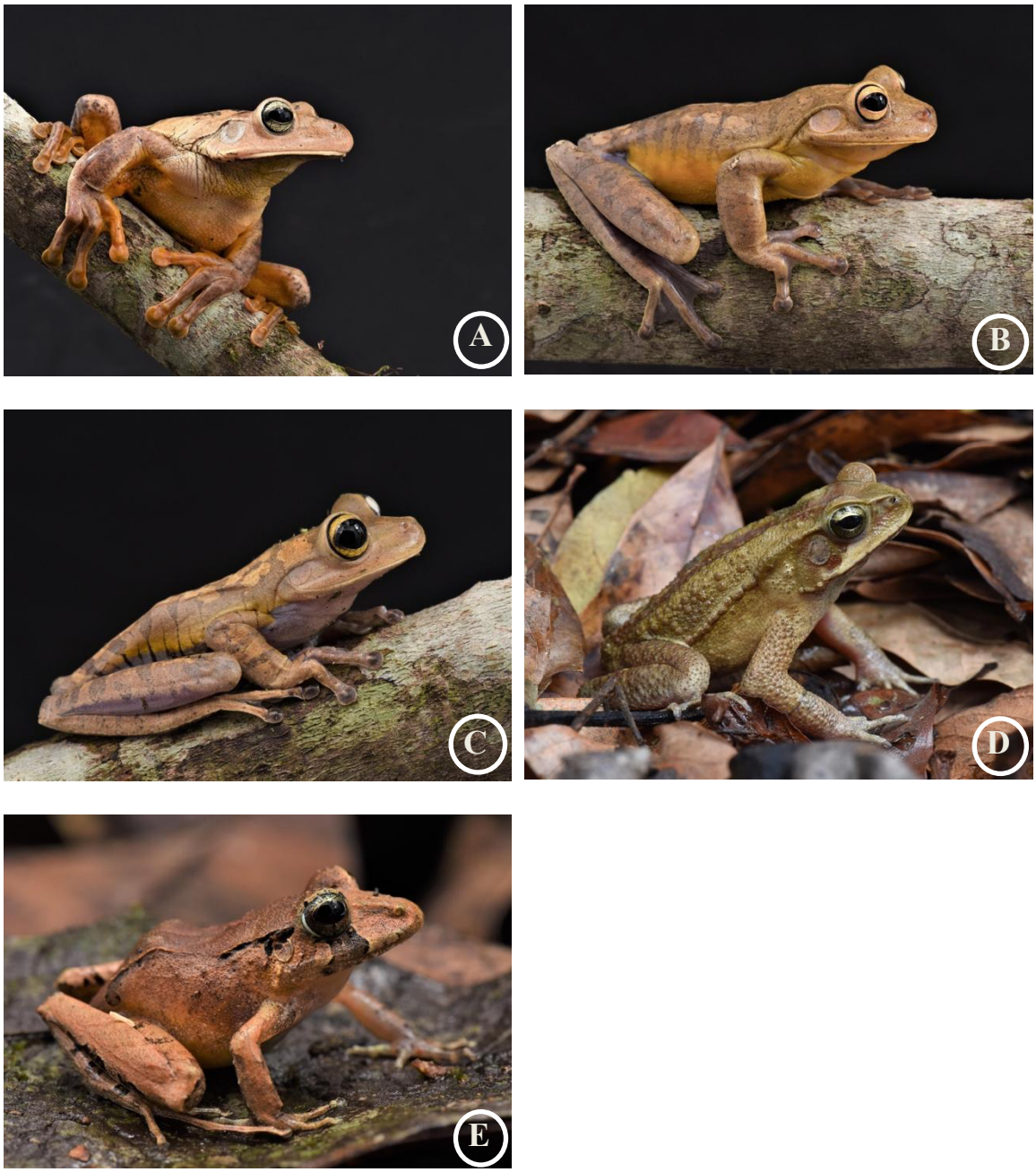


Fig. S1. The five focal amphibian species sampled in our study. (A) *Boana faber*; (B) *Bokermannohyla circumdata*; (C) *Bokermannohyla hylax*; (D) *Rhinella ornata*; and (E) *Ischnocnema henselii*.

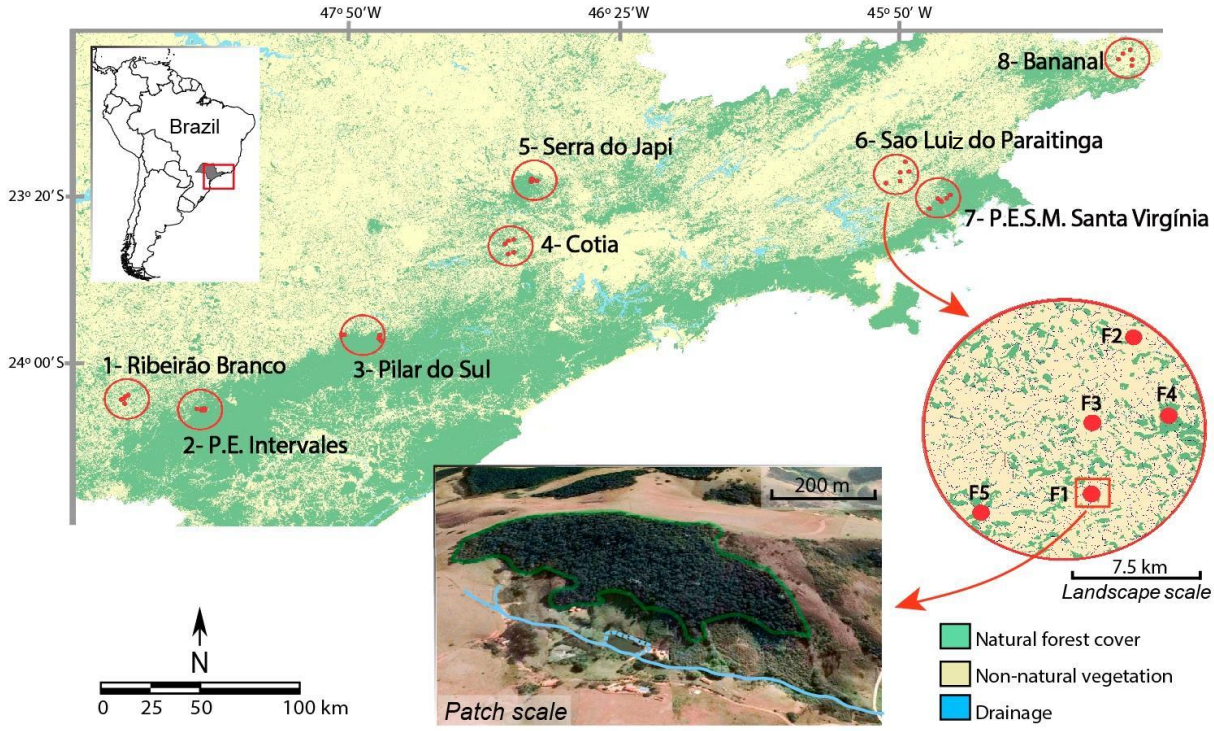


Fig. S2. Eight focal landscapes (red dots) of Atlantic Forest in the State of São Paulo, southeastern Brazil. Numbers correspond to each sampling landscape: 1, Ribeirão Branco; 2, Parque Estadual Intervales; 3, Pilar do Sul; 4, Cotia; 5, Serra do Japi; 6, São Luiz do Paraitinga; 7, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia; and 8, Bananal. The large red circle (15 km diameter) depicts one of our eight sampling landscapes, with five sampling sites (forest fragments F1–F5) highlighted. The patch scale image represents one of our focal forest fragments disconnected from the drainage network (blue line: drainage stream, dashed line: associated pond).

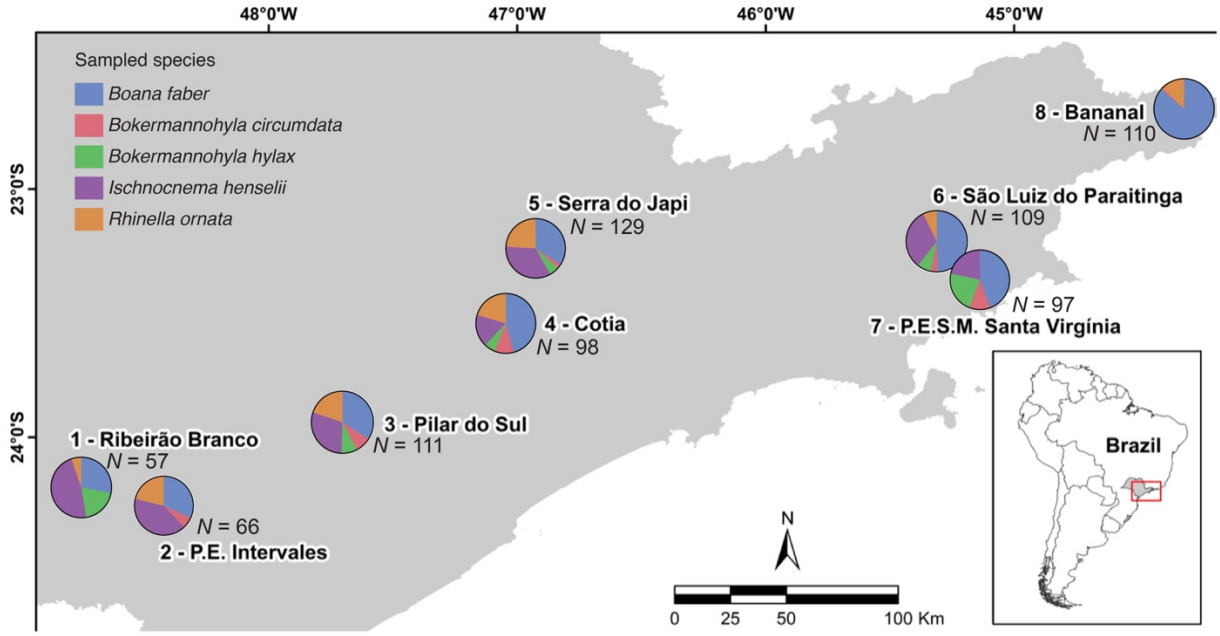


Fig. S3. Occurrence of five focal amphibian species in eight focal landscapes in the Atlantic Forest in the State of São Paulo, southeastern Brazil. Pie charts indicate relative abundance of species sampled within each landscape; total number of captures in each landscape is also given. Landscapes are (1) Ribeirão Branco, (2) Parque Estadual Intervales, (3) Pilar do Sul, (4) Cotia, (5) Serra do Japi, (6) São Luiz do Paraitinga, (7) Parque Estadual da Serra do Mar – Núcleo Santa Virgínia, and (8) Bananal.

APPENDIX S1. SUPPLEMENTARY METHODS

(1) Study landscapes and sampling design

We sampled five amphibian species in eight Atlantic Forest landscapes (15 km buffer) ranging between 13.55% and 81.32% in the degree of deforestation. Within each of these eight focal landscapes, amphibians were sampled in five natural forest fragments and continuous forest sites, totaling 40 sampling sites (Fig. S2). Relative number of captures by species and total number of captures is provided for each focal landscape in Fig. S3. Each landscape was sampled twice during four consecutive months; we revisited each landscape after our first round of sampling. In order to control for potentially confounding geoclimatic factors, we selected landscapes based on high similarity in (*i*) latitude, (*ii*) seasonal rainfall, (*iii*) topographic complexity and (*iv*) elevation. Our eight focal landscapes included both protected parks and private lands in São Paulo State, Brazil: Bananal (22º 47' S 44º 21' W), Parque Estadual Intervales (24º 16' S 48º 21' W), Serra do Japi (23º 14' S 46º 58' W), Cotia (23º 42' S 47º 05' W), Parque Estadual da Serra do Mar – Núcleo Santa Virgínia (23º 20' S 45º 08' W), Pilar do Sul (23º 54' S 47º 41' W), Ribeirão Branco (24º 09' S 48º 42' W), and São Luiz do Paraitinga (23º 13' S 45º 19' W; Fig. S1).

Hydrological and land use/land cover (LULC) data sets were obtained from Fundação Brasileira Para o Desenvolvimento Sustentável (Brazilian Foundation for Sustainable Development), comprising classified land cover layers (5 × 5 m resolution) generated from RapidEye imagery at the scale of 1:10,000 (Fundação Brasileira Para o Desenvolvimento Sustentável, 2020). LULC data include agriculture, urban, natural forest, silviculture, and drainage network including streams, lakes and swamps. Among the extracted spatial metrics, we defined natural habitat cover at the landscape scale as the per cent natural forest within our 15-km buffer. At the local scale, we quantified habitat split as the average minimum distance between the edge of each focal natural forest fragment and the nearest drainage network at north, south, east and west. Small focal forest fragments of comparable size (in the three most heavily fragmented landscapes of São Luiz do Paraitinga, Bananal, and Ribeirão Branco) often had contrasting levels of habitat split, which allowed us to tease apart the effects of habitat fragmentation from habitat split in our analysis. We also extracted the size of each focal forest fragment in hectares. All spatial metrics were calculated using ArcGIS 10.1 and Fragstats 4.0 (ArcGIS, 2012).

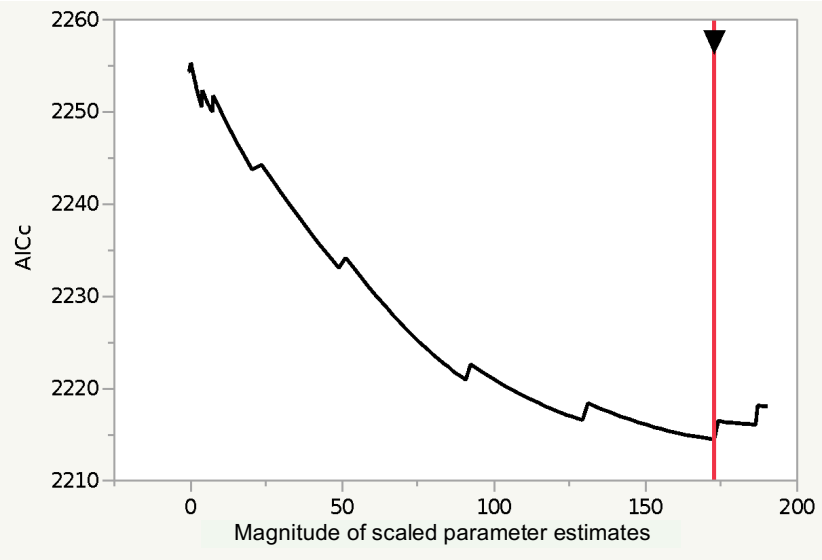
Sampling took place at the onset of the breeding season of most local anurans during the Austral spring (breeding season), from 21 September to 6 November 2018. Landscapes were sampled haphazardly to avoid potentially confounding seasonal factors in our spatiotemporal surveys.

(2) Study species

We sampled five forest-associated and wide-ranging anuran species endemic to Brazil's Atlantic forest (Haddad *et al.*, 2013): *Boana faber*, *Bokermannohyla circumdata*, *Bokermannohyla hylax* (Hylidae), *Rhinella ornata* (Bufonidae), and *Ischnocnema henselii* (Brachycephalidae) (Fig. S1). We captured a total of 777 individuals: *B. faber* ($N = 356$), *I. henselii* ($N = 204$), *R. ornata* ($N = 112$), *B. hylax* ($N = 63$), and *B. circumdata* ($N = 42$, Fig. S3). We rinsed all individuals with distilled water upon capture, before swabbing the skin with sterile MedicalWire Swabs MW-113 following standard protocols (Hyatt *et al.*, 2007). Swabs were kept on ice in 1.5 ml cryovials. After swabbing, all individual frogs were released at their capture location. Recapture rates are extremely low in amphibian communities in São Paulo due to high abundance and high predation rates (Haddad et al., 2013), thus we did not mark individual frogs upon release.

(3) Statistical analyses

We analysed our data using zero inflated negative binomial (ZINB) Akaike information criterion with small sample correction (AICc) model selection where *Batrachochytrium dendrobatidis* (Bd) infection intensity was treated as the response variable, and the following environmental, biotic and temporal metrics were included as explanatory variables: habitat split, natural habitat cover, forest fragment size, species, longitude, and Julian date. Studies suggest that per cent natural habitat cover at the landscape scale should be included in models looking at patch-scale processes, but landscape- and patch-scale metrics are often cross-correlated (Pardini *et al.*, 2010; Banks-Leite, Ewers & Metzger, 2013). Although landscape-scale natural habitat cover and habitat split were negatively correlated among our 40 sampling sites ($r = 0.482$; $P = 0.002$), this correlation was below the multicollinearity threshold commonly used in spatially oriented studies (Zuur *et al.*, 2009), and we also ruled out multicollinearity by checking the variance inflationary factor (VIF) in the most parsimonious model. We report results from the most parsimonious model based on delta AIC. To account for potential spatial autocorrelation in our models, we performed a Mantel test (5000 permutations) using Euclidian distance among our 40 sampling sites and included the resulting residuals in the best-fit models. We also tested these residuals for spatial autocorrelation using Moran's *I*. For these analyses, we used a matrix of inverse distance weighting (IDW), using the centroid of each sampling site. This test is a widely accepted method to test for autocorrelation and spatial dependence (Dale & Fortin, 2002). Both for Mantel and Moran's *I,* results indicated non-significant autocorrelation. Statistical analyses were performed in R (R Core Team, 2018) and JMP 15 (JMP, 2019).



Variable	*N*	DF	Wald X^2	*P*	
Natural habitat cover	1	1	34.955	<.0001	
Habitat split	1	1	18.217	<.0001	
Species	4	3	20.980	0.000	Levels remov.: 1
Julian date	1	1	6.397	0.011	
Longitude	1	1	5.887	0.015	
Forest fragment size	1	0	0.001	1.000	Removed

Fig. S4. Zero inflated negative binomial (ZINB) regression model selection. The variable 'Fragment size' was removed based on adaptive Lasso Akaike information criterion with small sample correction (AICc) validation. Levels remov.: number of levels within a categorical variable (1 out of 5 species) removed during stepwise model pruning.

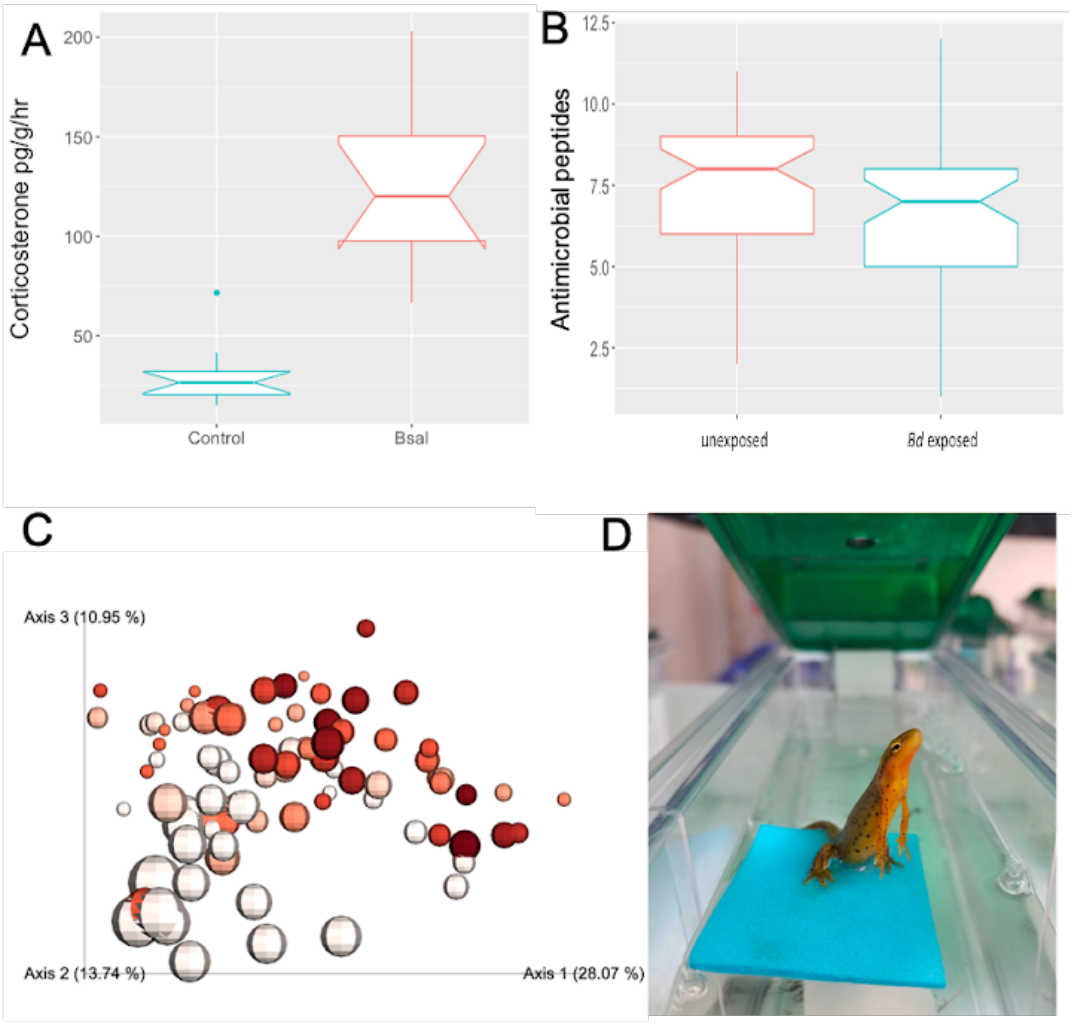


Fig. S5. The acute-phase response to infection in amphibians involves several interrelated components (Lopes *et al.*, 2021). (A) Activation of the hypothalamic–pituitary–interrenal axis stimulates an immediate corticosterone response, here accumulating in water rinses within the first hour of exposure to the fungal pathogen *Batrachochytrium salamandrivorans* (Bsal) as quantified from spotted salamanders, *Ambystoma maculatum* (data from Barnhart *et al.*, 2020). Activation of acute phase proteins including complement proteins from hepatocytes also takes place (not shown; reviewed in Jain *et al*., 2011; Rodriguez & Voyles, 2020). Catecholamines may also be stimulated by infection (Rollins-Smith, 2017). (B) Heat-killed *Aeromonas hydrophila* subcutaneous injection induced sickness responses including expression of skin defence antimicrobial peptides (AMPs) within 15 min of inoculation. More AMPs were secreted from 'unstressed' frogs (recently metamorphosed *Rana temporaria*), not previously exposed to the fungal pathogen *Batrachochytrium dendrobatidis* (Bd; Poisson distribution of AMP count analysed with the *nlme* package in R controlling for frog mass; $\chi^2_1 = 4.0907$, $P = 0.043$; data from Kupfer, 2012). Horizontal lines within box plots in panels A and B represent the median; top and bottom lines delimit the first and third quartile; the vertical lines delimit the maximum and minimum values, except for the outlier which is represented by a dot. (C) Skin bacterial communities of eastern red-spotted newts, *Notophthalmus viridescens*, depicted in a principal coordinates analysis (PCoA) showing samples coloured by Bd infection load (white = 0 to red = 3.1×10^6), and points scaled by predicted anti-Bd function of the microbiome of each individual (data from Carter *et al.*, 2021). Shifts in amphibian skin microbiome communities are a typical response to infection, although the mechanism and timing of the response remains under investigation (Jani & Briggs, 2014). Baseline corticosterone, immune defences, and microbiome can change seasonally and with circadian rhythms in amphibians and in other vertebrates (Martinez-Bakker & Helm, 2015; Le Sage *et al.*, 2021). (D) Sickness behaviours in *N. viridescens* in

response to Bsal infection include lack of appetite, unusual shedding patterns (reviewed in Grogan *et al.*, 2018*b*) and body posturing perhaps functioning to dry the skin or inducing movement away from the water potentially inhabited by conspecifics (photograph credit: Julia McCartney). This combination of acute-phase responses may direct infection outcomes differentially depending on anthropogenic habitat split.

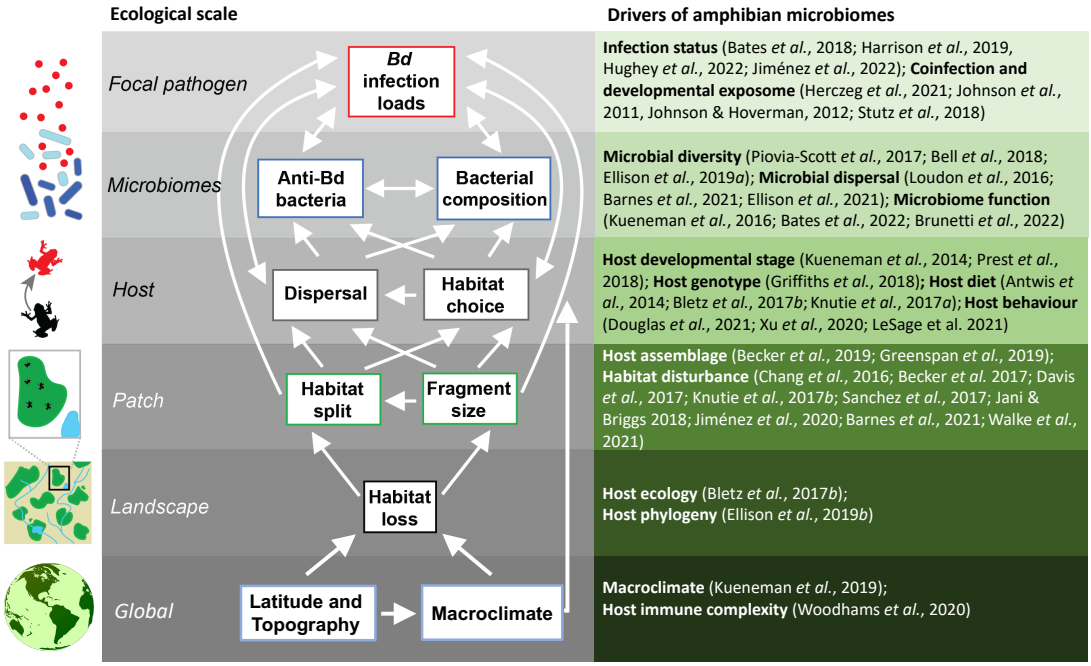


Fig. S6. Drivers of amphibian microbiomes across multiple ecological scales, with representative references. Bd, *Batrachochytrium dendrobatidis*.

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