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AMPHIBIANS DECLINE IN PENINSULAR ITALY: IS THE CHYTRID PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* PLAYING A ROLE?

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INDEX

PREMESSA

Questa tesi si basa sullo studio del ruolo del patogeno epidemico *Batrchochytrium dendrobatidis* nel declino delle popolazioni italiane di anfibi. Nel capitolo 1 e 2 vengono introdotte tematiche generali inerenti i patogeni epidemici emergenti ed il ruolo nel declino globale degli anfibi. Il capitolo 3, 4 e 5 sono in ogni loro parte conformi al manoscritto recentemente inviato per la pubblicazione su rivista (3, 4), o attualmente in corso di invio. Infine, nel capitolo 6 viene presentata una discussione generale dei risultati ottenuti.

PREFACE

This study is based on the study of the role of the panzootic pathogen *Batrachochytrium dendrobatidis* in amphibian populations decline in Italy. Chapters 1 and 2 introduce general themes about emerging infectious diseases and their role in the worldwide amphibian decline. Chapters 3, 4 and 5 are accordant with the original manuscripts submitted for publication (3, 4), or under submission. Finally, in chapter 6 a general discussion of the results achieved is presented.

INTRODUCTION

Emerging infectious diseases (EIDs) are defined as diseases that increase their pathogenic activity, spread to new geographic areas or affect new host populations. EIDs are increasingly recognized as constituting a major threat to biodiversity. Recently, many newly emerging or re-emerging pathogens have been shown to be involved in wildlife mortality events (Daszak et al., 2000). Nevertheless, pathogens are not the only factors affecting host populations; in particular, the emergence or re-emergence of infectious disease following environmental changes has frequently been observed (Morse, 1995; Harvell et al., 2002;).

In recent years, the wildlife EID chytridiomycosis, has been extensively studied by herpetologists, ecologists, epidemiologists and conservations biologists (Berger et al., 1998; reviewed in Fisher et al., 2009b; Kilpatrick et al., 2010). The disease is linked to worldwide amphibian decline, which is a well-documented phenomenon observed since the 1980s (Stuart et al., 2004; Beebee and Griffiths, 2005). The most dramatic aspect of amphibian decline is the high rate of population disappearances and species extinction. Many explanations for this pattern have been suggested, for example, habitat destruction or alteration, climate change, environmental pollution, disease, increased UV radiation, exploitation, and introduced species (Blaustein and Kiesecker, 2002; Collins and Storfer, 2003; Blaustein et al., 2011). Among these, chytridiomycosis is thought to play a prominent role (Daszak et al., 1999; 2003).

Chytridiomycosis is a fungal disease caused by the chytrid pathogen *Batrachochytrium dendrobatidis* − the only pathogen belonging to the phylum Chytridiomycota that has been found to affect a vertebrate class (Berger et al., 1998; Longcore et al., 1999). *B. dendrobatidis* affects several amphibian species across all continents where amphibians occur. The outcome of the disease is variable, and depends on host species, host populations, and geographic areas. Chytrid-related mass mortalities have been observed in various continents, mainly in mountainous

areas. However the origin, spread dynamics, and mortality outbreak patterns of *B. dendrobatidis* remain unclear (reviewed in Fisher et al., 2009b; Kilpatrick et al., 2010).

Two main hypotheses have been proposed to explain *B. dendrobatidis* epidemic dynamics (Rachowicz et al., 2005). The 'novel pathogen hypothesis' considers *B. dendrobatidis* as a recently spread pathogen, acting as a 'lone killer', and affecting 'naive', susceptible hosts (Laurence et al., 1996; Lips, 1999). Meanwhile, the 'endemic pathogen hypothesis' suggests a role for recent environmental changes in driving increased virulence of an endemic pathogen, or increased susceptibility of hosts, or both (Rachowicz et al., 2005). Nevertheless, these two hypotheses cannot be considered mutually exclusive, because environmental factors may affect host-pathogen interactions irrespective of pathogen endemicity or recent arrival.

Amphibian population declines in peninsular Italy has been reported for several species, and a number of different causes have been proposed (Barbieri et al., 2004; Lanza et al., 2007; D'Amen and Bombi, 2009). The most dramatic population declines concern the yellow-bellied toad, *Bombina pachypus* – an endemic Italian species, which has its range along the Apennine chain (Barbieri et al., 2004; Andreone et al., 2009). Populations of *B. pachypus* have been in decline since the 1990s, especially in northern and central Italy, and the species is now considered as endangered by the IUCN (Barbieri et al., 2004; Andreone et al., 2009). Chytridiomycosis has been diagnosed in captive dying specimens of *B. pachypus* collected in Bologna province (in the northern part of its range), and therefore *B. dendrobatidis* has been suggested to play a key role in the decline of the species (Stagni et al., 2004).

In the present study, we assessed the role of *B. dendrobatidis* in amphibian declines observed in peninsular Italy. Our objective was to evaluate the potential threat of this pathogen to the conservation of Italian amphibian species. We believe that our findings will contribute to the ongoing debate on chytrid epidemiology, through analysing the pattern of occurrence among Italian amphibians, and

considering potential interactions with other factors. Moreover, a better understanding of the processes and factors involved in population declines will provide useful insights for planning effective conservation strategies.

Our experimental strategy comprised 3 steps: 1) assessment of chytrid occurrence on *B. pachypus* in historical and contemporary samples; 2) evaluation of chytrid occurrence on co-distributed amphibian species; and 3) elucidation of the role of climate change in *B. pachypus* population declines.

In the first step, we used diagnostic PCR to screen historical and contemporary samples of *B. pachypus* from its entire range, and thereby assess the occurrence, spatial distribution, and historical presence of *B. dendrobatidis*. Our objective was to evaluate the spread dynamics of *B. dendrobatidis* and elucidate its role in *B. pachypus* population declines. By comparing the occurrence of *B. dendrobatidis* with the spatial and temporal dynamics of *B. pachypus* population declines, we aimed to demonstrate whether the pathogen acts as a 'lone killer' or whether it interacts with other factors.

In the second step, we used diagnostic PCR to evaluate chytrid occurrence in other amphibian species, with ranges overlapping that of *B. pachypus*. The species selected for analysis were *Salamandra salamandra gigliolii*, *Rana italica*, and *Mesotriton alpestris apuanus*. Our objective was to assess the occurrence and potential effects of *B. dendrobatidis* on species showing a lower extent of decline.

In the final step, we assessed the effect of climate change−another driver of amphibian decline, suggested to play a role either alone or as a co-factor (Blaustein et al., 2010), on the endangered *B. pachypus*. For this purpose, we used species distribution modelling (SDM) tools to generate models of habitat bioclimatic suitability for *B. pachypus*, under current and future climatic conditions. Our objective was to identify the areas and populations likely to be most affected by climate change.

CHAPTER 1

EMERGING INFECTIOUS DISEASES

Emerging infectious diseases are defined as diseases that appear for the first time in a population, re-emerge after periods of latency, increase in prevalence, move into new geographic areas, move into new host species, or are caused by newly-evolved pathogens.

Several processes can drive the emergence or re-emergence of infectious diseases, possibly leading to epidemics or pandemics. These processes include mutational or recombination events in the pathogen genome, changes in demography and/or behaviour, range shift of host species or vectors, and environmental changes.

Historically, the most investigated emerging infectious diseases have been those affecting humans. Nevertheless, there is increasing interest about in infectious diseases that affect wild animals and plants, because they constitute a severe risk to biodiversity conservation, ecosystems health, and human health as well.

Historical notes

Host-pathogen interaction has a long evolutionary history. However, the best known infectious diseases, from a historical perspective, are those affecting humans. Anthropogenic activities and technological development have deeply altered the environment, disturbing the ecological balance among hosts, pathogens, and the environment that was achieved in evolutionary times. The outcome of these alterations has been the establishment of new ecological interactions that sometimes have increased the spread or virulence of pathogens. In this way, humans have influenced the outbreak of epidemic diseases and epidemics have become part of human history affecting and shaping the rise and fall of civilisations.

There are several historical examples that demonstrate a link between disease outbreaks and technological achievements, development of societies, demographic trends, structuring of urban agglomerations, or changes in habits and customs (Nikiforuk, 1991).

One of the earliest achievements of human ingenuity leading to the birth of civilisation and favouring the emerging of infectious diseases was the Neolithic agricultural revolution (approximately 10,000 years ago). Agricultural practices are believed to have favoured the spread and increased incidence of malaria. Indeed, deforestation and the creation of irrigation systems with water-filled and sunlight-exposed grooves created the optimal habitat for *Anopheles* sp. mosquitoes, which are the vectors of the protozoan *Plasmodium* sp., the aetiological agent of malaria (Carter and Mendis, 2002; Rich et al., 2009).

The agricultural revolution not only led to the domestication of plants but also of animals. This increased the contact rate between humans and livestock pathogens, which could have led to the spread of human diseases such as measles, smallpox, tuberculosis, and whooping cough (Diamond, 1997).

War and famine are known to have historically set suitable conditions for epidemic outbreaks. For instance, in the 14th century, war and famine, along with climate change, long-range trade, and the spread of rats and fleas throughout towns, set the stage for the Black Death, the plague caused by the bacteria *Yersinia pestis* that flagellated Europeans until the 18th century (Nikiforuk, 1991).

Another example of the tight link among environmental changes, social changes, and emerging pathogens is demonstrated by the outbreaks of cholera during the Industrial Revolution. *Vibrio cholerae*, the aetiological agent of this disease, has spread in several outbreaks since the 19th century because of the conjunction of suitable environmental conditions and poor sanitary conditions such as those present in crowded London in the mid-19th century (Barua and Greenough, 1992).

Epidemics have not only followed historical changes but also have paved the way toward historical transitions. For instance, the decline of the Sacred Roman

Empire was accompanied by the Plague of Justinian. This plague resulted in the abandonment of major population centres toward rural communities, sealing the beginning of feudalism and the transition to the Middle Ages (Little, 2007). Another notable transition was initiated by the Black Death, which aroused distrust toward religious institutions, setting the stage for the Protestant Reformation (Nikiforuk, 1991).

Emerging infectious diseases have also determined the outcome of wars and colonisations. Smallpox, brought by European conquerors to the naive populations of the New World, contributed to the extermination of local populations anticipating the advance of the armies. In this case, the unintended use of a virus, to which Europeans were resistant because of a long co-evolutionary history dating back to the early cattle farms, proved to be a more efficient weapon than gunpowder against populations that had not evolved immunological defences to this pathogen. This resulted in centuries of colonialism and exploitation (Diamond, 1997).

This brief historical overview is obviously non-exhaustive and shows just a few examples of how various types of environmental and social changes led to the emergence of infectious diseases, and how emerging infectious diseases have influenced human history. History is repeating itself and new infectious diseases have been spreading in recent decades, such as acquired immunodeficiency syndrome (AIDS) or haemorrhagic fevers caused by some viruses (Nikiforuk, 1991). Moreover, there is a recent growing interest in wildlife infectious diseases because of their implications for human health, as in the case of several zoonotic viruses causing influenza, or because of expected economic damage following the loss of biological resources or collapse of ecosystems.

Ecology of emerging infectious diseases

Disease ecology is a discipline that examines the dynamics and mechanisms underlying the spread of pathogens and parasites between and within natural populations and how these affect populations and communities (Hudson et al., 2002;

Collinge and Ray, 2006). An ecological approach to epidemiology integrates multiple processes at different scales and emphasises the role of the environment in determining the outcome of host-pathogen interactions.

The role of environmental and other biotic factors is frequently overlooked in the belief that the only sufficient cause to determine the onset of a disease is the microbe itself. This view derives from the 'germ theory' and dates back to the end of the 19th century, when scientists such as Louis Pasteur and Robert Koch made important discoveries about the role of microbes in the aetiology of infectious diseases. The application of this conception to epidemiological studies is an example of Reductionism. In epistemology, the aim of Reductionism is to explain complex phenomena by reducing them to a minimum and investigating the processes of causality. However, in the past, eminent thinkers and scientists such as Hippocrates or Rudolf Virchow already understood that environmental factors play an important role in regulating host-pathogen interactions.

Host-pathogen interactions do not constitute an isolated system, but they occur within an environment in which conditions vary in space and time and within biotic communities that are susceptible to changes in space and time as well. The combination of these factors, and especially their interactions, can shift the balance of host-pathogen dynamics in unpredictable ways. For instance, outbreaks of cholera in Bangladesh are not only attributable to the presence of *V. Cholerae*; in fact, it can remain in a dormant state for years off the coast, but they also are linked to an increase in temperature, phosphorus, and nitrogen in seawater. These environmental conditions lead to algal blooms on which zooplankton feeds. Zooplankton itself flourishes and, with its chitin shell, is in turn the nourishment of the *Vibrio*. In this way, *V. cholerae* proliferates in seawater and monsoons may facilitate the spread of the pathogen in inland waters, where local human populations have their water reserves (Colwell, 1996). Another example of how complex interactions may set the stage for outbreaks of disease is the link between potato blight and death from typhus during the Irish Potato Famine of the 19th century and the role of multiple factors. In

this case, the unfortunate coincidence of particular climatic conditions and genetic erosion of potato crops made a common fungus usually present on various Solanaceae more aggressive toward its host. This deprived the Irish population of their main food source, and malnutrition weakened the immune systems and physical condition of the Irish people (Woodham-Smith, 1962). Deplorable sanitary conditions also contributed to the development of *Rickettsia* and the typhus epidemic (Crawford, 1984; Nikiforuk, 1991).

Such examples and considerations emphasise the importance of a holistic approach to the problem of emerging infectious diseases. The investigation of a single cause, in the case of a disease outbreak, could result in an underestimation of the role of other factors and in an overestimation of the factor under investigation because it may have a correlative relationship with the disease outbreak instead of a causative one. Furthermore, because of emergent properties, several factors may behave differently when interacting than when acting alone. Deeper analysis assessing the dynamics of pathogen occurrence in space and time, host characteristics, and ecological interactions is the most suitable method to study the epidemiology of infectious diseases in natural populations.

Host-pathogen interactions

A pathogen may be defined as a microorganism capable of causing damage in a host organism, whereas a host is a biological entity within which or upon which microorganisms reside and/or reproduce themselves. In this context, host damage is defined as an impairment of homeostatic mechanisms that causes an alteration of cell, tissue, and organ functions following the entry, establishment, proliferation, or secretion of the pathogen. Damage is a continuum and disease occurs when it exceeds a certain threshold and impairs host homeostasis.

The characteristic properties of pathogens are infectivity and virulence. Infectivity is a measure of the ability of a pathogen to colonise a susceptible host, giving rise to infection, and virulence is a measure of the ability of a pathogen to

cause damage to the host. On the other hand, host characteristics are defined according to the outcome of the interaction established with the pathogen; there are susceptible, resistant, or tolerant hosts. Host susceptibility to a pathogen is determined by an immune response that is insufficient to prevent the onset of disease. In contrast, resistance is characterised by an effective immune response against the infection that avoids the permanent establishment of the pathogen. Finally, tolerance is a condition in which a host is infected (i.e. it carries the pathogen) but does not develop the disease.

Given a certain pathogen and a certain host, the outcome of the interaction is not necessarily determined but may be influenced by several factors. For instance, different strains of a pathogenic species may show different virulence profiles or may show differences in resistance to drugs because of differences in their genomes. This is especially true in bacteria such as *V. cholerae*, in which the high mutation rate and the possibility of horizontal gene transfer have led to the spread of serotypes with different characteristics of virulence and resistance (Kitaoka et al., 2011). Another example is the hybridisation between strains of allopatric pathogenic fungi, which can result in a hyper-virulent strain or in an increase of host species number, as has been observed for the plant pathogen *Phytophthora* spp. (Brasier et al., 1999).

The genetic diversity of host populations is another important factor affecting the outcome of host-pathogen interaction. Indeed, several studies have shown that genetically homogeneous populations are more susceptible to disease than more genetically heterogeneous populations (Acevedo-Whitehouse et al., 2003; Spielman et al., 2004a; Hughes and Boomsma, 2004; Pearman and Garner, 2005). Pearman and Garner (2005), for instance, have shown that the less genetically variable western populations of Italian *Rana latastei* were more susceptible to *Ranavirus* than genetically variable oriental populations. Similarly, Acevedo-Whitehouse et al. (2003) highlighted the increased susceptibility to pathogens in sea lions due to inbreeding.

Spread dynamics

Spreading disease dynamics depend both on the spread of the pathogen through the environment and across different geographic areas and on the transmission of the pathogen, that is, contacts between susceptible hosts and infected hosts, pathogens and pathogen vectors.

Pathogens can be spread by several means. Some pathogens can persist in the environment, waiting to encounter a susceptible host. This phenomenon has been observed for some pathogenic fungi with saprophytic stage of the cell cycle (Fisher et al., 2012). Others actively move and search for a host, such as helminth parasites at the larval stage (Bush et al., 2001). Many pathogens can be transported over short and long distances by biological vectors such as insects or migratory birds (Kilpatrick and Randolph, 2012; Hubalek, 2004). Pathogens can also be spread by fomites (Boone and Gerba, 2007). Polymenaku et al. (2008), for instance, observed long-distance transport of aerosolised microbes after desert storms in North Africa.

Some pathogens can persist for a long time and spread while in the resting stages. The production of resting stages enables these organisms to survive under unfavourable environmental conditions, pending restoration of optimal conditions. For instance, the anthrax bacteria, *Bacillus anthracis*, can survive as an endospore in soil for a long time (Nicholson, 2002), infecting herbivorous animals that graze on the ground.

Transmission of pathogens can be divided into vertical and horizontal; the former is a direct transmission from parents to offspring, and the latter is a transmission mode that involves contact between individuals or with contaminants. There are various types of horizontal transmission: direct contact, ingestion of contaminated food or liquids, inhalation of contaminated particles, injection into tissues through a bite or saliva, invasion through open wounds, and active penetration into the host (Grenfell and Dobson, 1995). In epidemiology, the basic reproduction number (R_0) is a parameter that gives a measure of the transmission rate. R_0 can be defined as the average number of secondary cases of infection arising from a primary

case introduced in a population of susceptible individuals (Grenfell and Dobson, 1995).

Several models have been developed to predict the spread of diseases. These models provide useful insights; however, the difficulty of obtaining all the necessary data, the complexity of the parameterisation, and the onset of emerging properties due to the interaction of factors make it difficult to implement these models. For instance, simple deterministic models have been established to study the spread of species-specific pathogens with direct transmission and density-dependent trends, suggesting that disease alone cannot lead a host population to extinction. According to these models, disease will disappear from a population when it drops below a limit of critical density. Nevertheless, a population with a size that has fallen to a very low level may face extinction because of stochastic demographic events, the Allee effect, genetic erosion, or inbreeding depression (De Castro and Bolker, 2005). The loss of genetic diversity could in turn increase the susceptibility to additional diseases. O'Brien et al. (1985), for example, argue that a drastic reduction of genetic diversity in cheetahs, probably due to recent bottlenecks, may have increased their susceptibility to infectious peritonitis caused by feline coronavirus. Models have also been developed for non-specialised pathogens or pathogens with indirect transmission. In these cases, disease could lead to extinction because both pathogens that have multiple host species surviving in reservoir species and pathogens that can survive in the environment (e.g. as saprophytes) override density-dependent constraints. Haydon et al. (2002), for instance, have put in evidence the role of the domestic dog as a reservoir species contributing to the spread of rabies and canine distemper virus that threaten populations of the Ethiopian wolf (*Canis simensis*). Richards et al. (1999) showed that the nucleopolyhedrovirus infecting the polyphagous orgy moth (*Orgyia antiqua*) persists for a long time outside the host in the undergrowth, protected from solar radiation, and may lead host populations to extinction.

Other important issues affecting disease dynamics are superspreaders and key hosts (Hawley and Altizer, 2011). Superspreaders are individuals who disproportionately contribute to transmission of disease. A historical example of a superspreader is Mary Mallon, better known as Typhoid Mary, a cook who was an asymptomatic carrier of the typhoid bacterium *Salmonella enterica* and infected at least 54 people (Soper, 1939). Key hosts are those groups that mediate most of the transmissions. For instance, Perkins et al. (2003) showed that larger, sexually mature male yellow-necked mice (*Apodemus flavicollis*) have higher aggregation of the tick *Ixodes ricinus*, which acts as a carrier for the encephalitis virus, and their potential transmission of encephalitis conforms to the 20/80 rule according to which 20% of the host population accounts for 80% of transmission (Woolhouse et al., 1997).

Another issue concerning the spreading dynamics of diseases is related to seasonality. Seasonal patterns of diseases may depend on both climatic factors affecting the life cycle of the pathogen and seasonal changes affecting the host immune system. One example is the seasonality of influenza affecting humans in temperate regions. Cannell et al. (2006) observed that minor exposure to sunlight during the winter months, and therefore lower synthesis of vitamin D, increases susceptibility to infections of the respiratory system. In several animals, there is a periodic impairment of immune responses because of a shift of energetic resources towards other types of activity such as reproduction or migration (Nelson et al., 2002). Owen and Moore (2006), for instance, found that birds undergoing long migrations are immunocompromised during this period.

The role of environment and ecological interactions

The environment in which individuals live and move, along with the occurrence and the interaction with other organisms, influence the emergence and the spread of infectious diseases.

Climate, through trends in temperatures, precipitation, humidity, and currents, determines whether a given area is suitable for the occurrence of a certain pathogen.

Furthermore, climatic factors can affect the outcome of host-pathogen interaction by increasing the rate of reproduction, development, and transmission of the pathogen, reducing its inactivity periods or mortality during unfavourable seasons and modulating the susceptibility of the host (Harvell, 2002). For instance, the malarial protozoan *Plasmodium falciparum* has an incubation time in *Anopheles* mosquitoes of 26 days at 20°C, but incubation requires just 13 days at 25°C. This increase in the rate of development of the parasite at higher temperatures favours the completion of its life cycle within vector hosts that have a lifespan of only a few weeks. Moreover, high temperatures increase reproduction in mosquitoes and the frequency of blood meals, increasing the chance of *Plasmodium* transmission (Githeko et al., 2000; Githeko et al., 2001). Climatic conditions linked to cyclical patterns of oceanic currents, such as El Niño-Southern Oscillation, have been associated with spreading dynamics of various diseases, such as Rift Valley Fever in Kenya or outbreaks of cholera in Bangladesh (Linthicum et al., 1999; Pascual, 2000). In addition, extreme weather events can contribute greatly to the emergence of infectious diseases, setting optimal conditions for the proliferation of pathogens and vectors or weakening hosts. For example, heavy rainfall and flooding after Hurricane Mitch in 1998 led to an increase in outbreaks of cholera, malaria, and dengue in Honduras (Epstein, 1999).

The chemical and physical environment also plays a role in setting suitable conditions for the onset of infectious diseases. The range of values of parameters such as pH in aqueous medium or soil or the concentration of both organic and inorganic chemicals such as metals may establish optimal conditions for the proliferation of a pathogen or unsuitable conditions for host immune responses. Irradiation and the distribution of shaded patches and canopy may also play a role.

In the wild, biotic interactions can affect host-pathogen interactions and may in turn be affected by these. Competition, both intraspecific and interspecific, may affect hosts by reducing access to food and hence weakening their immune systems because of malnutrition. The outcome of competition can in turn be influenced or reversed by infections (Hudson and Greenman, 1998). Indeed, Park (1948) observed

that when two tenebrionid beetles (*Tribolium confusum* and *Tribolium castaneum*) were infected by the sporozoan *Adelina tribolii*, *T. confusum*, the species that normally outcompetes the other in the absence of the parasite, was more susceptible to the pathogenic action of *A. tribolii*. In this case, the parasite reduces the competitive ability of the dominant species and favours the persistence of the normally less competitive species. Competition may also affect pathogens, which can be disadvantaged or inhibited by the presence of other microorganisms.

Host-pathogen interactions can also influence predator-prey dynamics. Some parasites in which life cycle includes phases in both the prey and the predator, for example, can alter the behaviour of the prey to increase its capture by the predator and hence its transmission in the next host. Lafferty and Morris (1996) observed behavioural changes in the fishes *Fundulus parvipinnis* that were parasitized by larvae of the trematode *Euhaplorchis californiensis*. In this case, parasitized fishes were more susceptible to capture by a predator bird than non-parasitized fishes. The effects of infectious diseases on food chains can also be indirect. Zuberogoitia and Martinez (2001), for instance, observed severe declines in the populations of the eagle owl (*Bubo bubo*) in a region in Spain after the spread of the rabbit haemorrhagic disease that decimated its main prey.

Co-infections are another important case. The simultaneous occurrence of multiple infections caused by different pathogens can result in different outcomes. Pathogens can compete for physical space, or the consequences of physical and physiological damage caused by a pathogen can make the host unsuitable for another pathogen, or the immune response to a pathogen may intensify the response to another pathogen because they share some antigens, or, finally, the infection with one pathogen can favour other infecting organisms via general immunosuppression (Grenfell and Dobson, 1995). Immune responses against intracellular and extracellular pathogens involve different mechanisms and different cell types. Specifically, Th1 lymphocytes act against intracellular pathogens, whereas Th2 lymphocytes act against extracellular pathogens. These two ways are also

distinguished by the use of different signalling molecules. Some of these molecules, called cytokines, may act not only by activating or enhancing one particular pathway but also by inhibiting the other. In this way, the immune response to an intracellular pathogen can favour an extracellular pathogen and vice versa (Graham et al., 2007a). In this regard, Jolles et al. (2008), for instance, observed a negative association between helminth infestation and *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). They used empirical data and dynamic models and suggested that the observed pattern was due to immunosuppression and increased mortality in co-infected hosts.

Behaviour is another factor that plays an important role in the dynamics of infectious disease. The behaviour of sick individuals may be of concern both in spreading and limiting the transmission of diseases. Typical behaviours such as lethargy, anorexia, decreased libido, and abnormal posture that are related to an infectious state and are collectively called 'sickness behaviour' can derive from host adaptations to disease with the aim of favouring the immune response (Hart, 1988). Behavioural changes can also constitute a sign through which healthy individuals can recognise and avoid sick individuals to reduce the chance of infection. Other signals involved in avoidance behaviour can be chemical. Kiesecker et al. (1999) observed that healthy tadpoles of the bullfrog (*Lithobates catesbeianus*) avoid conspecific tadpoles infected with *Candida humicola*, a fungus that reduces their growth and kills them. Similarly, it has been observed that healthy Caribbean lobsters (*Panulirus argus*), which are normally gregarious, avoid conspecific lobsters infected with the lethal virus *Panulirus argus* virus 1 (Behringer et al., 2006). In social groups strongly influenced by kin selection, sick individuals can spontaneously move away from the colony to avoid infecting conspecific individuals. For instance, departure from the colony of dying ants in the species *Temnothorax unifasciatus* can reduce the likelihood of transmission of the pathogenic fungus *Metarhizium anisopliae* (Heinze and Walter, 2010). Sickness behaviours may also facilitate the transmission of pathogens. In this regard, Bouwman and Hawley (2010) observed that in the bird

Carpodacus mexicanus, male birds infected with the directly transmitted pathogen *Mycoplasma gallisepticum* show reduced aggressiveness and healthy male birds tend to forage more frequently in their proximity, increasing the risk of infection.

Anthropic factors

Human actions can deeply affect the environment and relationships among organisms. They can also affect the dynamics of host-pathogen interaction and the spread of infectious diseases.

Translocation of hosts, pathogens, or both from areas where they are endemic to areas where they are allochthonous can contribute to the emergence of infectious diseases (Cunningham, 1996). Transport of animals and plants takes place on a global scale and is conducted for different purposes, such as trade in livestock, pets, plants for decoration, and agriculture. Introduction of hosts into new geographical areas may expose them to endemic pathogens with which they lack co-evolutionary history and against which they have not evolved immune responses. Similarly, introduction of pathogens in new areas, for instance through the translocation of carrier hosts, can pave the way for new colonisation by these pathogens affecting new host populations or, possibly, new host species. For example, the global spread of *Varroa* affecting honey bees could have occurred as a result of importation of the western bee (*Apis mellifera*) to Asia and the following contact between *A. mellifera* and the eastern bee (*Apis cerana*), which is the natural host of *Varroa destructor*, the mite that causes the varroatosis disease (Rosenkranz et al., 2010). Similarly, the introduction of chestnut trees from Asia to North America and Europe seems to have contributed to the spread of *Cryphonectria parasitica*, the pathogenic fungus causing chestnut blight, a disease that devastates chestnut populations (Anangnostakis, 1987; Dutech et al., 2012). Livestock has also played a role in several outbreaks. An example is the outbreak of Nipah virus in Malaysia at the end of the 1990s. This zoonotic virus belonging to the Paramyxoviridae family has its natural host in bats of the Pteropodidae family, but

the intensive rearing of pigs seems to have played a role as a reservoir, favouring the spread of this virus and its transition to humans (Ksiazek, 2011).

Overexploitation of target species for food or business may indirectly affect its increase in susceptibility to disease. Overhunting and overfishing, for example, can reduce the size of a population, exposing it not only to extinction by random demographic processes but also to a severe loss of genetic diversity. Genetic erosion by genetic drift following a bottleneck event could result in the loss of resistance alleles from the gene pool of the population, leading to an increased susceptibility to pathogens (O'Brien and Evermann, 1988).

Human activities that transform the environment, such as urbanisation, agriculture, and deforestation, lead to the destruction or alteration of habitats and possibly create suitable conditions for the occurrence of pathogens and vectors. One way in which such changes may affect disease dynamics is through the reduction of host species range, which can increase host density, favouring the contact rate and transmission of pathogens. Another way is habitat fragmentation, which dramatically reduces migration of individuals between different populations of host species. Although reduction of dispersion and contacts between individuals belonging to neighbouring populations may reduce the spread of pathogens, McCallum and Dobson (2002) pointed out that despite the increased risk of infection, the maintenance of corridors through gene flow may be a more beneficial factor than isolation. An example of the influence of habitat alteration on disease dynamics is the effect of forest fragmentation on human exposure to Lyme disease in the eastern United States (Allan et al., 2003). Here, fragmentation of forests has led to a sharp decline in several mammal species except the rodent *Peromyscus leucopus*, which is the natural reservoir of *Borrelia burgdorferi*, the bacterium that causes Lyme disease. *P. leucopus* has greatly increased in density; as a consequence, the tick *Ixodes scapularis*, a vector of the disease, feeds more frequently on it, increasing the likelihood of becoming infected and spreading the bacterium. Other examples related to the role of environmental changes are the effects of the construction of irrigation

canals on population growth and the spread of mosquito vectors of malaria or the effects of construction of dams on outbreaks of schistosomiasis (Patz et al., 2000).

Pollution may contribute to the spread of infectious diseases, especially by weakening the immune system of the host. Chemicals that have immunotoxic effects include several environmental pollutants such as pesticides and heavy metals (Krzystyniak et al., 1995). Linzey et al. (2003), working on two amphibian species in Bermuda, *Bufo marinus* and *Eleutherodactylus johnstonei*, observed that individuals with high hepatic levels of cadmium, chromium, copper, zinc, and a by-product of dichlorodiphenyltrichloroethane decomposition showed altered morphology of the liver and spleen, a reduced immune response induced by B lymphocytes, and increased infections by trematodes. Pollutants such as carbon monoxide, nitrogen oxide, and soot released into the atmosphere by the consumption of fossil fuels may directly or indirectly cause asthma in humans, increasing susceptibility to allergies and other serious respiratory difficulties (Brunekreef and Holgate, 2002).

Eutrophication, caused by the release of phosphorus and nitrogen in aquatic systems as a consequence of agriculture and livestock, can favour proliferation of parasites. Johnson et al. (2007) showed in mesocosm experiments that the infestation rate of the trematode *Ribeiroia ondatrae* in *Rana clamitans* increased following an increase of growth, reproduction, and survival of gasteropod first intermediate hosts in a eutrophicated environment.

Changes in the intensity of incident ultraviolet (UV) radiation can affect host-pathogen interactions. The incidence of UV radiation can vary depending on several factors such as latitude, altitude, and cloudiness and is also affected by human activities such as deforestation, reducing canopy, and pollution due to halogenated hydrocarbons, contributing to the depletion of stratospheric ozone (Paul and Gwynn-Jones, 2003). Kiesecker et al. (2001) observed that lowering of the water depth at oviposition sites of the western toad *Bufo boreas*, as a consequence of changes in the precipitation pattern due to El Niño events, exposes the eggs to increased UV

radiation. This increased exposure to UV radiation increases susceptibility to the pathogenic oomycete *Saprolegnia ferax,* causing high embryonic mortality.

One result of medical progress achieved during the course of the past century that concerns the emergence and re-emergence of infectious diseases is the increased antibiotic resistance shown by many pathogenic bacteria. The excessive use of antibiotics has contributed, over time, to select strains of bacteria that are able to metabolise antibiotics and render them ineffective (Neu, 1992). The evolution of antibiotic resistance is facilitated not only by the fast generation rate and the high mutation rate but also by the easy exchange of genetic information between individuals through plasmids. Some important pathogenic bacteria such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* have been shown to exhibit resistance to several antibiotic drugs (Levy and Marshall, 2004).

Another anthropogenic factor that influences host-pathogen interactions and may result in the emergence of infectious diseases is global warming. According to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC, 2007), the average global surface temperature has increased by 0.74 ± 0.18°C during the 20th century. An additional increase in temperature is predicted during the 21st century, ranging from 1.1 to 6.4°C, depending on the prediction scenario. The increase in temperature has been associated with the increased emission of greenhouse gases in the atmosphere following the Industrial Revolution and deforestation. Climate change in the form of altered temperature range, precipitation pattern, and frequency of extreme weather events not only threatens the persistence and survival of species within their habitat, because bioclimatic parameters can shift toward undesirable values, but also affects the occurrence and spread of pathogens and their interactions with hosts (Harvell et al., 2002). Plenty of evidence suggests that climate change has played a role in increasing the incidence and geographical extent of various diseases in recent decades, such as malaria, yellow fever, and dengue in humans and bluetongue virus in cattle (Gratz et al., 1999; Purse et al., 2005). In England, Sutherland et al. (1997) observed that defoliation in elm trees

caused by the ascomycete *Ophiostoma novo-ulmi*, the pathogenic agent of Dutch elm disease, was more severe during periods of hot weather. Variations in oceanic temperature also affect the dynamics of infectious diseases. Cook et al. (1997) observed in the early 1990s, near the east coast of the United States, a northward shift of the disease caused by the protozoan *Perkinsus marinus* affecting the oyster *Crassostrea virginica*. They suggested that the expansion of this pathogen was related to warm winters and higher oceanic temperatures.

CHAPTER 2

CHYTRIDIOMICOSIS

Chytridiomycosis is an emerging infectious disease and is considered the main cause of the global decline of amphibian populations observed in recent decades (Berger et al., 1998; Daszak et al., 1999; 2003; Fisher et al., 2009b; Kilpatrick et al., 2010). The causative agent of this disease is *Batrachochytrium dendrobatidis*, a pathogenic fungus that belongs to the phylum Chytridiomycota (Longcore et al., 1999). The first evidence of chytridiomycosis in the literature dates back to 1998 (Berger et al., 1998); since then, a multitude of studies conducted in several laboratories have tried to clarify different aspects related to pathogen biology, epidemiology, interactions with hosts, and possible interactions with other factors. To date, chytrid has been observed to occur in many amphibian species on every continent where there are amphibians. However, many questions remain unanswered and there is not a clear view about the epidemiology of the chytrid and its role in the decline of amphibian populations.

Batrachochytrium dendrobatidis

The aetiological agent of chytridiomycosis was described for the first time in 1999 by Longcore et al. (1999) and was called *Batrachochytrium dendrobatidis*. The generic name is derived from the Greek word *batracho*, which means frog, and *chytr*, which means earthen pot, while the specific name is derived from *Dendrobates*, the name of the amphibian genera from which it was isolated for the first time (Longcore et al., 1999).

B. dendrobatidis belongs to the phylum Chytridiomycota, class Chytridiomycetes, in the order Rhizophydiales (Longcore et al., 1999). Chytrids belong to an ancient group of ubiquitous fungi that reproduce through mobile

zoospores (Barr, 1990). They are found in both the aquatic and terrestrial environment and mostly are saprobic on substrates such as pollen, chitin, keratin, and cellulose, while others are parasites of algae, plankton, and vascular plants (Barr, 1990). *B. dendrobatidis* is the only chytrid that is known to parasitize vertebrates.

Morphologically, *B. dendrobatidis* is characterised by a thallus called zoosporangium, from which filamentous rhizoids extend (Longcore et al., 1999). Zoosporangia show nonoperculate discharge papillae that release the flagellated zoospores. Zoospores are spherical or slightly oval and have a single flagellum located posteriorly. Zoosporangium is generally monocentric, but when it is internally divided by septa, it can develop in colonies, with a discharge papilla for each sporangium (Longcore et al., 1999). When cytoplasm grows, the zoosporangium becomes more complex and multinucleated because of mitotic divisions, and then it divides and matures in flagellated zoospores (Berger et al., 2005a). Zoospores are released through discharge papillae that extend through the membrane and are generally opened outward onto the surface of host cells (Berger et al., 2005a). As a result, the sporangium remains empty and can become a site of infection by opportunistic bacteria.

The *B. dendrobatidis* cell cycle has been described as a simple progression between two stages; flagellated zoospores encyst in host epidermal cells and mature into the thallic form, zoosporangium, which produces and releases new zoospores (Berger et al., 2005a). The duration of the cell cycle ranges from 4 to 5 days at 22°C *in vitro*. Studies performed in culture have shown that zoospores travel short distances (they swim less than 2 cm in water) and for a short time (less than 5% zoospores swim for more than 24 hours) before they encyst (Piotrowski et al., 2004). However, chemotaxis toward attractive chemicals, such as keratin or other nutrients (Moss et al., 2008), may extend this motility. Zoosporangia infect cells of the stratum granulosum and stratum corneum of the amphibian epidermis, and their maturation follows the progression of cell development in this tissue (Berger et al., 2005a). Immature zoosporangia can be observed within viable cells of the deeper layers,

while mature and empty sporangia are found in keratinised cells of the outermost layers.

Variations from asexual clonal reproduction have not yet been directly observed, but it is known that chytrids can also reproduce sexually, giving rise to resting spores (Sparrow, 1960). Genetic data suggest that recombination events and sexual reproduction may, in fact, occur in *B. dendrobatidis* (Farrer et al., 2011; Schloegel et al., 2012). In addition, Di Rosa et al. (2007) observed a possible resting stage of *B. dendrobatidis* on the skin of water frogs. These observations certainly deserve further investigation, because both genetic mixing due to sexual reproduction and the ability to form resting stages are issues of great importance from an epidemiological point of view.

From a physiological perspective, studies performed *in vitro* have shown that *B. dendrobatidis* grows and reproduces at temperatures between 4°C and 25°C and pH conditions between 4 and 8 (Piotrowski et al., 2004). However, the optimal condition for its growth is a temperature between 17°C and 25°C and pH between 6 and 7 (Piotrowski et al., 2004).

Several chemicals, such as 70% alcohol, 1% Virkon, bleach (sodium hypochlorite), and DDAC have been shown to have killing activity on *B. dendrobatidis* at various concentrations and exposure times (Johnson et al., 2003). UV irradiation, often used in sterilisation, has been observed to be ineffective against *B. dendrobatidis*, but drying and heating at temperatures greater than 32°C are effective. Indeed, studies performed *in vitro* have shown that *B. dendrobatidis* die after 96 hours at 32°C and 4 hours at 37°C (Johnson et al., 2003).

Little is known about the possible persistence and the ecology of *B. dendrobatidis* outside its host. Johnson and Speare (2003) found that chytrid zoospores may remain viable for up to 7 weeks in autoclaved lake water and can survive and remain in an infectious state for up to 12 weeks in moist and sterile river sand (Johnson and Speare, 2005). The role of the chytrid in aquatic food chains and its possible predators among zooplankton is poorly understood. Buck et al. (2011),

for instance, observed that *B. dendrobatidis* is a prey of the waterflea *Daphnia magna*.

Pathogenesis

The most frequent symptoms observed in amphibians affected by chytridiomycosis are anorexia, lethargy, abnormal posture, and loss of the righting reflex (Daszak et al., 1999; Nichols et al., 2001).

B. dendrobatidis infects the epidermis of its host by localising in the cytoplasm of keratinocytes (Pessier et al., 1999). Morphologically, it has a circular or oval thallus and is typically 7-15 µm in diameter (Pessier et al., 1999). The distribution of the thalli can be clustered because zoospores tend to encyst near their original zoosporangium (Berger et al., 2005b). The occurrence of chytrid on keratinised tissues is reflected in its distribution on the body surface of the host. Indeed, in tadpoles, it is found only in mouthparts, which is the only keratinised tissue at this stage, although it may occur throughout the epidermis of adults (Marantelli et al., 2004). However, in anurans, it has been observed to occur mainly in ventral surfaces, hindlimbs, and interdigital webbing (Berger et al., 2005b).

The histopathology of chytridiomycosis is characterised by hyperkeratosis and hyperplasia of skin layers (Pessier et al., 1999; Berger et al., 2005b). Other signs include disordered epidermal cell layers, spongiosis, and occasional erosion and ulceration of skin (Berger et al., 2005b). Among the most prominent clinical signs observed by Nichols et al. (2001) in experimental infections performed in two species of Dendrobatidae was an excess of shedding skin, while lesions were visible as moderate thickening of the skin and discoloured areas on the hindlimbs and ventral surface.

Two non-mutually exclusive hypotheses have been proposed about the mechanisms that cause death in infected hosts. The first hypothesis suggests possible secretion of toxins or proteolytic enzymes by the pathogen, and the second hypothesis suggests a role for electrolytic imbalance caused by disruption of osmotic

functionality in the skin (Berger et al., 1998; Pessier et al., 1999; Berger et al., 2005a).

In studies performed on *Litoria caerulea* infected with *B. dendrobatidis*, Voyles et al. (2007; 2009) showed that chytridiomycosis actually affects the osmotic functionality of amphibian skin, impairing transport of electrolytes. In particular, they observed a reduction of plasmatic levels of sodium and potassium by 20% and 50%, respectively, leading to asystolic cardiac arrest (Voyles et al., 2007; 2009).

However, some studies have shown that the *B. dendrobatidis* genome presents a marked expansion of gene families codifying for proteases and that this feature does not occur in other analysed chytrids, including its closest known relative, the non-pathogenic chytrid *Homolaphlyctis polyrhiza* (Rosenblum et al., 2008; Joneson et al., 2011). The proteases families in which genomic expansion has been observed include metallo-proteases, serine-proteases, and aspartyl-proteases (Rosenblum et al., 2008; Joneson et al., 2011). Rosenblum et al. (2008) also observed differential expression of metallo-protease genes between two stages of the *B. dendrobatidis* cell cycle, with prevalent expression (72% of the genes) at the zoosporangium stage. Nevertheless, even thought the expansion of protease genes has been observed in other fungi that are pathogens of vertebrates (Burmester et al., 2011; Jousson et al., 2004; Sharpton et al., 2009), and though aspartyl-proteases reportedly play a role in the invasion of host tissues by the human pathogenic fungi *Candida* spp. (Kaur et al., 2007; Monod and Borg-von Zeppelin, 2002), no direct evidence has been found, until now, that elucidates the role of proteases in the pathogenic activity of *B. dendrobatidis* (Joneson et al., 2011).

Diagnostic techniques

The earliest screenings to detect *B. dendrobatidis* infections were performed by histological analyses (Pessier et al., 1999). This method involves the microscopic examination of skin sections stained with hematoxylin and eosin, and a sample is considered positive when typical sporangia 7-15 µm in diameter can be observed

inside keratinocytes (Pessier et al., 1999). The main advantage of histological analysis is the direct observation of the severity of infection; nevertheless, because of its poor sensitivity (only a small skin patch of the host is examined), it was replaced by molecular techniques based on the polymerase chain reaction (PCR) (Annies et al., 2004; Boyle et al., 2004; Goka et al., 2009).

Three different PCR approaches have been developed to detect the presence of *B. dendrobatidis* in analysed samples. Annies et al. (2004) developed a single-round PCR amplifying specifically the ITS1-5.8S-ITS2 region of *B. dendrobatidis* ribosomal DNA to detect the chytrid genome in a heterogeneous pool of DNA. Boyle et al. (2004) developed a method based on quantitative PCR (qPCR) that increases sensitivity and, most importantly, allows a quantitative measure of the level of infection in analysed individuals. Lastly, Goka et al. (2009) introduced a nested PCR assay which further increases sensitivity and specificity but without the advantage of quantification. The limits of detection for these assays are 0.1 pg of DNA for single-round PCR, 0.01 pg for qPCR and 0.001 pg for nested PCR (Goka et al., 2009).

The main advantages of PCR techniques compared with histological techniques, are the increase in detection sensitivity and the chance to perform non-invasive sampling, because pathogen DNA can be extracted from skin swabs without harming the host (Kriger et al., 2006; Hyatt et al., 2007). The choice of diagnostic technique therefore depends on the aims of the analysis: evaluation of pathogenicity (histological analysis), measurement of the level of infection (qPCR), or simply detection of pathogen occurrence (PCR, nested PCR).

Interaction with host

B. dendrobatidis is the only known chytrid that parasitizes a class of vertebrates; it affects amphibians but is not a species-specific parasite. Varying levels of infection have been reported in hundreds of amphibian species among both anurans and urodeles. In many cases, the chytrid is considered the main cause of worldwide declines in the amphibian populations and possible extinction, for

instance, of several species of harlequin frogs, genus *Atelopus*, in Latin America (La Marca et al., 2005). Nevertheless, the occurrence of the chytrid does not necessarily imply disease, decline, and extinction, but a certain degree of variability has been observed in the outcome of the interaction between this parasite and its hosts.

The outcome of this interaction may vary according to differential host susceptibility, which can differ among both species and populations of the same species (Tobler and Schmidt, 2010). As a consequence, there can be scenarios in which different syntopic species are infected but with different intensity and/or prevalence, in which affected and declining species are syntopic with resistant or tolerant species, or in which populations of a given species are affected and declining in parts of their range but show less or no infection in other parts even if a chytrid occurs in the area. Differences may also eventually occur within a population, depending on the stage of development and the size at the time of metamorphosis (Garner et al., 2009; Tobler and Schmidt, 2010).

Environmental and ecological factors are likely to play an important role in determining this variability. Studies performed with experimental infections have also shown that time from exposure to death is influenced by the dose of infectious zoospores, duration of exposure, and body size of the host (Carey et al., 2006). Moreover, observed differences between syntopic species also suggest an important role for other factors intrinsic to the hosts, such as differences in immune responses, secretion of different antimicrobial peptides, and different commensal skin bacteria. On the other hand, genetic variability may explain different outcomes between populations of the same species.

Little is known about the immune responses of amphibians to *B. dendrobatidis* infection and about possible mechanisms of immune evasion by the pathogen. Studies performed by Rosenblum et al. (2009) on gene expression of the susceptible host species Silurana tropicalis after exposure to *B. dendrobatidis* showed no evidence for a strong immune response. On the contrary, they observed a reduction of expression for some genes involved in immune defences, such as those involved in

the complement pathway (Rosenblum et al., 2009). Ramsey et al. (2010), however, examined the immune system of *Xenopus laevis*, a species in which *B. dendrobatidis* does not give rise to lethal infections, and observed effective immune responses; this shows evidence for involvement of both innate and adaptive responses. In particular, in the secretion of mucous membranes, they found antimicrobial peptides that effectively inhibit the growth of *B. dendrobatidis* and immunoglobulin IgM, IgY and IgX were able to bind to chytrid antigens (Ramsey et al., 2010).

Great attention has been directed at determining the role of various antimicrobial peptides in the defence against chytridiomycosis. In amphibians, these peptides are secreted by granular glands of the dermis as a defence against predators and pathogenic microorganisms (Simmaco et al., 1998). There is great variability among peptides secreted by amphibian species. Many of these peptides may have the ability to inhibit the growth of *B. dendrobatidis* when they are present at levels greater than a certain concentration; moreover, they are usually secreted as a heterogeneous mix, with the additional advantage of the synergic action (Rollins-Smith and Conlon, 2005). Woodhams et al. (2006; 2007b) showed a positive relationship between the anti-chytrid efficacy of antimicrobial peptides secreted by a given species and both the ability of that species to persist in the wild and the survival rate following experimental infections (Woodhams et al., 2006; 2007b).

The microbial community of amphibian skin is another factor that may contribute to host defences against *B. dendrobatidis* (Woodhams et al., 2007a; Harris et al., 2009). In many cases, symbiotic microbes of hosts may play a protective and beneficial role against pathogens (Dethlefsen et al., 2007). Several genera of bacteria, isolated from the skin of many amphibian species, have been shown to inhibit growth of *B. dendrobatidis in vitro* (Harris et al., 2006). Harris et al. (2009) showed that inoculation of *Janthinobacterium lividum*, a bacterium found in several amphibian species, prevented morbidity and mortality caused by *B. dendrobatidis* in *Rana muscosa*. One of the mechanisms through which skin bacteria inhibit the growth of

the chytrid seems to be the production of antifungal metabolites (Brucker et al., 2008).

On the other hand, the role of the genetic diversity of the host as a defence against *B. dendrobatidis* is still poorly understood. Luquet et al. (2012), in a study performed with experimental infections on tadpoles of *Hyla arborea*, observed that those from populations with low genetic diversity had a smaller body mass and shorter duration of post-metamorphic life than those from more genetically diverse populations, even if both did not show detectable levels of chytrid infection at the end of the experiment. According to them, genetic erosion, although it does not prevent effective resistance against the pathogen, reduces the ability to simultaneously accomplish resistance, the growth rate of the tadpoles, and metamorphosis (Luquet et al., 2012). In another study performed on *Lithobates yavapaiensis*, Zamudio and Savage (2011) assessed genetic diversity at one locus of the major histocompatibility complex (MHC), which plays an important role in immune responses. In this case, they found a positive relationship between heterozygosity at the MHC class IIB locus and the survival rate among infected individuals and also identified one allele associated with reduction of mortality, which revealed evidence of recent positive selection (Savage and Zamudio, 2011).

Epidemiology of chytridiomycosis

Chytridiomycosis was diagnosed for the first time in 1997, when amphibians were dying off in Australia and Central America (Berger et al., 1998). Since then, many efforts have focused on trying to understand the spreading dynamics of the causative agent of this emerging infectious disease. Important questions that are still not completely answered concern the distribution chytrid, origin, and mechanisms of spread, transmission, and persistence of the chytrid.

Following early observations of chytridiomycosis in Australia and Central America, the chytrid has been detected elsewhere in the Americas, Europe, Asia, and Africa, that is, on all the continents where amphibians exist (www.bd-maps.net).

Given the wide distribution and the variable outcomes on host species, depending on species and geographic areas, a detailed understanding of the epidemiology of chytridiomycosis is of utmost importance.

Two hypotheses have been initially advanced to explain the emergence and occurrence of chytridiomycosis (Rachowicz et al., 2005). The first hypothesis, called the 'novel pathogen hypothesis' states that *B. dendrobatidis* originated in a particular area and then spread mainly through international trade of amphibians (Laurence et al., 1996; Lips, 1999; Garner et al., 2006). According to this hypothesis, the pathogen arrives in new areas through human-mediated transport and begins to expand in a wave-like manner, causing severe dying offs of naïve hosts that lack immunity defences against this new threat. This type of scenario has been proposed, for instance, for the spreading pattern of chytridiomycosis in Central America (Lips, 1999; Lips et al., 2006; Cheng et al., 2011). According to some researchers (Mazzoni et al., 2003; Daszak et al., 2004; Weldon e al., 2004; Garner et al., 2006), *Lithobates catesbeianus* and *X. laevis* are the species that mostly contribute to the spread of *B. dendrobatidis*, because they are tolerant to infection and are extensively traded worldwide.

The second hypothesis or the 'endemic pathogen hypothesis' instead argues that *B. dendrobatidis* was already widely spread and that environmental changes such as climate change have altered the balance of host-pathogen interactions, resulting in an emerging epidemic disease (Rachowicz et al., 2005). According to this scenario, environmental changes may act on both the host (for instance, causing sub-lethal stress conditions that could weaken its immune system) and the pathogen (establishing optimal conditions for its growth and its transmission) (Pounds et al., 1999; 2006; Raffel et al., 2012).

Although this dualism is emphasised in the literature, these two hypotheses are not mutually exclusive. Regardless of the endemic occurrence or the recent arrival of a pathogen, alterations of environmental factors can shift the balance of host-pathogen interactions. To disentangle this issue, it would be of utmost importance to

not only investigate the affected geographical areas but also to widen epidemiological surveys to a temporal dimension, assessing how long the chytrid is present in a given region and the relationship between its endemicity or its arrival and the pattern of decline in the amphibian populations.

Until now, the oldest occurrences of *B. dendrobatidis* were found in Cameroon in a specimen of *Xenopus fraseri* collected in 1933 (Soto-Azat et al., 2010), in Uganda in a specimen of *X. laevis* collected in 1934 (Soto-Azat et al., 2010), in South Africa in a specimen of *X. laevis* collected in 1938 (Weldon et al., 2004), and in Canada in 2 specimens of *R. clamitans* collected in 1961 (Ouellet et al., 2005). The observed historical presence of *B. dendrobatidis* in Africa, especially its occurrence on the species *X. laevis*, which is a tolerant species widely traded since the 1930s because of its use as a pregnancy test and as a model species in biological studies, has inspired the hypothesis of the 'African Origin' of the chytrid (Weldon et al., 2004). According to this hypothesis, *B. dendrobatidis* is endemic to Africa and African species such as *X. laevis* would develop tolerance as a result of a long co-evolutionary history. Worldwide trade of *X. laevis* would then lead to the spread of the chytrid out of Africa toward other continents. Subsequently, the contact between *B. dendrobatidis* and naïve amphibian species would have induced waves of mortality, while its contact with tolerant and commercially important species such as *L. catesbeianus* would have further increased spread of the chytrid. However, this hypothesis has not yet found the support of genetic data. In fact, if *B. dendrobatidis* originated in Africa, African strains would have exhibited higher levels of genetic diversity than those found elsewhere, but there is no evidence for this (James et al., 2009). Furthermore, in an unpublished study performed in Japan, *B. dendrobatidis* was detected on specimens of *Andrias japonicus* collected in 1902 (Une et al., unpublished data), that is long before *X. laevis* became a globally traded species.

Other unsolved questions about the epidemiology of the chytrid concern other means of dispersion, its transmission, and its possible persistence in the environment. In addition to dispersion of the chytrid through water courses, natural movement,

infected host translocation, and anthropogenic transport of infectious material, other means may contribute to the spread of the pathogen at different scales. For instance, Garmyn et al. (2012) reported the presence of *B. dendrobatidis* on toes of waterfowls, which could carry it across several ponds within a few tens of kilometres.

The only known means of transmission of the chytrid are direct contact between infected hosts and contact between hosts and freely swimming zoospores. However, zoospores show poor dispersal ability (Piotrowski et al., 2004). Furthermore, pathogens with direct transmission are usually regulated by density-dependent mechanisms and the pattern of chytrid-related local extinction of some populations suggests the involvement of more complex dynamics (Mitchell et al., 2008), which requires the presence of reservoir species, saprophytic stages, or resting stages. Larval stages showing reduced mortality compared with adults and syntopic-tolerant species have been suggested as likely reservoirs of infection (Daszak et al., 1999; 2003; Schloegel et al., 2010), and a possible resting stage of the chytrid has actually been observed (Di Rosa et al., 2007). Nevertheless, little is known about the ecology of the chytrid out of its host. A better understanding of the life cycle and ecology of the chytrid and its relationship with environmental factors such as climate is of utmost importance in the epidemiological analysis of chytridiomycosis. Moreover, genetic studies will be valuable in shedding light on the origin and spread of this emerging infectious disease.

Climatic factors

Among the environmental factors that may play a role in the emergence and spread of chytridiomycosis, climate change is one of the most stressed. Some studies have shown that chytrid-related die-offs are more frequent in highlands (Walker et al., 2010) and in cold seasons (Berger et al., 2004) and that *B. dendrobatidis* increases its pathogenicity at low temperatures (Berger et al., 2004). Low temperatures, in fact, are optimal conditions for growth of *B. dendrobatidis*, which occurs slowly at 4°C, reaches the optimum at approximately 23°C and stops at 29°C,

and it does not survive at higher temperatures (Piotrowski et al., 2004). Based on these observations, even if global warming has been linked to the emergence of other infectious diseases, it would seem difficult to reconcile a role of warm temperature with the outbreaks of chytridiomycosis. However, evidence for a relationship among climate change, rising temperatures, and chytridiomycosis has been found, such as in Spain (Bosch et al. 2006), where the earliest episodes of chytrid-related mass mortality in Europe were also recorded. Moreover, in Italy, a relationship has been observed between an extreme weather event, the heat-wave of 2003, and outbreaks of *B. dendrobatidis* and *Amphibiocystidium ranae*, affecting water frogs (Di Rosa et al., 2007).

Pounds et al. (2006) observed a highly significant association between global warming and the disappearance of populations of *Bufo periglens* and *Atelopus* spp. in Costa Rica and advanced the hypothesis of the 'chytrid thermal optimum'. According to this hypothesis, the warming of the atmosphere and ocean surface would contribute to an increase in cloudiness and to a shift of clouds toward higher altitudes. The cloud cover, on the one hand, shielding the solar radiation, would contribute to the lowering of daytime temperatures; on the other hand, it would reduce the loss of heat, contributing to increasing night time temperatures (Pounds et al., 1999). In many areas, indeed, minimum temperatures are increasing faster than maximum temperatures (Dai et al., 1999). The increase in cloudiness due to global warming, increasing minimum temperatures, lowering maximum temperatures, and favouring humidity conditions, would create, in this way, an optimal context for the survival, growth, and reproduction of *B. dendrobatidis* (Pounds et al., 2006).

Climate change can act not only by setting optimal conditions for the growth of the pathogen but also by affecting the immune responses of the hosts. Andre et al. (2008), through experimental infections on *R. muscosa*, observed a mortality rate of 95% in frogs held at 17°C and 50% in frogs held at 22°C. Because both temperatures fall within the optimum for the growth of *B. dendrobatidis*, they hypothesized that the difference in mortality was attributable to differences in the host immune

response at different temperatures (Andre et al., 2008). Studying gene expression, Ribas et al. (2009) noted that infected *S. tropicalis* lost the ability to express genes codifying for a group of antimicrobial peptides at 18°C. Furthermore, Raffel et al. (2006) showed that, in *Notophthalmus viridescens*, the parameters of the immune system were influenced by changes of temperature and seasonality in natural conditions.

In addition to the effects on the growth of the pathogen and the host's defences, climate change may act by shifting the host-pathogen interaction. Assessing the relationship between climatic factors and the decline of *Atelopus* spp., Rohr and Raffel (2010) observed that the variability of temperatures was related to this decline, suggesting the 'hypothesis of climate variability'. In a subsequent study, Raffel et al. (2012) showed evidence supporting this theoretical framework according to which fast and unpredictable fluctuations of temperatures, both monthly and diurnal, accentuated by climate change, make amphibians more susceptible to the action of *B. dendrobatidis* because the pathogen is able to acclimate to changes faster than hosts, which then show reduced resistance.

Epidemiology and genetics

To shed light on the origin and the spread dynamics of *B. dendrobatidis*, many laboratories have performed genetic analysis of the various globally dispersed strains. Berger et al. (2005c) and Retallick and Miera (2007), through experimental infections, found that variations in virulence could arise using different strains of chytrid even if, according to them, different stories of passages *in vitro* may have contributed to the attenuation of virulence of certain strains. To investigate possible differences between different strains, Fisher et al. (2009a) used both molecular tools (genetics and proteomics) and phenotypic observations (morphology, virulence, and drug resistance to a fungicide). Their study showed that different isolates have different proteomic profiles, related to the genetic distance, and different morphological characteristics, such as size of sporangia. They also reported

differences in virulence, while no changes were observed in the resistance to the fungicide (Fisher et al., 2009a).

Despite the observation of these differences, early works on the genetic characterization of *B. dendrobatidis* strains showed little genetic diversity, providing evidence in favour of the hypothesis of a single clone spreading globally. In particular, Morehouse et al. (2003) analyzed 10 loci, an overall of 5918 bp, finding variability on only 5 nucleotide positions. Furthermore, they observed that the frequencies of heterozygous genotypes were quite fixed, concluding that *B. dendrobatidis* is a diploid organism reproducing clonally. In a following study, Morgan et al. (2007) analysed the genetic structure of populations of *B. dendrobatidis* strains from the Sierra Nevada (California, USA), using 12 microsatellite loci and 3 of the variable loci derived from Morehouse et al. (2003). They found low genetic diversity and poor correlation between genotypes and geography. Discussing their data, they concluded by assuming a recent introduction of the chytrid in the area, supporting the hypothesis of the novel pathogen, but pointing out that signals of recombination events detected at the local scale suggested a sort of following endemism at sites of persistence and a likely sexual reproduction. Other data and assumptions supporting the expansion of a novel pathogen reproducing clonally have emerged from the work of James et al. (2009) in which, analysing the sequences of 17 loci on 59 globally distributed strains, they observed an excess of heterozygosity in half of the loci and a reduced genetic diversity, with a maximum of 2 alleles per locus, and hypothesized a severe bottleneck due to the recent expansion of a single diploid line. Furthermore, to explain the high genotypic diversity (44 unique genotypes of an overall of 59 strains), they have proposed a mechanism of progressive loss of heterozygosity by mitotic recombination. Similarly, Velo-Anton et al. (2011) observed a progressive loss of heterozygosity of *B. dendrobatidis* along the presumed spreading wave from North America to Central America (Lips et al., 2008; Cheng et al., 2011), suggesting repeated founder effect and mitotic recombination.

Contrary to these findings, Goka et al. (2009), analysing the sequences of the ITS region of the 5.8S ribosomal RNA gene (the same region that is amplified in diagnostics screening) on *B. dendrobatidis* sampled in Japan, found a higher genetic diversity, revealing 26 haplotypes among their samples. The analysis was conducted by isolating DNA from skin swabbing performed on native and exotic species from pet shops, farms, and natural populations. The largest diversity of haplotypes was observed on the bullfrog, but interestingly, they also observed haplotypes apparently endemic to Japan. In particular, they identified 3 haplotypes that were found only on the Japanese giant salamander *A. japonicus*. Because the phylogenetic analysis showed that these 3 strains were genetically isolated from the others, and *A. japonicus* showed no signs of disease, they suggested a commensal relationship between these strains and their host species as a result of a long period of co-evolution. Similarly, the phylogenetic analysis performed in a later study conducted in China using the same marker showed that another haplotype, derived from the Chinese endemic species *Bobina pleuraden*, formed a distinct clade together with the 3 Japanese haplotypes, supporting the evidence for an endemic lineage in Asia (Bai et al., 2012). New haplotypes in the ITS region continue to be identified (Kaiser and Pollinger, 2012), but the short length of the fragment and the possible heterogeneity of haplotypes within the same sample make analysis of this region scarcely suitable to investigate the origin and propagation of the various lines (Bai et al., 2012; Schloegel et al., 2012).

New-generation sequencing, allowing the comparison of entire genomes, has increased the resolution of the genetic analysis and may contribute greatly to disentangle questions about the genealogy of the different lineages of *B. dendrobatidis*. Farrer et al. (2011) used a SOLiD (ABI) platform to sequence and compare 20 different strains of *B. dendrobatidis* from various areas of the world. Although sampling was not uniform at a global level (in fact there were no strains from Asia), the study showed significant results. Firstly, among these 20 strains, the presence of 3 distinct lineages was found: a globally distributed lineage, a lineage

present in South Africa and Mallorca (Spain), and a lineage present in Switzerland. They also observed morphological differences between the lineages and a difference in virulence between the South African / Majorcan lineages and the globally distributed one, with the latter more virulent and called ‘global pandemic lineage’. A result of great interest was the observation of the hallmarks of genetic recombination on the genome of pandemic lineage, and they hypothesized that the emergence of the global hypervirulent lineage was the consequence of a hybridisation event. Later, Schloegel et al. (2012) reported evidence for a hybridisation event between the global pandemic lineage and a Brazilian lineage, with evidence of sexual reproduction and possible differences in the ploidy of the different lineages.

From the results collected so far, therefore, *B. dendrobatidis* has been shown to not be a single clone reproducing only asexually, but instead consists of several lineages variously distributed and can reproduce sexually. The occurrence of several genetically diverse lineages is an additional factor affecting the outcome of host-pathogen interaction. Genetic characterization of *B. dendrobatidis* strains will be of great consequence in understanding the epidemiology of chytridiomycosis and will help to shed light on the origin and evolution of this pathogen.

CHAPTER 3

WIDESPREAD OCCURRENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN CONTEMPORARY AND HISTORICAL SAMPLES OF THE ENDANGERED *BOMBINA PACHYPUS* ALONG THE ITALIAN PENINSULA

Daniele Canestrelli, Mauro Zampiglia & Giuseppe Nascetti

Abstract

Batrachochytrium dendrobatidis is considered a main driver of the worldwide declines and extinctions of amphibian populations. Nonetheless, fundamental questions about its epidemiology, including whether it acts mainly as a 'lone killer' or in conjunction with other factors, remain largely open. In this paper we analysed contemporary and historical samples of the endangered Apennine yellow-bellied toad (*Bombina pachypus*) along the Italian peninsula, in order to assess the presence of the pathogen and its spreading dynamics. Once common throughout its range, *B. pachypus* started to decline after the mid-1990s in the northern and central regions, whereas no declines have been observed so far in the southern region. We show that *Batrachochytrium dendrobatidis* is currently widespread along the entire peninsula, and that this was already so at least as early as the late 1970s, i.e. well before the beginning of the observed declines. This temporal mismatch between pathogen occurrence and host decline, as well as the spatial pattern of the declines, suggests that the pathogen has not acted as a 'lone killer', but in conjunction with other factors. Among the potentially interacting factors, we identified two as the most

probable, genetic diversity of host populations and recent climate changes. We discuss the plausibility of this scenario and its implications on the conservation of B. pachypus populations.

3.1 Introduction

Chytridiomycosis is an emerging infectious disease threatening amphibian populations and several authors have suggested that it is a primary driver of the worldwide amphibian declines (Berger et al., 1998; Daszak et al., 1999; Pessier et al., 1999; Daszak et al., 2003; Skerratt et al., 2007). The chytrid fungus *Batrachochytrium dendrobatidis* is the causative agent of this disease (Longcore et al., 1999) and has been intensively studied in recent years (reviewed in Fisher et al., 2009b; Kilpatrick et al., 2010). Nevertheless, many aspects of its epidemiology still remain unresolved, including its area of origin and spread dynamics (Weldon et al., 2004; Fisher and Garner, 2007; Goka et al., 2009; Farrer et al., 2011).

Two main hypotheses have been proposed to explain chytridiomycosis outbreaks and disease dynamics (Rachowicz et al., 2005). The first hypothesis, called 'novel pathogen hypothesis', suggests a recent diffusion of the pathogen into novel geographic areas, where it has encountered 'naïve' hosts susceptible to infection (Laurance et al., 1996; Lips, 1999). Under this scenario, a crucial causative agent of the disease spread would have been the human-driven introduction of infected individuals belonging to reservoir species (Garner et al., 2006). The second hypothesis, called 'endemic pathogen hypothesis', suggests that recent outbreaks of *B. dendrobatidis* were driven by recent environmental changes, which would have affected the virulence of an endemic pathogen, host susceptibility or a combination of such factors (Rachowicz et al., 2005). Finally, since these two hypotheses are not mutually exclusive, a third scenario can be suggested, i.e. that the pathogen spread

(either natural or human-driven) and various environmental changes could act as interacting factors in favouring disease outbreaks (Pounds and Masters, 2009; Blaustein et al., 2010; Blaustein et al., 2011).

In the last decades, the first two hypotheses have been intensively explored (e.g., Rachowicz et al., 2005; Walker et al., 2010; Lips et al., 2008). To disentangle among them, most studies have focused on the identification of possible patterns of wave-like die-offs of amphibian populations (Lips et al., 2008; Vredenburg et al., 2010; Cheng et al., 2011; but see Phillips et al., 2012), genetic imprints of a recent demographic/range expansion of the pathogen (Morehouse et al., 2003; Morgan et al., 2007; James et al., 2009; Velo-Antón et al., 2012), and correlations between chytridiomycosis outbreaks and climate-related environmental factors (Pounds et al., 2006; Bosch et al., 2007; Rohr and Raffel, 2010; Raffel et al., 2012). Nevertheless, and in spite of the significant achievements in these fields, whether a single or multiple causes can account for the worldwide emergence of chytrid outbreaks remains disputed (McCallum, 2005). In light of this, growing emphasis has been recently placed on the comparative analysis of historical (i.e. pre-decline) vs. contemporary samples (e.g., Weldon et al., 2004; Ouellet et al., 2005; Soto-Azat et al., 2010; Bodinof et al., 2011). Indeed, evidence of the chytrid's presence for a number of years in pre-decline (apparently healthy) amphibian populations would suggest that this fungus did not act as a 'lone killer' (contrary to the 'novel pathogen' hypothesis), but would support a role for other 'conspiring' factors in promoting declines, regardless of whether the pathogen is native or introduced.

Some of the first evidence for the occurrence of *B. dendrobatidis* in a declining amphibian in Europe concerned the Apennine yellow-bellied toad *Bombina pachypus* (Stagni et al., 2004), a species endemic to the Italian peninsula. Once abundant throughout its range (it was categorized as ‘Least Concern’ in the IUCN Red List of Threatened Species until 2009, see Andreone et al., 2009), *B. pachypus* populations have been reported to decline in most parts of its natural range, with the exception of the southernmost portion (the Calabria region), since about the last 15 years

(Andreone et al., 2009; Barbieri et al., 2004). For instance, during a survey carried out in the years 1999-2000 (Barbieri et al., 2004), 82% of known sites of presence were confirmed within the Calabria region, whereas 54% were confirmed throughout the rest of the peninsula. Moreover, 64 new sites of presence were discovered in Calabria since 1996, while just 5 new sites were discovered in the other regions (Barbieri et al., 2004). Consequently, the species is now considered as 'Endangered' by the IUCN (Andreone et al., 2009), mostly owing to its susceptibility to chytridiomycosis. It is listed in the Annexes II and IV of the EU Habitat Directive, in the Appendix II of the Bern Convention and is protected by several national and regional laws in Italy. Nevertheless, no studies have been carried out to date in order to assess the distribution of *B. dendrobatidis* among populations neither of this species nor of other co-distributed amphibians.

In this work, using the recently described nested-PCR procedure (Goka et al., 2009), we carried out diagnostic tests for the occurrence of *B. dendrobatidis* on individuals of *B. pachypus* sampled throughout its range. Our aims were: 1) to assess the current distribution of *B. dendrobatidis* along the Italian peninsula and 2) to contribute to the above mentioned debate about disease dynamics by assessing whether *B. dendrobatidis* was already present in the area before the beginning of the observed decline or if its spread in the area could be reliably assumed to have occurred close to the time of the observed declines. With this aim, we analyzed individuals sampled recently (2003-2012) and earlier (1978-1981), in both cases spanning the range of the study species along the Italian peninsula.

3.2 Materials and methods

Ethics statement

Bombina pachypus individuals sampled recently were captured under a permit from the Italian Ministry of Environment (Direzione Generale per la Protezione della

Natura). No permits were needed for sampling activities at the time of the first sampling session (1978-1981). We collected tissue samples from toe-tips, after anaesthetizing toads by submerging them in a 0.02% solution of MS222 (3-aminobenzoic acid ethyl ester). After the completion of the sampling procedure, the individuals were released at the collection site. Tissue samples were stored in 96% ethanol until further analyses.

Sampling and laboratory procedures

We analysed 136 individuals of *B. pachypus* from 15 sampling sites (17 samples, as two sites were sampled in two different years), 8 in the northern and central portion of the peninsula (sites 1 to 8; 82 individuals overall) and 7 from the Calabria region (southern Italy; sites 9 to 15; 54 individuals overall). Sampling sessions were carried out from late April to mid June. Sampling locations - spanning the entire Apennine peninsula -, as well as the number of individuals sampled at each site and year are presented in Table 1 and Figure 1. Among the tested individuals, 56 were collected during a sampling campaign carried out between 1978 and 1981 (38 were from Calabria and 18 were from populations located more to the north), while the remaining 80 samples were drawn between 2003 and 2012 (16 were from Calabria and 64 were from populations located more to the north).

Genomic DNA was extracted from single toes stored at -80°C, following the protocol by Boyle et al. (Boyle et al., 2004) with some modifications. Skin swabbing, although more sensitive than toe-clipping as sampling method (Hyatt et al., 2007), was not used for this study since whole bodies were not available for the historical samples. We added 50 µL of PrepMan Ultra (Applied Biosystems) to each tissue sample, with 30-40 mg of glass beads measuring 0.4-0.6 mm diameter (Sartorius). Samples were homogenized for 1 min at 3000 rpm in a Mikro-Dismembrator S (Sartorius) and centrifuged for 30 sec at 13000 × g in a microfuge (Eppendorf Centrifuge 5415 D). The homogenization and centrifugation procedure was repeated

twice. Samples were then boiled for 10 min, cooled at room temperature for 2 min, and centrifuged at 13000 × g for 3 min. Next, 20 µL of supernatant were retained and stored at -80°C until further analysis.

In order to assess the presence/absence of *B. dendrobatidis* DNA within the extracted DNA, we used the nested-PCR protocol recently developed by Goka et al. (Goka et al., 2009). This method has been shown to increase specificity and sensitivity by a factor of 10 and 100 (see Goka et al., 2009), compared to real-time TaqMan PCR assays (Boyle et al., 2004) and single round PCR assays respectively (Annis et al., 2004). The target DNA was amplified twice using 2 different pairs of primers. The first PCR was performed using the primer pair Bd18SF1 (5'-TTTGTACACACCGCCCGTCGC-3') and Bd28SR1 (5'-ATATGCTTAAGTTCAGCGGG-3'). The reaction mix (25 µL) contained: 5 µL of template DNA diluted 1:10 with distilled water, 0.5 µM of each primer, 2 mM MgCl2, 0.2 mM of each dNTP, Colorless GoTaq Reaction Buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling conditions were as follows: an initial denaturation step for 9 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at 50°C and 2 min at 72°C and a final extension step of 7 min at 72°C (Goka et al., 2009). The second PCR was performed using the primer pair Bd1a (5'CAGTGTGCCATATGTCACG-3') and Bd2a (5'-CATGGTTCATATCTGTCCAG-3') (Annis et al., 2004). The reaction mix (25 µL) contained: 2 µL of the first PCR product used as template, 0.5 µM of each primer, 2 mM MgCl2, 0.2 mM of each dNTP, Colorless GoTaq Reaction Buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling was as follows: an initial denaturation for 9 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at 65°C and 30 sec at 72°C and a final extension step of 7 min at 72°C (Goka et al., 2009).

Each PCR assay was performed in duplicate, and we considered the test positive when PCR products of the expected size (approximately 300 bp) were observed at least once. We included in each assay a positive control with DNA

extracted from *B. dendrobatidis* zoospores (JEL423 kindly provided by Prof. Joyce Longcore, University of Maine) and a negative control of DNA-free distilled water. PCR products (if any) were separated and visualized on a 1% agarose gel. To confirm that the amplification products were from the *B. dendrobatidis* genome, a randomly selected 13% of the PCR products were double-sequenced (n = 11) and compared with reference sequences in GenBank. Sequences were carried out by Macrogen inc. (www.macrogen.com) using an ABI3730XL.

3.3 Results

Nested PCR diagnostic tests for *B. dendrobatidis* presence/absence yielded positive results in 85 out of 136 individuals analysed (62.5%). In all cases the diagnostic PCR bands were of the expected size (approximately 300 bp; see Goka et al., 2009; Annis et al., 2004). Among the 11 PCR products that were sequenced, percent identity with *B. dendrobatidis* sequences available in GenBank was always 100%, and in no case organisms other than *B. dendrobatidis* were among the first 100 blast hits.

The geographic and temporal distributions of the individuals tested positive are shown in Table 1 and Fig.1. *B. dendrobatidis* positives were found in all sampled populations, including the oldest samples analysed (sample 1 and 12; year 1978). Moreover, the prevalence of *B. dendrobatidis* positivity was never below 12%, with frequencies ≥70% observed in most samples (samples 3, 7, 8, 11, 13, 14 and 15).

Prevalence of *B. dendrobatidis* significantly increased over time. Indeed, 51.8% of individuals analyzed in 1978-1981 tested positive, compared to 70% in 2003-2012 (Fisher exact test, P = 0.0472). Nevertheless, this pattern was not apparent throughout the species range. For the Calabrian localities (9-15), the difference between the two periods is striking: 47.4% in 1978-1981, compared to 93.8% in 2003-2012 (Fisher exact test, P = 0.0017). However, for the northern localities (1-8),

overall prevalence of the chytrid did not differ significantly between the two periods: 61.1% in 1978-1981 versus 64.1% in 2003-2012 (Fisher exact test, P = 1.00).

Finally, differences in *B. dendrobatidis* prevalence among northern (i.e. declining) and Calabrian (i.e. non-declining) populations appeared to have accumulated over the study period. Indeed, in the early sampling (1978-1981), there appears to be no effect of latitude: 61.1% in the north versus 47.4% in Calabria (Fisher exact test, P = 0.3990). However, in the recent sampling (2003-2012) there is a significant difference between northern and Calabrian populations: 64.1% vs. 93.8% respectively (Fisher exact test, P = 0.0300).

3.4 Discussion

Since the first evidence for the occurrence of *B. dendrobatidis* infections in an amphibian species in Italy (Stagni et al., 2004), several investigations have been carried out to assess its presence/absence at specific locations, mostly in northern Italy (Garner et al., 2006; Simoncelli et al., 2005; Federici et al., 2009; Ficetola et al., 2011). This is the first study carried out using range-wide sampling, spanning the entire peninsula and using both historical and contemporary samples. Our results clearly showed that *B. dendrobatidis* has a widespread distribution along the Italian peninsula, and that it was already present throughout this area, at least since 1978.

Among the studied populations of *B. pachypus*, the occurrence of *B. dendrobatidis* was conspicuous: it was found in all the sampled populations and with high overall infection prevalence (62.5% of the individuals analysed). Moreover, chytrid prevalence could be even higher than suggested by our data (due to the likelihood of false negatives associated with the toe-clipping method; see Hyatt et al., 2007). When combined with previous observations of chytrid-driven mortality in captive individuals, a role for *B. dendrobatidis* in the widespread decline of *B. pachypus* appears especially plausible (Stagni et al., 2004). Nevertheless, at least two

main issues emerging from our data indicate that the pathogen's distribution alone cannot explain the patterns of decline: (i) the chytrid was already present and widespread along the peninsula well before the first evidence of the decline of *B. pachypus*, and (ii) the geographic distribution of the declines does not match that of the chytrid (in historical or contemporary samples).

In suitable habitats along the Apennine chain, *B. pachypus* was considered as a common species until the mid 1990's, when population declines and disappearances begun to be reported (Andreone et al., 2009; Barbieri et al., 2004). Nevertheless, our results date back the presence of the pathogen to at least 1978. According to the 'novel pathogen hypothesis', the chytrid would undergo wave-like spreads, causing mass mortality of 'naïve' susceptible species as it arrives (Skerratt et al., 2007; Rachowicz et al., 2005; Lips et al., 2008; Vredenburg et al., 2010; Cheng et al., 2011). If this was the case, we should have observed waves of mortality long before the mid 1990's, particularly when considering the estimated rates of spread of *B. dendrobatidis* (e.g. Lips et al., 2008; but see also Phillips et al., 2012). On the contrary, a time lag of more than 15 years separates the first evidence of chytrid occurrence from the observed declines. On the basis of our data, we cannot speculate about how and when the chytrid became established in the area (i.e. if it is endemic or if it was introduced once or many times, owing to anthropogenic transport or via natural long-distance dispersal). Nevertheless, these data allow us to exclude wave-like mortality of *B. pachypus* soon after chytrid arrival. Therefore, regardless of whether *B. dendrobatidis* is native or exotic to the region and when it arrived, it has not acted as a 'lone killer' (see also Di Rosa et al., 2007; Pounds and Coloma, 2008).

The spatial mismatch between chytrid occurrence and the decline of *B. pachypus* populations provides further evidence against the hypothesis that *B. dendrobatidis* acts as a 'lone killer'. Indeed, the decline is affecting *B. pachypus* populations from the northern and central regions of its range, while no evidence of decline has been reported for the Calabria region. Nevertheless, our data showed that *B. dendrobatidis* is present in Calabria as well, and it was already there in the late

‘70s, indicating that southern populations are coping with chytrid infections for at least 35 years, without detectable signs of dying-offs or negative demographic trends (Andreone et al., 2009; Barbieri et al., 2004).

On the whole, the observations above have at least two important implications concerning the disease dynamics: 1) *B. dendrobatidis* did not cause extirpation of *B. pachypus* populations immediately after its arrival, but affected them during outbreaks in areas where it had previously become established, probably under the influence of other factors and 2) these factors are affecting *B. pachypus* populations differently along the species range. Noteworthy, spatial and temporal patterns were not only observed at the level of *B. pachypus* population declines, but also of *B. dendrobatidis* prevalence, although the latter should be taken with some caution owing to the higher probability of false negatives in historical samples.

Several factors have been proposed as ‘cooperating’ with *B. dendrobatidis* in promoting the decline of amphibians (reviewed in Blaustein et al., 2010; Blaustein et al., 2011; Blaustein and Kiesecker, 2002; Collins and Storfer, 2003; Beebee and Griffiths, 2005). A role for factors that have a local action, e.g. changes in land-use or environmental contaminants, cannot be excluded with regard to the decline of single *B. pachypus* populations. Nevertheless, they are unlikely to explain the spatial and temporal pattern of declines, as they are managed at a local scale and not suitable to account for the extent of the observed process.

Although both field and laboratory experiments will be needed to reliably assess the nature of those factors interacting with *B. dendrobatidis* in priming the observed declines, we suggest that two of such factors could be the genetic diversity of host populations, that could account for the spatial pattern, and the recent climate change, that could account for the temporal pattern of declines.

The spatial pattern of the decline of *B. pachypus* is strikingly paralleled by the range-wide pattern of population genetic diversity. In a recent survey of the genetic variation among *B. pachypus* populations (Canestrelli et al., 2006), the Calabria region was identified as the hotspot of intraspecific genetic diversity and the glacial

refugium for this species, from where northern areas were re-colonized since the end of the last glaciation. Northern populations, although slightly differentiated from the southern ones, appeared genetically invariable, as expected for 'recently' founded populations (see also Canestrelli et al., 2008; Canestrelli et al., 2010; Canestrelli et al., 2012) and references therein). Populations with lower levels of genetic diversity have a higher probability of becoming genetically inbred, with potential consequences of lowered fitness and increased probability of facing higher extinction risks (Spielman et al., 2004a; Spielman et al., 2004b; Frankham, 2005). Furthermore, it is well established that low levels of genetic variation can be accompanied by higher susceptibility to emerging pathogens (Spielman et al., 2004a; Acevedo-Whitehouse et al., 2003; Hughes and Boomsma, 2004; Pearman and Garner, 2005). In the present case, the pattern of population genetic diversity across the species range would comfortably accommodate the spatial pattern of declines, suggesting higher susceptibility to *B. dendrobatidis* of northern populations owing to their reduced adaptive potential (see also Luquet et al., 2012).

Nonetheless, a hypothesis of a interaction between *B. dendrobatidis* and the reduced genetic diversity of northern populations in promoting population declines would left the temporal framework unexplained (i.e. the time laps since the first evidence of chytrid occurrence in the area and the observed declines). Among the factors that could help explaining such framework, climate is the only one that would act on a sufficiently large geographic scale, with marked and well-documented changes in recent years and an increasingly apparent impact on living systems (Blaustein et al., 2010; Blaustein et al., 2011). Along the Italian peninsula, mean temperatures have raised in the last decades, intensity and duration of heat-waves have increased, and the number of wet days has decreased (Brunetti et al., 2004; Toreti and Desiato, 2008). Changes in temperature regimes and precipitation patterns, as well as extreme climatic events, could have altered disease dynamics in the last decades, promoting disease outbreaks, as already suggested both for other chytrid-driven amphibian declines (Pounds et al., 2006; Bosch et al., 2007; Rohr and Raffel,

2010; Raffel et al., 2012) and for a wide range of distinct host-pathogen systems (e.g. Harvell et al., 2002).

3.5 Concluding remarks

The assessment of the spatial and temporal distribution of emerging pathogens is of utmost importance in epidemiological research. Indeed, such an approach is providing increasingly useful insights into host-pathogen-environment relationships and disease dynamics.

In this work, analysis of the spatial and temporal distributions of *B. dendrobatidis* among samples spanning the whole Italian peninsula allowed us to exclude *B. dendrobatidis* as the 'lone killer' of the endangered species *B. pachypus*, as a scenario of wave-like mortalities soon after *B. dendrobatidis* arrival does not fit the spatial and temporal occurrence data gathered. Moreover, the temporal mismatch between the occurrence of *B. dendrobatidis* and the decline of *B. pachypus*, as well as different demographic trends of its southern vs. northern populations, indicate that other factors interact with *B. dendrobatidis* causing the observed declines.

Among the potentially interacting factors, we indicated two as the most plausible ones: 1) genetic diversity of host populations, which could have formed the basis for the spatial component of the observed declines, and 2) recent climate changes, which could have triggered changes in the outcome of host-pathogen interactions.

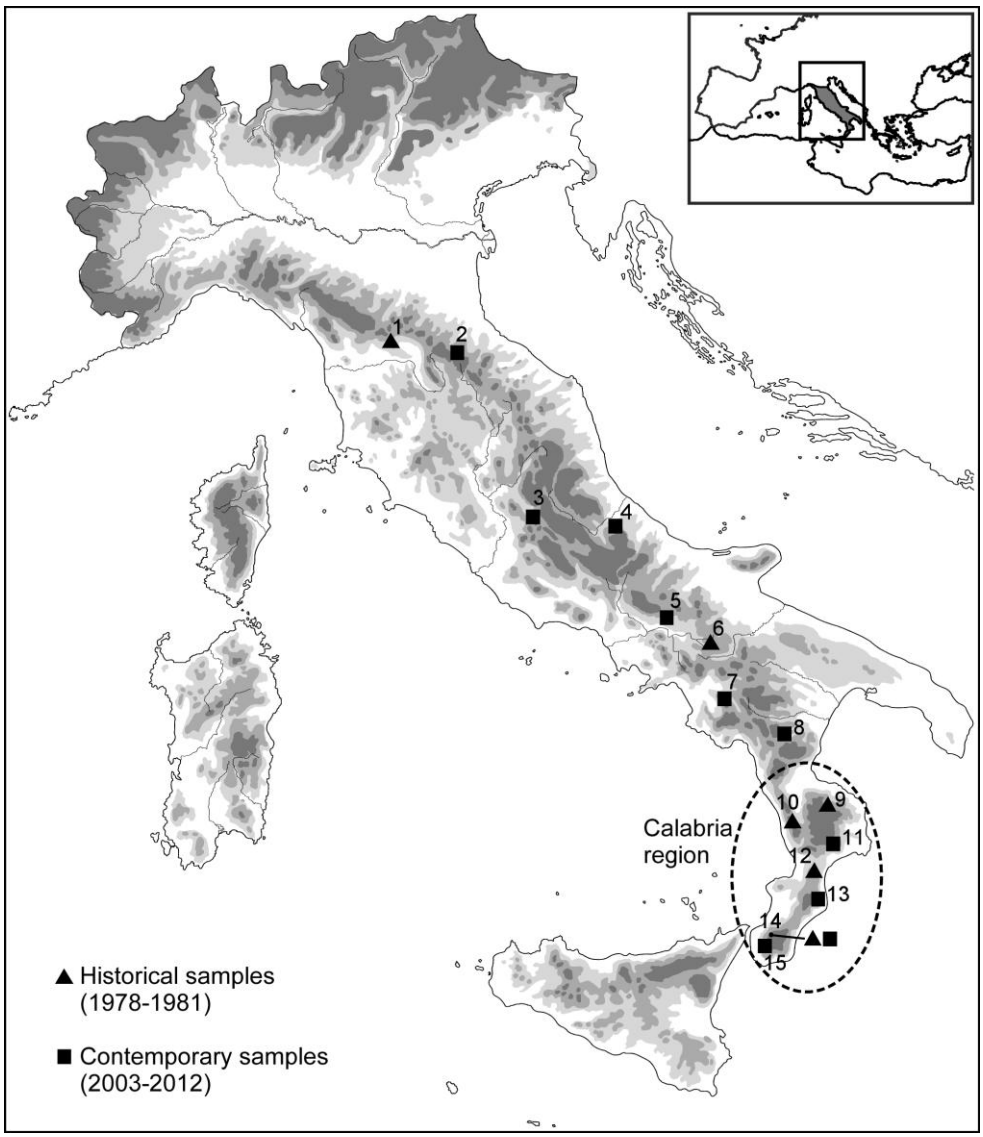
Both field and laboratory experiments are ongoing to test this hypothesis. Indeed, once validated, it would have at least one major implication of both theoretical and applied importance: management programs aimed at the genetic rescue of northern *B. pachypus* populations using genetic resources from southern populations, could have the potential to revert the observed negative demographic trend.

3.6 Tables and figures

Table 1 Geographic location and sampling year of the 15 populations of *Bombina pachypus* analysed, number of individuals analysed (n), and number of individuals testing positive for the presence of *Batrachochytrium dendrobatidis* (p).

	Site	Latitude N	Longitude E	Year	n	p
1	Rio a Buti	43° 56’	11° 6’	1978	13	9
2	Bagno di Romagna	43° 51’	11° 57’	2004	13	4
				2012	9	6
3	Stipes	42° 15’	12° 58’	2003	5	5
4	Rocca Caramanico	42° 09’	14° 01’	2003	6	3
5	San Lupo	41° 16’	14° 39’	2003	10	6
6	Carife	41° 01’	15° 12’	1981	5	2
7	S. Angelo a Fasanella	40° 27’	15° 20’	2003	19	15
8	S. Severino Lucano	40° 05’	16° 04’	2003	2	2
9	Macchia Longa	39° 22'	16° 36'	1981	5	2
10	Monte Cocuzzo	39° 14'	16° 10'	1981	8	1
11	Taverna	39° 01'	16° 35'	2003	2	2
12	Cardinale	38° 44'	16° 24'	1978	15	7
13	Stilo	38° 30'	16° 25'	2003	11	10
14	Gambarie	38° 10'	15° 50'	1981	10	8
				2003	2	2
15	Cardeto	38° 05'	15° 46'	2006	1	1

Figure 1 Geographic distribution of the 15 sampled populations of *Bombina pachypus*. The inset shows the species range along the Italian peninsula.



CHAPTER 4

GEOGRAPHIC DISTRIBUTION OF THE CHYTRID PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* AMONG MOUNTAIN AMPHIBIANS ALONG THE ITALIAN PENINSULA

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Abstract

The amphibian chytrid pathogen *Batrachochytrium dendrobatidis* is considered a main driver of amphibian population declines, particularly in montane areas. In peninsular Italy, its occurrence has been reported in populations spanning the entire range of the endemic and declining yellow-bellied toad *Bombina pachypus*. Here, we investigate the presence and distribution of *B. dendrobatidis* among populations of 3 mid- to high-altitude species spanning the entire peninsula (486 individuals from 39 sites overall): the stream frog *Rana italica*, the fire salamander *Salamandra salamandra gigliolii*, and the alpine newt *Mesotriton alpestris apuanus*. We found *B. dendrobatidis* in all of the analyzed species. Despite the widespread distribution of the pathogen, its overall prevalence (6%, 9% and 19%, respectively) was lower than previously reported for the endangered *B. pachypus* (62.5%). Moreover, contrary to that result for the latter species, several populations of the species studied here were not affected, even in sites known to have *B. dendrobatidis*. When coupled with the lack of evidence for chytrid-related mortalities in these species in peninsular Italy, these results suggest that mechanisms of resistance and/or tolerance are protecting populations of these species from the pathogenic activity of *B. dendrobatidis.*

Nevertheless, in light of the dynamic pattern of chytrid-host interaction reported in other studies and the ongoing climate changes in montane environments, we suggest that the occurrence of *B. dendrobatidis* should be considered a potential threat to the long-term persistence of these species, and urge the implementation of monitoring and conservation plans.

4.1 Introduction

Amphibians are declining worldwide and chytridiomycosis is among the main causes (Berger et al., 1998; Daszak et al., 1999; 2003; Fisher et al., 2009; Kilpatrick et al., 2010). This emerging infectious disease is caused by the chytrid fungus *Batrachochytrium dendrobatidis*, which affects the keratinized epidermal cells of its amphibian hosts (Longcore et al., 1999; Pessier et al., 1999). In recent years, many studies have been devoted to assessing the global distribution of *B. dendrobatidis* and the susceptibility of different amphibian species and populations to decline following infection (e.g. Berger et al., 1998; Bosch et al., 2001; Muths et al., 2003; Schloegel et al., 2006; Goka et al., 2009).

Despite these efforts, many aspects of its epidemiology are still far poorly understood (Rachowicz et al., 2005; McCallum, 2005; Fisher et al., 2009). The geographic origin of *B. dendrobatidis*, its spread dynamics, interaction with other factors, and variability in the outcome of infection are among the main issues that are still unclear (Weldon et al., 2004; Rachowicz et al., 2005; Pounds et al., 2006; Blaustein et al., 2011). Indeed, field observations show that, despite its wide distribution and the many species it infects, the outcome of the infection varies not only between different species, but also between populations within species and between geographic areas (e.g. Tobler and Schimdt, 2010; Walker et al., 2010).

Altitude is one of the geographic factors apparently related to the severity of disease; the most severe mass mortalities have been observed in mountain species (Berger et al., 1998; Fellers et al., 2001; Bosch et al., 2001). Early observations of chytridiomycosis were reported from montane rain forests in Australia and Central America (Berger et al., 1998). Severe die-offs at high altitudes are also been observed in temperate regions in North America and Europe (Fellers et al., 2001; Bosch et al., 2001). In Europe, mass mortalities of 3 common species (*Alytes obstetricans*, *Salamandra salamandra,* and *Bufo bufo*) were observed only at a high altitude in a protected area (Bosch et al., 2001; Bosch and Martínez-Solano, 2006), and a strong association between altitude and fatal chytridiomycosis was found (Walker et al., 2010).

In a previous assessment of *B. dendrobatidis* occurrence along the Apennine Mountains (Italy), we found evidence of this pathogen's widespread presence in the endangered yellow-bellied toad *Bombina pachypus*, a species with a mid- to high-altitude range (Canestrelli et al., 2013). This Italian endemic species has experienced severe population declines in most of its range, with the exception of the southernmost area, over the last 15 years (Barbieri et al., 2004; Andreone et al., 2009), and it has been proposed that chytrid-related mortality has played a role in this (Stagni et al., 2004). However, we found a delay of at least 1 decade between the first evidence of chytrid occurrence and the beginning of the *B. pachypus* decline. Moreover, we observed spatial heterogeneity in the relationship between chytrid-carrying populations and population declines (Canestrelli et al., 2013). On the whole, these observations suggests that 1) *B. dendrobatidis* did not acted as a 'lone killer', 2) *B. dendrobatidis* can occur in susceptible host species without signs of decline until triggered by other factors, and 3) other factors can influence the outcome of the host-pathogen interaction, resulting in a temporally and spatially heterogeneous pattern.

In this study, we extended the assessment of *B. dendrobatidis* occurrence along the Italian peninsula to 3 other species with a mid- to high-altitude range, which are sympatric and frequently syntopic with *B. pachypus* along the Apennine Mountains.

We carried out diagnostic tests using a nested PCR approach to check for the presence of *B. dendrobatidis* on individuals of the Italian fire salamander *Salamandra salamandra gigliolii*, the Italian endemic stream frog *Rana italica*, and the Italian alpine newt *Mesotriton alpestris apuanus*. These species are not considered to be as severely endangered as *B. pachypus*, although population declines have been recently reported (Lanza et al., 2007). In light of the widespread occurrence of the pathogen along their range and their frequent syntopy with a highly infected species, our aims were 1) to assess the *B. dendrobatidis* infection status for these species in the wild and 2) to evaluate if *B. dendrobatidis* is or could become a risk factor for these species from a conservation perspective.

4.2 Materials and methods

We analyzed a total of 486: 77 *S. salamandra*, 136 *R. italica*, and 273 *M. alpestris*. Sampling locations, as well as the number of individuals sampled in each site and year are presented in Table 1 and Figure 1. Sampling was carried out using fine-tipped swabs (Medical Wire & Equipment Co. MW 113) for samples collected after 2010 and toe clipping for samples collected before 2010.

Genomic DNA was extracted from swabs following the protocol of Boyle et al. (2004) with some modifications. We placed a single swab in a 2 ml tube, adding 30-40 mg of glass beads measuring 0.4-0.6 mm in diameter (Sartorius) and 70 µl of PrepMan Ultra (Applied Biosystems). Tubes were vortexed for 1 min at 2400 rpm in a vortex and centrifuged for 30 s at 13000 × *g* in a microfuge (Eppendorf Centrifuge 5415 D). The vortex and centrifugation procedure was repeated twice. Next, samples were boiled for 10 min, cooled at room temperature for 2 min, and centrifuged at 13000 × *g* for 3 min. 20 µl of supernatant were retained and stored at -20°C until further analysis.

Genomic DNA was extracted from tissue samples following the standard cetyltrimethyl ammonium bromide (CTAB) protocol (Sambrook et al., 1989) with a final elution in 30 µl in order to gain a concentrated DNA solution.

The molecular diagnostic screening to test for the presence of chytrid DNA within the pool of extracted DNA was conducted using the nested-PCR protocol developed by Goka et al. (2009) with some modifications. The target DNA was amplified twice using 2 different pairs of primers.

The first PCR was performed using the primer pair Bd18SF1 (5'-TTTGTACACACCGCCCGTCGC-3') and Bd28SR1 (5'-ATATGCTTAAGTTCAGCGGG-3'). The reaction mix (25 µl) contained: 2 µl of template DNA (DNA extracted from swabs using PrepMan Ultra was diluted 1:10 with distilled water), 0.5 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, Colorless GoTaq Reaction Buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling process was as follows: an initial denaturation step for 9 min at 95°C, 30 cycles of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C and a final extension step of 7 min at 72°C (Goka et al., 2009).

The second PCR was performed using the primer pair Bd1a (5'CAGTGTGCCATATGTCACG-3') and Bd2a (5'-CATGGTTCATATCTGTCCAG-3') (Annies et al., 2004). The reaction mix (25 µl) contained: 2 µl of the first PCR product used as template, 0.5 µM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, Colorless GoTaq Reaction Buffer 1× and 1 U of GoTaq Polymerase (Promega). The PCR cycling was conducted with a touchdown program. After an initial denaturation for 5 min at 95°C, 35 cycles were performed as follows: 30 s at 94°C, 30 s at 65°C with a decrement of 0.2°C at each cycle, and 30 s at 72 °C. These cycles were followed by a final extension of 7 min at 72°C. Each assay included a positive control with DNA extracted from *B. dendrobatidis* zoospores (JEL423 kindly provided by Prof. Joyce Longcore, University of Maine) and a negative control of DNA-free distilled water.

PCR products (if any) were separated and visualized on a 1% agarose gel. We considered as positive result to occur if a sample returned an amplification product of the correct size (approximately 300 bp). To confirm that the amplification products were from the *B. dendrobatidis* genome, a randomly selected 18% of the PCR products were double-sequenced (n = 12) and compared with reference sequences in GenBank. Sequencing were carried out by Macrogen Inc. (www.macrogen.com) using an ABI3730XL.

4.3 Results

From 486 total screened samples the diagnostic nested PCR assay yielded 66 positive results, showing a PCR band of the expected size (approximately 300 bp). Among these, the highest chytrid prevalence was observed in *M. a. apuanus*, in which 51 out of 273 samples (19%) tested positive, while the observed prevalence in other analyzed species was 7 out of 77 (9%) in *S. s. gigliolii*, and 8 out of 136 (6%) in *R. italica*. All the sequenced PCR products showed 100% identity with the *B. dendrobatidis* sequences available on GenBank.

Numbers of positive and tested individuals in each population, along with geographic information on the sampling site and year are reported in Tab. 1. The pathogen was widespread geographically among the analyzed species (Fig. 1). Among them, *M. a. apuanus* showed a wider chytrid distribution, with positive individuals detected in 50% of the sampling sites, distributed throughout its entire range. *S. s. gigliolii* and *R. italica* showed *B. dendrobatidis* occurrence in 36% and 30% of the sampling sites, respectively.

A significant increase in *B. dendrobatidis* prevalence with altitude was observed in *M. a. apuanus* in which 22% individuals tested positive from populations sampled at sites above 800 m a.s.l., while 5% individuals tested positive from populations sampled at sites below 800 m a.s.l. (Fisher exact test, P = 0.002). In *S. s.*

gigliolii and *R. italica* we did not observed significant differences in chytrid prevalence with altitude. The prevalence of chytrid occurrence in individuals from *S. s. gigliolii* populations sampled above and below 800 m a.s.l was 8% and 15%, respectively, (Fisher exact test, P = 0.336), and 6% of individuals tested positive from *R. italica* populations sampled both above and below 800 m a.s.l. (Fisher exact test, P = 1).

Finally, contrary to what was previously observed for *B. pachypus* (Canestrelli et al., 2013), we did not find significant differences in chytrid prevalence according to latitude. The prevalence of *B. dendrobatidis* in *S. s. gigliolii* was 9% among both northern and central populations and populations from Calabria (Fisher exact test, P = 1), while its prevalence in *R. italica* was 11% among northern and central populations and 3% in populations from Calabria (Fisher exact test, P = 0.139).

4.4 Discussion

Our results are in agreement with previous findings in showing a wide distribution of *B. dendrobatidis* along the Apennine Mountains (see Canestrelli et al., 2013). Nevertheless, both the infection prevalence and the proportion of the affected populations in each species were lower in the 3 species analyzed here than in *B. pachypus*. Moreover, several of the populations testing negative were sampled at sites where individuals from other species tested positive (sites 13, 29, 32, 37, and 38) or at sites where *B. pachypus* populations were previously observed to be highly infected (sites 14, 29, 35, 38, and 39). Furthermore, despite some reports of population declines (e.g., for *S. s. gigliolii* in the north-central Apennines and for *M. a. apuanus* in the southern areas; see Ambrogio and Gilli 1998; Bologna et al., 2000; Canestrelli et al., 2006; Lanza et al., 2007) no evidence of chytrid-related die-offs have been reported for these species in Italy, and other factors have been indicated as the main drivers, such as the introduction of fishes, eutrophication due to intensive

farming, water catchment, climate changes, geographic isolation, and reduced genetic variability. On the other hand, some populations testing positive did not appear demographically impoverished or unstable over multiple years of observation (e.g., *M. a. apuanus* at site 13; Canestrelli, pers. obs.).

On the whole, contrary to what was previously suggested for *B. pachypus*, it does not seem that the chytrid pathogen is severely threatening populations of *S. s. gigliolii*, *R. italica*, or *M. a. apuanus* in Italy. In turn, this suggests that mechanisms of resistance or tolerance are protecting populations of these species from the pathogenic activity of *B. dendrobatidis*.

Nevertheless, we suggest that the widespread distribution of *B. dendrobatidis* along the Italian peninsula and its occurrence on the studied populations should be regarded as a serious threat for the long-term survival of *S. s. gigliolii*, *R. italica*, and *M. a. apuanus* in the area, for at least 2 reasons.

First, a time lag between chytrid occurrence and host population declines has been observed in other studies (Puschendorf et al., 2006; Canestrelli et al., 2013), indicating that currently resistant/tolerant populations can eventually become threatened in the future, when altered environmental conditions shift host-pathogen interactions toward the pathogen's optimum or increase host susceptibility. For instance, within Peñalara Natural Park in central Spain the fire salamander *Salamandra salamandra* and the common toad *Bufo bufo* underwent mass mortalities 4 years after chytridiomycosis almost extirpated the midwife toad *Alytes obstetricans* from the same area (Bosch and Martínez-Solano, 2006; Bosch et al., 2001). In addition, in the case of the Apennine yellow-bellied toad *B. pachypus*, population declines were observed more than a decade after the first evidence of chytrid occurrence in the area (Canestrelli et al., 2013).

Second, most of the reported events of chytrid-related mass mortality are from montane areas, both in tropical and temperate regions, suggesting that the outcome of host-pathogen interactions could be more susceptible to environmental changes at high altitude (Berger et al., 1998; Fellers et al., 2001; Bosch et al., 2001). On the one

hand, most montane amphibians show prolonged aquatic life stages, giving them a prolonged exposure to chytrid zoospores (Catenazzi et al., 2013). On the other hand, the particularly severe impact of ongoing climate change on montane biodiversity (Diaz et al., 2003; Nogués-Bravo et al., 2007) could lead to a combination of suboptimal environmental conditions for host species due to the upward shift of the climatic niche and increased pathogenic activity as the 'chytrid thermal optimum' is approached (Pounds et al., 2006). Interestingly, we found a significantly higher prevalence of *B. dendrobatidis* among high altitude populations of *M. a. apuanus*, the species most strictly confined to montane habitats.

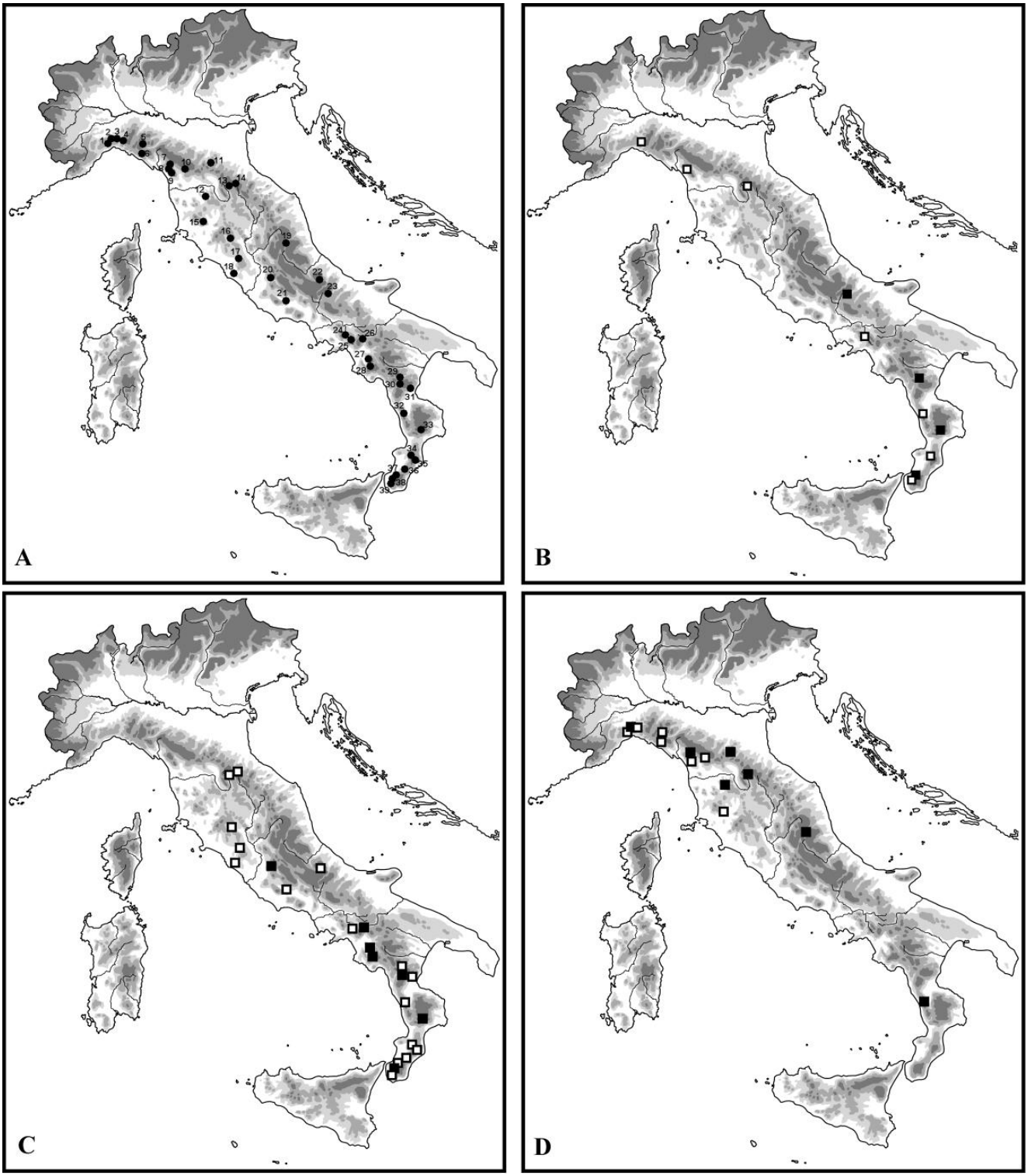
Unraveling the regional distribution of *B. dendrobatidis* is a first, highly necessary but insufficient step toward the appreciation of disease dynamics in the area and the implementation of management and conservation plans for its amphibian hosts (Woodhams et al., 2011). Here we have shown that this pathogen is widespread among montane amphibians throughout the Italian peninsula. Monitoring plans to assess chytrid prevalence and virulence and their variation over time among multiple host populations should be carried out in the future, in order to identify possible shifts of host-pathogen interactions toward increased pathogenicity. Moreover, it will be of the utmost importance to extend the study to the whole batrachofauna in the area, to better understand disease ecology and dynamics among different species and populations, and to fully appreciate the potential threats posed by *B. dendrobatidis* to the amphibians in the area. Finally, in light of recent advances in the development of strategies to mitigate chytridiomycosis (Woodhams et al., 2011; Bletz et al., 2013), it now appears reasonable to develop management plans that includw ex-situ conservation programs for host populations.

4.6 Tables and figures

Table 1 Geographic location, altitude and sampling year of the populations of *S. s. gigliolii*, *R. italica,* and *M. a. apuanus.* with number of individuals analysed (n), and number of individuals testing positive for the presence of *B. dendrobatidis* (p) are also reported.

	Site	Latitude N	Longitude E	Altitude (m a.s.l.)	*S. s. gigliolii*			*R. italica*			*M. a. apuanus*		
					Year	N_{tot}	p	year	N_{tot}	p	Year	N_{tot}	p
1	Pianpaludo	44°26'	8°35'	800							2010	10	0
2	Rossiglione	44°32'	8°37'	480							2010	16	1
3	Capanne di Marcarolo	44°33'	8°46'	780							2009	18	0
4	Vallecalda	44° 32’	8° 57’	580	2007	6	0						
5	Monte Penna	44°29'	9°29'	1460							2007	16	0
6	Lago di Bargone	44°19'	9°29'	850							2009	18	0
7	Minucciano	44°09'	10°14'	800							2009	20	5
8	Cipollaio	44° 03’	10° 15’	820	1981	3	0						
9	Stazzema	44°01'	10°18'	890							1984	5	0
10	Abetone	44°07'	10°37'	1800							2009	24	0
11	Monghidoro	44°13'	11°17'	830							2009	16	4
12	Greve in Chianti	43°33'	11°23'	500							1983	4	0
											2009	14	2
13	Camaldoli	43° 48’	11° 49’	1000							2001	5	0
											2004	12	12
					2007	10	0						
					2012	4	0	2012	1	0	2012	18	17
14	Bagno di Romagna	43° 50’	11° 57’	460				2007	7	0			
								2012	5	0			
15	Lama	43° 05’	11° 14’	260							2009	7	0
16	Monte Rufeno	42° 48’	11° 53’	500				2007	1	0			
17	Blera	42° 15’	12° 03’	270				2007	2	0			
18	Tolfa	42°07'	11°56'	400				2007	7	0			
19	Monti della Laga	42°42'	13°19'	1500							2000	3	0
											2003	23	5
											2008	18	1
											2012	2	0
20	Percile	42° 04’	12° 54’	580				2003	6	1			
21	Patrica	41°35'	13°14'	400				2007	2	0			
22	Palena	41° 59’	14° 08’	770				2003	1	0			
23	Pescolanciano	41° 43’	14° 20’	945	2003	2	1						
24	Mercogliano	40°54'	14°44'	600	2004	1	0						
25	Serino	40° 50’	15° 51’	420				2005	5	0			
26	Oppido	40°51'	15°10'	600				2009	2	1			
27	S. Angelo a Fasanella	40° 27’	15° 20’	520				2003	10	2			
28	Laurino	40°19'	15°20'	900				2006	3	1			
29	S. Severino Lucano	40° 03’	16° 07’	680	2003	6	2						
								2012	5	0			
30	Viggianello	39° 58'	16° 05'	550				2003	4	1			
31	S. Lorenzo Bellizzi	39° 53'	16° 20'	830				2003	6	0			
32	Fagnano Castello	39°33'	16°01'	1090	2012	6	0	2012	2	0	2010	19	2
											2012	5	2
33	Villaggio Mancuso	39° 04'	16° 33'	1200	2003	10	2	2003	2	1			
34	Serra S. Bruno	38° 35'	16° 20'	805	1998	5	0						
								2003	7	0			
					2010	3	0	2010	10	0			
					2012	1	0	2012	1	0			
35	Stilo	38° 29'	16° 28'	290				2003	4	0			
								2010	12	0			
								2012	9	0			
36	Zomaro	38° 20'	16° 09'	960				2006	9	0			
37	Carmelia	38° 14'	15° 55'	1220	2006	6	2	2006	3	0			
38	Gambarie	38° 10'	15° 50'	1310	2003	14	0	2003	1	1			
								2012	7	0			
39	Cardeto	38° 05'	15° 46'	680				2006	2	0			

Figure 1 Geographic distribution of the 39 sampling sites (A) and *B. dendrobatidis* occurrence on *S. s. gigliolii* (B), *R. italica* (C) and *M. a. apuanus* (D). Full squares indicate chytrid positive populations, empty squares indicate chytrid negative populations.



CHAPTER 5

FORECASTING THE IMPACTS OF CLIMATE CHANGE ON THE DISTRIBUTION OF THE ENDANGERED APENNINE YELLOW-BELLIED TOAD

5.1 Introduction

Anthropogenic climate change, resulting from increased emission of greenhouse gases and changes in land and water use, is recognized as a major threat to biodiversity conservation (Thomas et al., 2004). Average temperatures, sea level, and extreme climatic events are predicted to increase during the next decades (IPCC, 2007); the rate of these changes is forecast to be rapid on an evolutionary time scale.

Observations regarding changes in climatic parameters recorded during recent years confirm that the climate is already changing according to predictions (IPCC, 2007). Recent evidence indicates that these changes are having an immediate effect on ecosystems, which respond through changes in community assembly, phenology, and species range shifts (Walther et al., 2002; Parmesan, 2006). Species with poor dispersal ability or lower tolerance plasticity and adaptive potential are of key concern (Parmesan, 2006). Moreover, the dispersal ability of many species is strongly affected by natural geographical barriers or landscape fragmentations resulting from habitat destruction (Travis, 2003; Opdam and Wascher, 2004).

Many attempts have been made to predict the impact of climate change on species persistence, by evaluating the loss of habitat suitability under altered climatic conditions (Thomas et al., 2004; Araújo et al., 2006; Pereira et al., 2010). Such predictions are mainly derived by implementing the recently developed species distribution modelling (SDM) tools (reviewed in Elith and Leathwick, 2009). SDMs enable the correlation of species occurrence records at several sampling points with

the bioclimatic envelop at these points, and hence the construction of a prediction model for species occurrence in all suitable areas on a geographic range scale. The distribution model can be projected under future predicted climatic conditions, to evaluate the potential loss or gain of habitat suitability along the species range or range shift.

Along the Italian peninsula, a species that has recently been hypothesized to be negatively affected by climate change is the Apennine yellow-bellied toad, *Bombina pachypus* (Canestrelli et al., 2013). This endangered amphibian species has its range along the Apennine chain and has suffered from population decline since the 1990s (Barbieri et al., 2004; Andreone et al., 2009). Recently, this decline was reported to be mainly associated with the occurrence of the amphibian pathogenic chytrid, *Batrachochytrium dendrobatidis* (Canestrelli et al., 2013). However, the observed temporal and spatial pattern of decline is not explained solely by pathogen occurrence. The decline of *B. pachypus* started in the late 1990s and was only observed in the northern and central part of the range (Barbieri et al., 2004; Andreone et al., 2009). On the other hand, *B. dendrobatidis* has been shown to be present throughout the entire *B. pachypus* range, from at least 1970s (Canestrelli et al., 2013). Recent climate change has been suggested to play a role in the temporal mismatch between the arrival of *B. dendrobatidis* and the decline of *B. pachypus*.

In the present study, we investigated the potential impact of climatic factors on the decline of *B. pachypus*, by modelling the pattern of 'recent' habitat suitability based on bioclimatic variables (averaged from 1950 to 2000) along the species range. Our objective was to assess changes in habitat suitability between 'recent' and future climatic scenarios. Initially, we implemented a maximum entropy approach, to construct a *B. pachypus* distribution model based on 45 species presence records derived from the literature and 19 bioclimatic variables averaged from 1950 to 2000. Next, we projected the distribution model under the climatic conditions predicted by IPCC3 for 2050 and 2080, according to 2 different emission scenarios (IPCC, 2001).

Finally, we evaluated the loss of habitat suitability between 'recent' and predicted models.

5.2 Materials and Methods

We obtained occurrence records, and the corresponding latitude and longitude data, for *B. pachypus*, from the literature, and also from museum collection archives. In order to obtain a homogeneous distribution, and therefore avoid bias caused by data clustering, we selected a subset of 45 occurrence records from available data (see Appendix).

To model the species distribution, we used the maximum entropy method as implemented in MAXENT 3.3.3K (Phillips et al., 2006). We selected MAXENT because it was specifically developed to model species distribution by using presence-only data; furthermore, it has been shown to outperform other methods, particularly when few presence records are available (Graham et al., 2007b).

To model the current distribution of *B. pachypus*, we selected 19 bioclimatic variables (averaged from 1950 to 2000 at a resolution of 2.5 arc-min) from the WORLDCLIM database (www.worldclim.org) as environmental predictors. To model the future distribution of the species, we used the IPCC3 model Csiro with a2a and b2a emission scenarios, and the IPCC3 model HADCM3 with a2a and b2a emission scenarios (IPCC, 2001). We chose these 2 emission scenarios because they predict different climatic conditions according to different patterns of economic development (a2a predicts local oriented economic development, while b2a predicts local environmental sustainability). The distributions were modelled under the bioclimatic conditions predicted for 2050 and 2080. In order to verify the accuracy of the model generated, we withheld 25% of occurrences (selected at random), and used them to test the model performance as the area under the receiver operating characteristic curve (AUC) (Phillips et al., 2006).

We used DIVA-GIS 7.5.0 (www.diva-gis.org) to averaged the generated layers of predicted future distribution among the years and emission scenarios, and thereby obtain 4 predicted distribution layers: 2050 a2a, 2050 b2a, 2080 a2a and 2080 b2a. Finally, we used DIVA-GIS to subtract the current habitat suitability model from models projected for 2080, in order to evaluate the predicted areas of loss or gain of habitat suitability by that year.

5.3 Results and Discussion

The modelled distribution showed high accuracy (AUC = 0.916; SD = 0.029) according to the proposed classification of model performance (Fielding and Bell, 1997).

The *B. pachypus* distribution modelled under 'recent' bioclimatic conditions (Fig. 1) showed high habitat suitability throughout the actual species range, further indicating excellent model performance. An area with particularly high predicted values was the southernmost portion of the species range, corresponding to the Calabria region.

The predicted distributions under future bioclimatic conditions showed substantial changes in the geographic pattern of habitat suitability (Fig. 2). The pattern of these changes differed markedly between the 2 emission scenarios; however, their spatial and temporal directions were very similar. Emission scenarios a2a and b2a predict differing levels of greenhouse gases according to different patterns of economic development; a2a predicts a scenario with a continuously increasing human population and intensive consumption of fossil fuel, whereas b2a predicts a scenario with more sustainable economic development (IPCC, 2001). The prediction distributions generated according to both scenarios showed a wide loss of habitat suitability along the range of *B. pachypus*; however, according to the

expectations, the model generated under the a2a scenario showed more severe changes.

Comparison of the *B. pachypus* distribution modelled under 'recent' bioclimatic conditions with that modelled under the b2a future emission scenario reveals a predicted northward gain of areas with high habitat suitability by 2050, but an overall loss of habitat suitability by 2080, especially in the southern part of the species range (Fig. 2). Meanwhile, comparison of the *B. pachypus* distribution modelled under 'recent' bioclimatic conditions with that modelled under the a2a future emission scenario depicts a more severe pattern of change. In this case, the predicted northward gain of areas with high habitat suitability by 2050 holds true; however, the loss of habitat suitability in the southern part of the species range is already noticeable by 2050. Furthermore, the overall loss of habitat suitability observed by 2080 is higher than that observed under the b2a scenario, especially in Calabria (Fig. 2).

To further assess the overall effect of predicted climate changes on *B. pachypus* habitat suitability, we subtracted the current distribution layer from the 2 projections for 2080. Our results indicated that climate changes are predicted to have the most severe impact on southern populations of *B. pachypus* (Fig. 2). This pattern is evident according to the b2a and a2a scenarios; however, the loss of habitat suitability predicted under the a2a scenario is wider and more intense, and indeed almost complete.

Previous observations reported abundant and demographically stable populations of *B. pachypus* in Calabria, and declining populations mainly in the central and northern areas of the species range (Barbieri et al., 2004; Andreone et al., 2009). The geographic patterns of observed decline and population persistence overlap with the distribution patterns modelled under current conditions of minor and major habitat suitability, respectively. Hence, climatic factors could be hypothesized to play a role in setting the stage for the geographic pattern of *B. pachypus* population declines. Nevertheless, predictions regarding future climatic conditions show that the

current geographic distribution pattern of habitat suitability will be reverted, under both emission scenarios, by 2080.

This predicted pattern is a matter of considerable concern from a conservation standpoint, because the only *B. pachypus* populations that appear currently not to be in decline will be most threatened by climatic change. In addition, these populations have been shown to be the most genetically variable (Canestrelli et al., 2006). A recent study hypothesized that this high genetic variability may have conferred on such populations the ability to persist, despite the presence of the epidemic chytrid pathogen *Batrachochytrium dendrobatidis* (Canestrelli et al., 2013). Moreover, climate change has been hypothesized to play a role in driving the pathogenic activity of *B. dendrobatidis*, and thereby leading to *B. pachypus* population declines (Canestrelli et al., 2013). In this way, climate change and the subsequent loss of habitat suitability in the southernmost area of the species range would result in increased pathogen susceptibility and loss of adaptive potential.

Under natural conditions, a northward range shift from southernmost areas seems unlikely for *B. pachypus*, bacause of the considerable rapidity of the predicted loss of habitat suitability and the poor dispersal capability of the species. Moreover, *B. pachypus* is strongly influenced by habitat fragmentation, and this further reduces its dispersal capability (D'Amen and Bombi, 2009). In general, the only way to develop an efficient conservation strategy aimed at avoiding extinction of this endangered species would be to facilitate the northward range shift through restocking activities.

5.4 Figures

Figure 1 Habitat suitability predicted under climatic conditions averaged from 1950 to 2000 for *Bombina pachypus*. Higher habitat suitability is shown in warmer colours

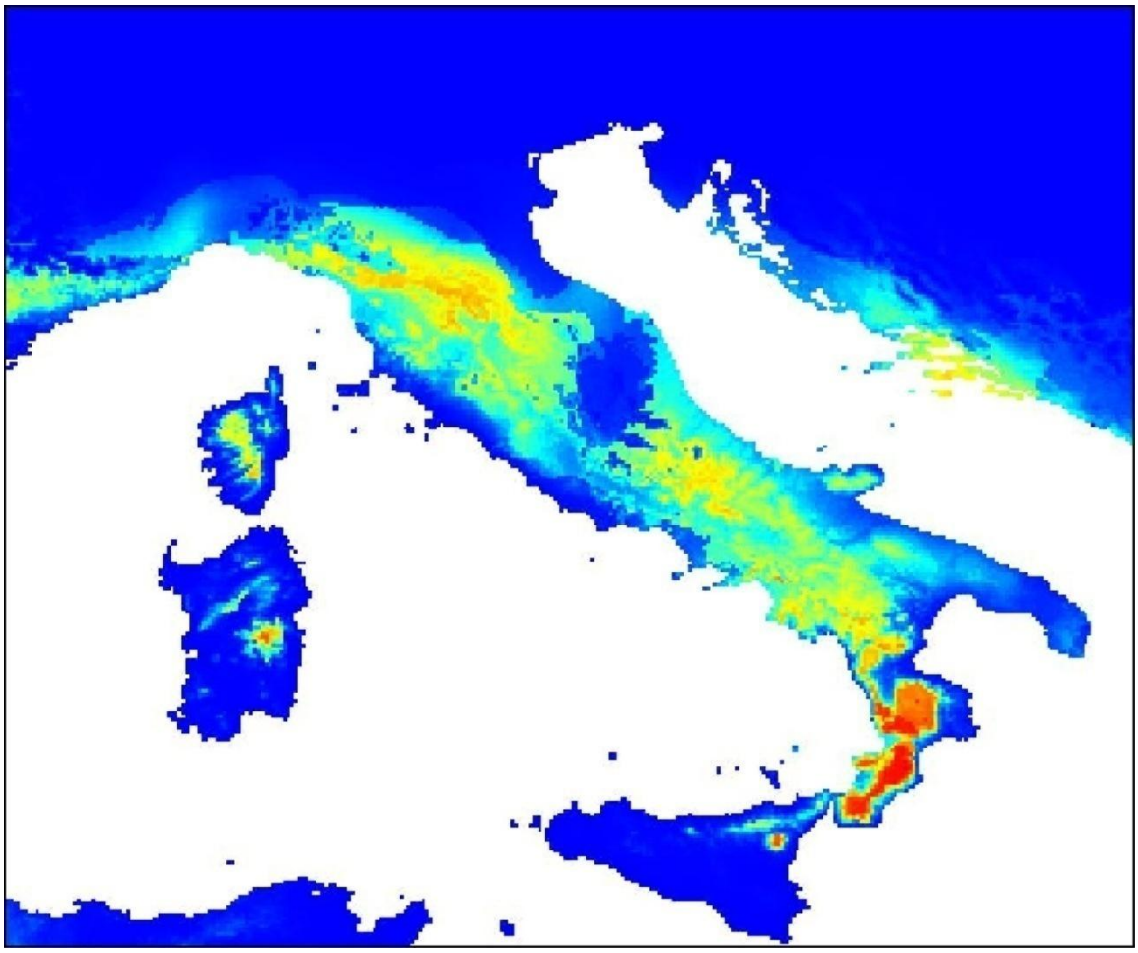
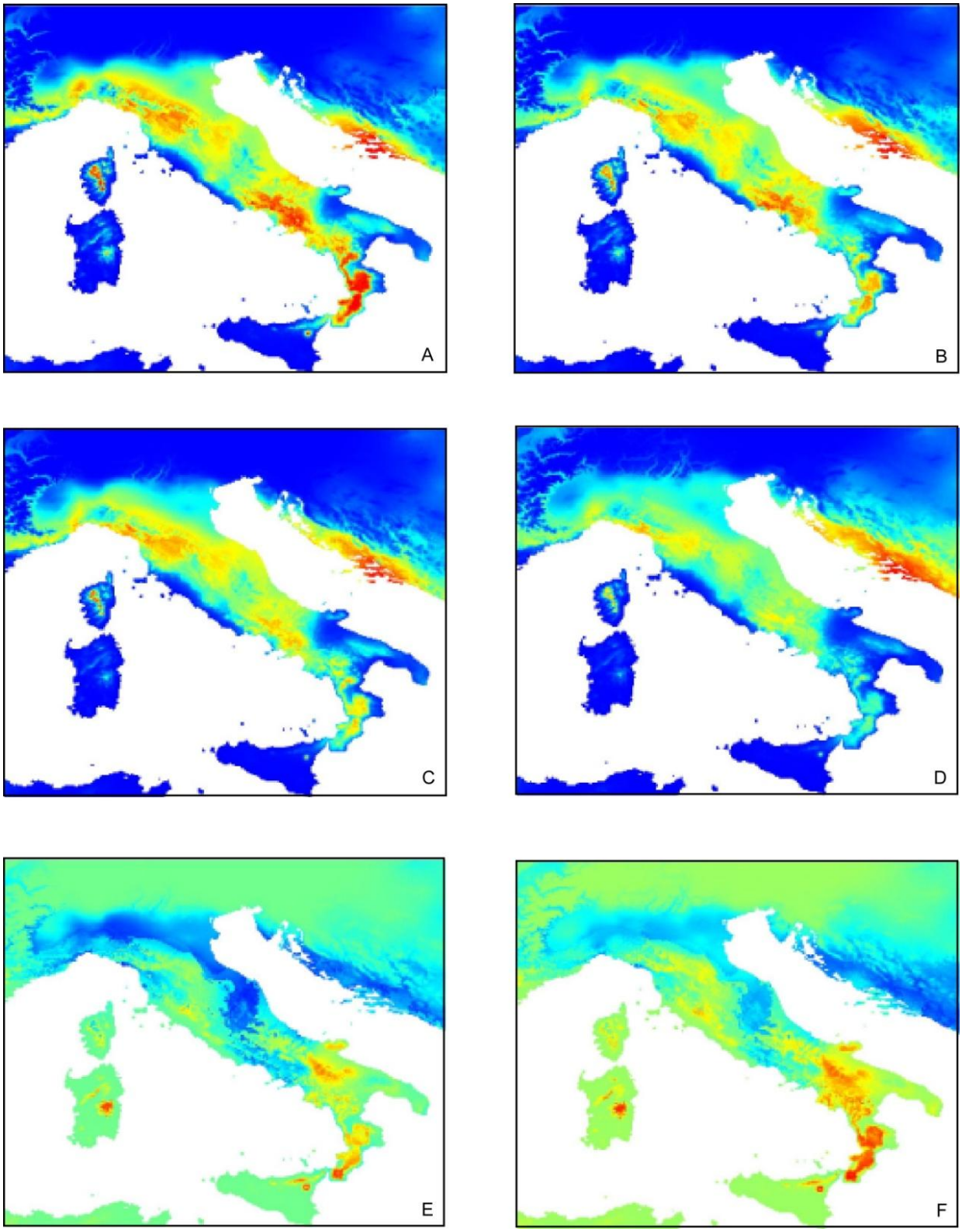


Figure 2 Predicted habitat suitability for *Bombina pachypus* under future climatic scenarios. Predictions for 2050 under b2a emission scenario averaged between IPCC3 Csiro and HADCM3 models (A); predictions for 2050 under a2a emission scenario averaged between IPCC3 Csiro and HADCM3 models (B); predictions for 2080 under b2a emission scenario averaged between IPCC3 Csiro and HADCM3 models (C); predictions for 2080 under a2a emission scenario averaged between IPCC3 Csiro and HADCM3 models (D); higher habitat suitability is shown in warmer colours (A-D). Predicted loss of habitat suitability by 2080 under b2a emission scenario (E); predicted loss of habitat suitability by 2080 under a2a emission scenario (F). Areas of higher loss of habitat suitability are shown in warmer colours (E-F).


A
B
C
D
E
F

5.5 Appendix

Data points used for Species Distribution Modelling

Site	Longitude	Latitude
1	11.949981	43.850156
2	11.1	43.933333
3	11.233333	43.783333
4	14.016667	42.15
5	12.967025	42.257736
6	13.9	41.616666
7	14.65	41.266667
8	15.203758	41.026180
9	15.329269	40.453330
10	16.073133	40.091936
11	16.147455	39.225364
12	16.6	39.366667
13	16.590522	39.021119
14	16.408647	38.727175
15	16.483830	38.820564
16	16.416667	38.5
17	15.836837	38.170219
18	14.183333	42.3
19	17.433333	40.533333
20	17.051822	40.617394
21	16.383617	38.635386
22	16.38305	38.652725
23	16.416667	38.816667
24	16.372153	38.64885
25	16.500039	39.250453
26	16.333333	38.6
27	11.963133	43.836566
28	12.895809	41.841408
29	9.6122222	44.271389
30	11.855717	43.662893
31	11.738625	43.771585
32	11.334449	43.591312
33	11.361654	43.552736
34	11.390555	43.573055
35	11.582844	43.848927
36	11.033333	43.7
37	11.633446	43.916857
38	11.7	42.666667
39	10.306445	44.046359
40	10.343906	44.167949
41	10.254854	44.018601
42	10.160538	44.052717
43	10.211870	44.083936
44	10.189722	44.054444
45	11.141389	43.918055

CHAPTER 6

DISCUSSION

Emerging infectious diseases (EIDs) affecting wildlife are considered to constitute a threat to biodiversity, by driving species decline and extinction (Daszak et al., 2000). One of the most researched EIDs is chytridiomycosis−the amphibian disease caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (reviewed in Fisher et al., 2009b; Kilpatrick et al., 2010). Since its discovery at the end of 1990s, the number of publications about chytridiomycosis and *B. dendrobatidis* has steeply increased. However, many investigations have considered the pathogen as a 'lone killer', regarding its presence or spread as sufficient reason for the decline of amphibian species. In this view, pathogen occurrence, disease, and decline are linked in a mechanistic chain, and any possible role of other factors − either as co-factors or as boundary conditions affecting host-pathogen interactions − are generally escluded.

The first evidence of *B. dendrobatidis* occurrence in Italy was found in captive dying specimens of the yellow-bellied toad *Bombina pachypus* (Stagni et al., 2004), an endemic Italian species, which has been in decline since the 1990s (Barbieri et al., 2004; Andreone et al., 2009). Therefore, in the present study, we investigated the historical presence and spread dynamics of *B. dendrobatidis* in the population declines of *B. pachypus*. *B. pachypus* has its range along the Apennine chain, and is considered as endangered by the IUCN (Andreone et al., 2009). However the decline of the species shows a geographic pattern, with declining populations observed only in the northern and central regions of its range, while southern populations appear to be demographically stable (Barbieri et al., 2004; Andreone et al., 2009). When we initiated our researh, the only known site of *B. dendrobatidis* occurrence on *B. pachypus* was in Bologna province, where evidence of chytrid-related mortalities was observed (Stagni et al., 2004). Our laboratory holds a rich collection of *B. pachypus*

samples, spanning the last 35 years, from sites distributed along the entire species range. To assess spatial and temporal occurrence of *B. dendrobatidis*, and thereby elucidate the role of the pathogen in *B. pachypus* population declines, we tested these samples for the presence of *B. dendrobatidis*, by using molecular diagnostic screening.

Our results showed the widespread occurrence of *B. dendrobatidis* along the entire range of *B. pachypus*, with all populations tested being infected. Furthermore, the positive populations included populations sampled in 1978, in northern and southern parts of the species range, i.e. more than a decade before the earliest evidences of population declines. These observations clearly contradict a pattern in which *B. dendrobatidis* acts as a 'lone killer' spreading in a wave-like manner. Our data insufficient to allow speculation regarding when *B. dendrobatidis* became established in Italy, or whether the pathogen is endemic to the country; however, they clearly indicate a time lag between the first evidence of *B. dendrobatidis* occurrence and the start of *B. pachypus* population declines. Thus, we exclude the hypothesis that *B. dendrobatidis* acts as the only factor driving *B. pachypus* population declines. Susceptibility to chytridiomycosis has previously been observed, and therefore we propose that a change in other factors may have altered the host-pathogen interaction during the last two decades.

Among the factors that may account for the increased pathogenic activity of *B. dendrobatidis*, we investigated those acting on a wide geographic scale. Factors with a local action, such as changes in land-use or environmental pollution, are managed at a local scale and may explain the decline of single populations; however, they cannot explain the extent of the observed decline. We propose climate change as the factor that most likely helps to explain the pattern of temporal change of disease dynamics on such a large scale. Rising mean temperatures, increasing intensity and duration of heat-waves, and a decreasing number of wet days have recently been reported along the Italian peninsula (Brunetti et al., 2004; Toreti and Desiato, 2008). Climate change would thus constitute a sufficiently large geographic scale of action,

with an increasingly apparent impact on living systems (Blaustein et al., 2010; Blaustein et al., 2011). Moreover, climate change has previously been suggested to affect the pathogenicity of *B. dendrobatidis*, by setting the 'thermal optimum' for the chytrid (Pounds et al., 2006) or favouring *B. dendrobatidis* in host-pathogen interactions through its fast acclimation to temperature variations (Raffel et al., 2012). Accordingly, it appears especially plausible that changes in temperature regimes and precipitation patterns, as well as extreme climatic events, may have played a role in altering disease dynamics and driving disease outbreaks during the last decades.

Our results further showed that the geographic distribution of *B. dendrobatidis* does not strictly overlap with the geographic pattern of *B. pachypus* population declines. Accordingly, at a certain time, northern and central populations began to decline following an alteration of host-pathogen interactions. By contrast, southernmost populations (i.e. those with their range in Calabria) persisted as demographically stable populations, despite being infected with *B. dendrobatidis* since at least 1978. This geographic mismatch further contradict the hypothesis that *B. dendrobatidis* acts as a 'lone killer', because it implies heterogeneous outcomes of host-pathogen interactions, according to other factors acting on a geographic scale. In this case, the geographic pattern of *B. pachypus* population declines appears to be strikingly paralleled by the range-wide pattern of population genetic diversity (Canestrelli et al., 2006). The southernmost area of the *B. pachypus* range has been shown to be the hotspot of intraspecific genetic diversity, and the glacial refugium from where re-colonization of northern areas occurred following the end of the last glaciation (Canestrelli et al., 2006). According to this pattern, northern populations have been shown to be less genetically variable, as expected for recently founded populations. Lower genetic variability is known to account for higher susceptibility to emerging pathogens, because of lower adaptive potential (Acevedo-Whitehouse et al., 2003; Spielman et al., 2004a; Hughes and Boomsma, 2004; Pearman and Garner, 2005); thus, the geographic pattern of genetic variability may comfortably

accommodate the spatial pattern of decline, suggesting higher susceptibility of northern populations.

Taken together, our results imply that *B. dendrobatidis* plays a role in the decline of *B. pachypus*; however, the pathogen is clearly not acting alone, but in conjunction with other factors that contribute to shaping the temporal and spatial pattern of declines. We hypothesize that these co-factors are climate change acting on a temporal scale, and host genetic diversity acting on a wide geographic scale.

Having assessed the role of *B. dendrobatidis* in *B. pachypus* population declines, we extended our screening to investigate *B. demdrobatidis* occurrence in another 4 species with their range along the Apennine chain, including many areas of sympatry with *B. pachypus*. Our objective was to evaluate the pathogen dynamics in different species having their range in areas of *B. dendrobatidis* occurrence, and to determine whether the chytrid pathogen constitutes a threat for these species. We included in this screening species with populations reaching a high-altitude range, because altitude is frequently associated with the severity of chytridiomycosis worldwide (Berger et al., 1998; Fellers et al., 2001; Bosch et al., 2001; Walker et al., 2010). The species selected for analysis were the Italian fire salamander (*Salamandra salamandra gigliolii*), the Italian endemic stream frog (*Rana italica*), and the Italian alpine newt (*Mesotriton alpestris apuanus*).

Our screening results showed an overall widespread occurrence of *B. dendrobatidis* among the 3 selected species, along the Apennine chain. However, the pattern of geographic occurrence depicted a lower extent of infection, with many of the species populations testing free from *B. dendrobatidis*, even when these populations were sympatric − or even syntopic − with infected populations of the other species analysed, or with highly infected and declining populations of *B. pachypus*. Moreover, the infection prevalence for each population was generally much lower than that observed for *B. pachypus*. This pattern of *B. dendrobatidis* occurrence suggests the presence of resistance mechanisms that limit the spread of infection among populations of the 4 species analysed, or tolerance mechanisms

whereby *B. dendrobatidis* occurs in higher prevalence, but in populations that appear to be demographically stable.

Our data are insufficient to allow speculation regarding the role of *B. dendrobatidis* in the decline of *S. s. gigliolii* in Central Italy, or the decline of the southernmost populations of *M. a. apuanus*; however, a role for previously suggested factors remains plausible. We observed the occurrence of *B. dendrobatidis* on these species, and the persistence of populations with low and high infection prevalence. However, major susceptibility to *B. dendrobatidis* infection among the declining populations because of other factors, such as lower genetic diversity, cannot be excluded, because detailed investigations of intraspecific genetic diversity among such populations are lacking. Nevertheless, from a conservation standpoint, the time lag observed between *B. dendrobatidis* occurrence and *B. pachypus* decline, as well as that observed in other studies (Puschendorf et al., 2006; Bosch and Martínez-Solano, 2006), implies that the occurrence of *B. dendrobatidis* on other species must be treated with concern. Even if the pathogen does not seem immediately to affect host species that are apparently resistant or tolerant, it may constitute a latent threat, with possible future implications when altered conditions set the stage for its pathogenic activity.

Climate change has been suggested to play a role as co-factor in driving *B. pachypus* population declines. Therefore, in the final step of our study, we assessed the role of bioclimatic habitat suitability in accommodating the observed geographic pattern of population declines, and the impact of predicted future climatic change on habitat suitability.

Our results showed that *B. pachypus* habitat suitability, modelled under current climatic conditions, strikingly overlaps the observed geographic pattern of population declines. The model shows higher suitability of the southernmost part of the species range (i.e. Calabria), where demographically stable populations persist, and lower habitat suitability in other areas of the species range. By contrast, the species distribution modelled under future expected climatic conditions shows a reversal of

this pattern according to the 2 emission scenarios. The predicted future distribution modelled under the b2a emission scenario (local environmental sustainability) shows an increased habitat suitability of the northern and central-southern parts of the species range compared with the current model by 2050 (especially in regions north of Calabria), but an overall loss of habitat suitability compared with current model by 2080 (mostly in Calabria). Meanwhile, the predicted future distribution modelled under the a2a emission scenario (regionally oriented economic development) is of more concern. In this case, the forecast initially shows a northward range shift, but a more dramatic loss of habitat suitability in the southernmost part of the species range by 2050. Furthermore, an overall greater loss of habitat suitability is expected by 2080, especially in Calabria, where the loss of habitat suitability appears to be almost complete.

According to the predicted distributions, climate change will likely shape the habitat suitability of *B. pachypus* along its range. Notably, the most dramatic consequences of these changes are predicted to occurr in the southernmost part of the species range, i.e. the hotspot of intraspecific genetic variability (Canestrelli et al., 2006) and the only area where populations decline has not previously been observed (Barbieri et al., 2004; Andreone et al., 2009). Under future conditions, the climate change affecting these populations is expected to be rapid. In addition, a northward range shift will be limited by the poor dispersal capability of *B. pachypus*, which will be further hampered by habitat fragmentation. Thus, there is a high probability that the only viable populations of *B. pachypus*, and also the reservoir of its genetic variability, will be lost in the near future.

The loss of this reservoir of genetic variability could have catastrophic consequences for the conservation of *B. pachypus*, because it will result in the loss of adaptive potential. Populations showing higher genetic variability, and thus higher adaptive potential, are known to be better able to cope with current and future threats or environmental changes. Meanwhile, populations with lower levels of genetic diversity have a higher probability of becoming genetically inbred, with the potential

consequences of lowered fitness and increased probability of facing higher extinction risks (Spielman et al., 2004a; Spielman et al., 2004b; Frankham, 2005). Furthermore, the higher genetic variability of the southernmost populations of *B. pachypus* has been suggested to be an important contributory factor for lower susceptibility to the pathogenic activity of *B. dendrobatidis*.

Based on our findings, we propose that the only way to avoid the likely extinction of *B. pachypus* is a restocking activity with a captive breeding program, aimed at increasing the genetic variability of northern populations. In this way, *B. pachypus* would benefit from a northward shift of habitat suitability, and also from increased adaptive potential in northern areas. Such a conservation strategy would enable the species to face future challenges.

Conclusion

The aim of the present study was to assess the role of *B. dendrobatidis* in amphibian decline in Italy. Initially, we assessed the spatial and temporal occurrence of this pathogen on the susceptible declining species, *B. pachypus*. Our results depicted a complex pattern, in which *B. dendrobatidis* appears to play a role in the decline of this species, but in conjunction with other factors, thereby resulting in temporal and spatial heterogeneous outcomes. Our findings provide a valuable contribution to the ongoing debate regarding *B. dendrobatidis* epidemiology, and substantially contradict the most accepted hypothesis, namely, that *B. dendrobatidis* acts as a 'lone killer' spreading in a wave-like manner. Nevertheless, our research raises new questions regarding the implications of such a widespread chytrid occurrence on other co-distributed amphibian species, and also the impact of the expected climate change, a co-factor that has been suggested to play a role in *B. pachypus* population declines. We subsequently determined that *B. dendrobatidis* has varying effects on different Italian amphibian species, and that the expected climate change is predicted to severely impact *B. pachypus* populations in the near future, especially in the area where they show the higher intraspecific genetic variability.

Based on our final observation, we propose the planning of a restocking program as a conservation priority, to avoid the extinction of this endangered species.

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