The Drivers of Variation in Susceptibility to the Amphibian-Killing Fungus, *Batrachochytrium dendrobatidis*

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ABSTRACT

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is regarded as a primary driver of the dramatic global declines experienced by amphibian populations in recent decades. The threat posed by *Bd* extends into pristine regions, decimating populations naïve to the pathogen. The patterns of genetic variation in amphibians in natural habitats, and the degree to which this influences traits such as response to novel pathogens, is therefore of particular relevance to conservation biology.

To examine the interplay between geographic context and population genetics, and the role that individual-level traits play in shaping critical components of host viability in the face of exposure to *Bd,* I focused on a set of mainland and isolated island common toad (*Bufo bufo*) populations situated on the edge of their range in northwest Scotland.

Microsatellites were used to characterise patterns of genetic variability across eleven populations. It was found that seawater serves to isolate demes, producing high levels of population differentiation, and that loss of genetic diversity parallels degree of isolation. Standardised laboratory experiments showed that *Bd*-induced mortality in amphibians with no prior history of exposure to the pathogen was predicted by population of origin and individual mass, but not by population-level allelic diversity or heterozygosity at microsatellite loci. Restriction site-associated DNA sequencing (RADseq) was then used to genotype individuals exposed to *Bd* at over 10,000 single nucleotide polymorphism (SNP) loci. This revealed clear genetic structure not discernible through microsatellite analysis. Outlier loci putatively related to local adaptation were identified, but it was not possible to characterise their function. No loci with a clear association to disease response were identified.

These findings underscore the importance of habitat in forming discrete populations, and how this can shape variation in fundamental phenotypic traits, including disease response. These responses are, however, rather idiosyncratic, and not predicted by factors such as level of population isolation or neutral genetic diversity. SNP datasets generated through RADseq hold great potential in further unravelling the dynamics of the amphibian-*Bd* system, but these are likely to remain complex and context-dependent.

CHAPTER 1 INTRODUCTION:
GLOBAL AMPHIBIAN DECLINES AND DRIVERS OF A DESTRUCTIVE DISEASE

1.1 THE BIODIVERSITY CRISIS OF THE ANTHROPOCENE: A SIXTH MASS EXTINCTION?

The history of life on planet Earth has been punctuated by a number of pulses of rapid and severe reductions in biodiversity. Evidence from the fossil record suggests that, over the past 540 million years, the biota of Earth have experienced at least five mass extinctions, episodes during which the number of species has reduced by 75% or more in a period of 2 million years or less (Barnosky et al., 2011; Raup & Sepkoski, 1982). These declines were driven by drastic changes in the planet's environment and climate (Barnosky et al., 2011), the possible causes for which vary depending on the event in question and include changes in the eccentricity of the Earth's orbit (Sutcliffe et al., 2000), asteroid impact (Schulte et al., 2010), volcanism (Hesselbo et al., 2007), and the release of large amounts of methane gas from methane hydrates (Berner, 2002). There is growing consensus that a sixth mass extinction is probably underway, one largely attributable to human impact (Barnosky et al., 2011; Ceballos et al., 2015; Dirzo et al., 2014; McCallum, 2015).

The extinctions in the late 17th and early 18th centuries of the dodo (*Raphus cucullatus*) and its close relative the Rodrigues solitaire (*Pezophaps solitaria*) contributed to an acknowledgement that a species could be extinguished forever, and that these disappearances could be driven at least in part by the activities of humans (Lyell, 1853). Since then, the accounts of extinctions have multiplied, as have the extinctions themselves. Within vertebrates, of a highly conservative estimate of 338 extinctions since 1500, 198 (59%) have occurred since 1900 (Ceballos et al., 2015). The true scale of species loss is likely to be far higher, with estimates varying between 50 and 36,000 extinctions per year (Costello et al., 2013; Mora et al., 2013). Assessing the degree to which this constitutes an exceptional loss of biodiversity requires careful evaluation of the fossil record to produce a calculation of what would be considered a normal "background rate" of extinction, as well as a focus on a well understood taxonomic group to form a reasonable estimate of modern extinction rates. Barnosky et al. (2011) used the fossil record of mammals to estimate an average background extinction rate of approximately 1.8 extinctions per million species-years (E/MSY, which can be thought of as the number of extinctions per 10,000 species per 100 years). In calculating what they described as an underestimate of the true scale of the current extinction crisis (i.e. one in which a high estimate of the background rate from the

fossil record was compared with a low estimate of current extinction rates), Ceballos et al. (2015) rounded this rate up to 2 E/MSY as a high reference point for vertebrates as a whole, and then compared it to modern extinction rates using an optimistic interpretation of IUCN Red List data. They argued that, even under these conservative assumptions, the loss of vertebrate species over the last century is up to 100 times higher than the background rate. In an understandable emphasis on species extinction, however, there is a risk of neglecting a critical component of biodiversity loss: that of declines in both the sizes and numbers of populations. Evaluating the situation at this level paints a yet more grimly severe picture. There has been an estimated overall decline of 60% in vertebrate population sizes between 1970 and 2014 (WWF, 2018). Approximately a third of vertebrate species are experiencing decreases in population size and range (Ceballos et al., 2017). Invertebrate animals are less well understood, but show similarly stark declines, with 67% of monitored populations showing a mean decline in abundance of 45% (Dirzo et al., 2014). This points to an even deeper crisis of biodiversity loss than a superficial reading of species extinctions would convey, a crisis that is likely to have profound negative effects on ecosystem functions and services, and thus, ultimately, on human well-being (Ceballos et al., 2017; Dirzo et al., 2014; Hooper et al., 2012a).

Arresting these declines represents a major challenge. Their drivers—land use change, over-exploitation, nitrogen deposition, atmospheric CO_2 concentration climate change, and the spread of exotic species (including pathogens)—arise from human activity (Sala et al., 2000) and conservation efforts, despite making an important contribution, remain insufficient to compensate for their impacts (Hoffmann et al., 2010).

1.2 AMPHIBIANS: THE MOST THREATENED VERTEBRATE GROUP

Amongst vertebrates, the loss of species incurred by amphibians has been particularly dramatic. 41% of amphibian species for which there is sufficient data to make an assessment are classed by the IUCN as threatened with extinction, as compared with 25% of mammalian species and 13% of avian species (IUCN, 2018). There is worrying evidence that the threat faced by this group is worsening: while 34 species of amphibians are estimated to have gone extinct since 1500 (as compared with 129 birds and 74 mammals), nine of these losses have occurred since 1980 (Stuart et al., 2004). More alarmingly, the number amphibian of species no longer found but not yet formally categorised as extinct (as this requires exhaustive surveys) number 126 (IUCN, 2018). The trends for those remaining look bleak. At least 43%

of assessed species are undergoing some form of population decrease, though this number is likely to be an underestimation as the trend us unknown for 29% of assessed species and 22% of described species are too poorly understood to assess (Stuart et al., 2004). The prospect, then, is for the near future to be as damaging to amphibian diversity as the recent past has been, an exceptionally destructive period itself: Ceballos et al. (2015) calculated that the species loss incurred by amphibians since 1900 would have taken up to 10,000 years to occur had normal historical background rates prevailed.

It first became apparent that amphibians were undergoing a major global crisis at the inaugural World Congress of Herpetology in 1989, at which researchers shared experiences of abrupt and unexplained population declines and extinctions from multiple disparate locations (Barinaga, 1990). Similar reports multiplied in the following years, culminating in the 2004 publication of the IUCN Global Amphibian Assessment, which provided a comprehensive view of the declines and threats faced by amphibian taxa worldwide (Stuart et al., 2004). While there have been incremental updates to the IUCN amphibian database since, there has not been a follow-up major assessment to the group (IUCN, 2018).

Declines have been rapid and severe, with specific regions losing significant proportions of their amphibian species (for example, a 40% loss in a montane region of Costa Rica, Pounds et al. 1997) and populations (for example, a >90% loss of historical populations of mountain yellow-legged frogs in California in under a decade, Vredenburg et al. 2007). The reductions have a global spread, occurring in all biogeographic regions inhabited by amphibians, and exceed what would be expected from natural population fluctuations (Ceballos et al., 2015; Houlahan et al., 2000; Pounds et al., 1997; Wake & Vredenburg, 2008). While over-exploitation and habitat loss have played a major role in many of these losses, almost half (47%) of the species defined as “rapidly declining” are not exposed to these pressures, with many so called “enigmatic declines” and extinctions occurring in protected and apparently pristine areas (Hero et al., 2004; Laurance et al., 1996; Pounds et al., 1997; Stuart et al., 2004). It became apparent by the late 1990s that the principal culprit for enigmatic amphibian declines was a previously-undescribed emerging infectious disease, chytridiomycosis.

1.3 CHYTRIDIOMYCOSIS: A WIDELY-DESTRUCTIVE DISEASE CAUSED BY A FUNGAL PATHOGEN, BATRACHOCHYTRIUM DENDROBATIDIS

Chytridiomycosis, an emerging infectious disease caused by the chytrid fungus *Batrachochrytrium dendrobatidis* (hereafter *Bd*), is now recognised as a primary driver of

amphibian extinction and declines worldwide, particularly in less disturbed habitats (Fisher et al. 2009; Skerratt et al. 2007; Becker & Zamudio 2011). *Bd* is an aquatic member of the phylum Chytridiomycota, a widespread and diverse group of fungi normally found as primary degraders and saprobes in soil and water (Berger et al., 1998; Longcore et al., 1999). Together with the recently-described *Batrachochytrium salamandrivorans*, now known to be responsible for declines of salamanders in Europe, it forms a genus unique amongst the Chytridiomycota for its pathogenicity to a vertebrate host (Berger et al., 1998; Martel et al., 2013). The genus diverged from the remainder of the Chytridiomycota an estimated 67 million years ago, and infection by both species is restricted to the amphibian epidermis in post-metamorphic individuals and keratinised mouthparts in larvae (Berger et al., 1998; Farrer et al., 2017; Martel et al., 2013). Pathogenicity appears to be associated with significant expansions of protease and cell wall gene families when compared with close non-pathogenic relatives, but the repertoire of putative virulence factors associated with these taxa remains to be characterised (Farrer et al., 2017).

In post-metamorphic individuals, *Bd* infection is restricted to the keratinised regions of the amphibian epidermis, which the fungus encounters during its motile waterborne stage as a flagellate zoospore (Berger et al., 2005a; Longcore et al., 1999). Once a zoospore encysts within an epidermal cell, the fungus enters the stationary stage of its lifecycle, the thallus, which begins to produce new zoospores and develops a reproductive structure, the zoosporangium. The timing of the development of the zoosporangium is matched to the maturation of the host skin cell such that recently-parasitised cells with immature zoosporangia are found a number of layers deep and take the developing zoosporangium with them as they progress towards the surface and keratinise. Once on the skin surface, the zoosporangia disperse zoospores through discharge tubes which form an opening in the cell membrane. The free-swimming zoospores can then either re-infect the same individual or encounter a new host (Berger et al., 2005a; Longcore et al., 1999).

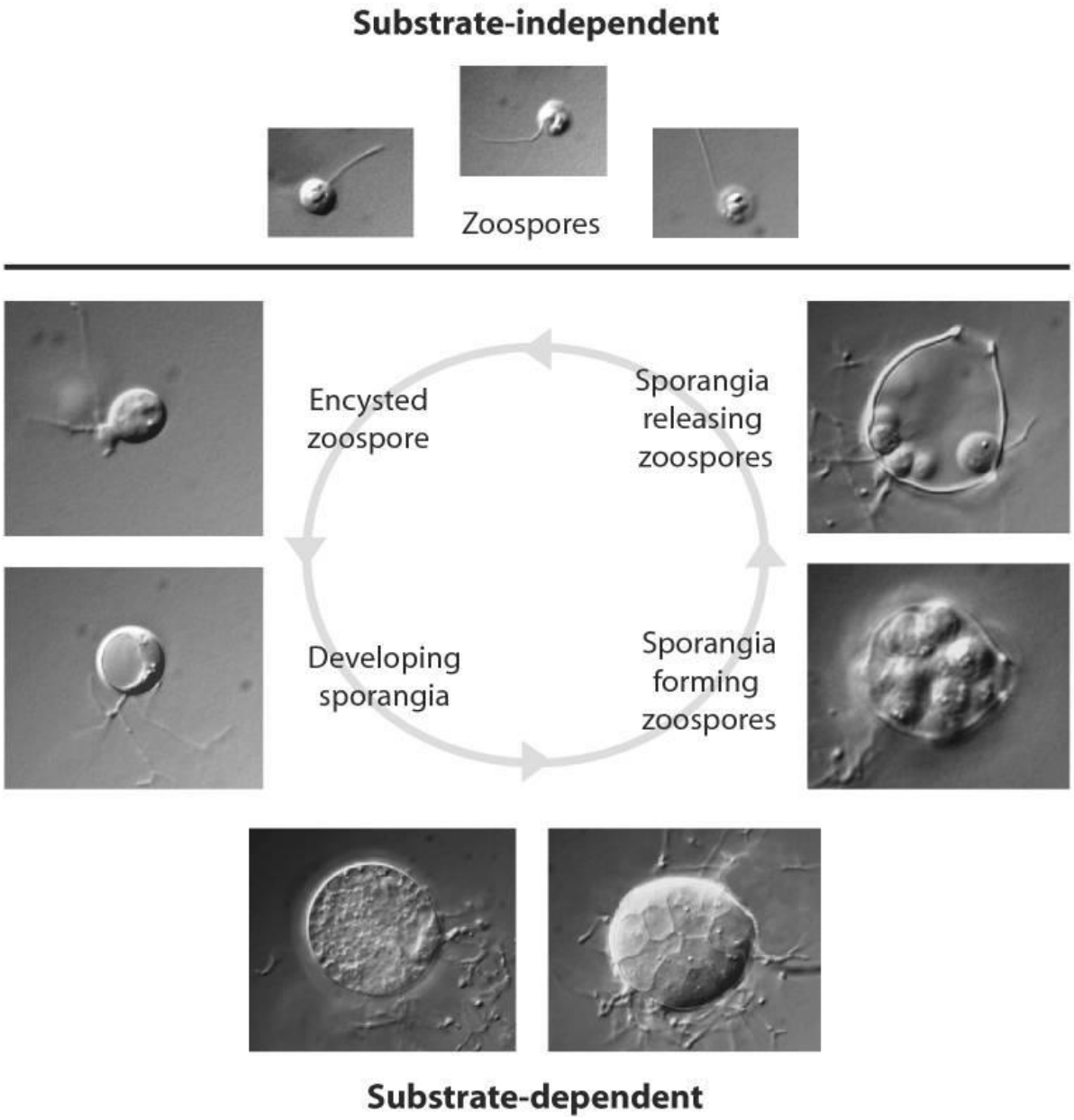

Figure 1.1 Life cycle of the fungal amphibian pathogen, *Batrachochytrium dendrobatidis.* In the flagellate zoospore stage, the fungus can move independently in water, transitioning to its stationary reproductive stage upon encounter of the keratinised regions of amphibian tissues. Figure reproduced from Rosenblum et al., (2008).

The exact mechanism by which *Bd* causes disease and mortality is still not fully understood. Clinical signs, if present, vary and can include lethargy, inappetance, decreased respiration rate, cutaneous erythema, irregular skin sloughing, abnormal posture, hyperpigmentation, and the loss of righting and diving reflexes (Parker et al., 2002; Voyles et al., 2007, 2009). The site of infection, the skin, is a critical component of the amphibian body, playing a crucial role in maintaining homeostasis through respiration and osmoregulation. By physically disrupting the structure of the epidermis, *Bd* can compromise osmoregulation, leading to

electrolyte imbalance, and eventual cardiac arrest and death (Campbell et al., 2012; Voyles et al., 2007, 2009). Glucocorticoid hormones play a role both in ion homeostasis and regulation of appetite and skin shedding, and elevated levels of corticosterone has been found to be associated with chytridiomycosis, and may be the link between its disparate symptoms (Peterson et al., 2013). Finally, *Bd* infection may simply impose a high energetic burden on its host: In addition to displaying increased skin sloughing and decreased appetite, *Bd*-infected individuals suffer an increased risk of ion loss and higher mass-specific metabolic rate (Wu et al., 2018).

Since its description in 1999 (Longcore et al., 1999), *Bd* has been detected on all continents apart from Antarctica, and infects a diverse, large, and growing assemblage of amphibian species - at least 520 of 1252 surveyed (42%, http://www.bd-maps.net/ [accessed 06/03/2019]). The fungus has been implicated in mass mortality events, population declines, and extinctions globally (Berger et al., 1998; Bosch & Martínez-Solano, 2006; Cheng et al., 2011; Fisher et al., 2009b; Rachowicz et al., 2006; Skerratt et al., 2007). However, not all species and populations are equally susceptible. While *Bd*-driven catastrophic amphibian declines have occurred in areas such as Australia and Central America (Berger et al., 1998; Crawford et al., 2010), amphibian communities in Brazil and Southeast Asia, for example, have persisted despite the presence of *Bd* for a century or more (Bataille et al., 2013; Fong et al., 2015; Rodriguez et al., 2014). There is also heterogeneity amongst sympatric species. In the Sierra Nevada in California, for example, the Pacific chorus frog has persisted, apparently unaffected by outbreaks of chytridiomycosis that have driven precipitous declines in two species of mountain yellow-legged frogs, and tolerates levels of *Bd* lethal to sympatric species in laboratory tests (Reeder et al., 2012). Similarly tolerant species include European water frogs in Switzerland (Woodhams et al., 2012), and *Litoria wilcoxii* in Australia (Retallick et al., 2004). There is also variation in susceptibility between different populations of the same species; in the lowland leopard frog, population-level variation in susceptibility in the wild has been replicated in laboratory infection experiments and linked to MHC genotype (Savage & Zamudio, 2011). Variation in response to exposure was also observed in laboratory infection experiments using European treefrogs derived from previously-unexposed populations with varying levels of genetic polymorphism (Luquet et al., 2012).

1.4 A VIRULENT GLOBAL PANZOOTIC LINEAGE OF BD EMERGING FROM POSSIBLE ASIAN ANCESTRY OF THE GROUP

Despite almost two decades of research, the origins and patterns of spread of this disease remain somewhat obscure. Initially, two leading hypotheses were put forward to explain how chytridiomycosis had emerged to have such a profound effect on such a diverse range of taxa and habitats in a comparatively short period of time. The novel pathogen hypothesis (NPH) proposed that *Bd* was a recent introduction to most of its geographic range, encountering naïve populations as it spread. Under this hypothesis, its global distribution was likely mediated by human activities such as the global trade in amphibians and other aquatic organisms (Rachowicz et al., 2005). Alternatively, the endemic pathogen hypothesis (EPH) proposed that *Bd* was endemic to affected habitats and populations, and that recent disruption had occurred in parasite-host dynamics so as to alter the pathogenicity of the fungus, thus leading to the recent emergence of chytridiomycosis. Such a disruption could be provoked by changing environmental conditions in an area, leading to immunosuppression in the host (for example as a consequence of the introduction of sub-lethal stressors), or more favourable conditions for pathogenesis in the parasite (for example through changed temperatures or eutrophication of water bodies, Rachowicz et al. 2005). As the epidemiology and evolutionary history of *Bd* continue to be characterised, however, framing the question in terms of these two competing hypotheses increasingly appears to be an overly simplistic dichotomy.

Bd has been well documented as a rapidly-spreading novel pathogen in parts of Central and South America (Cheng et al., 2011; Lips et al., 2008), California (Vredenburg et al., 2010), the Iberian Peninsula (Walker et al., 2010), and Australia (Laurance et al., 1996). Worldwide declines and epizootics have been linked to a single lineage of *Bd*, known as the Global Panzootic Lineage (*Bd*GPL), shown to have a widespread global distribution and increased virulence as compared with other strains (Farrer et al., 2011; O'Hanlon et al., 2018). However, retrospective analyses of museum amphibian specimens have confirmed that *Bd* has had a broad geographic distribution long before the supposed emergence of chytridiomycosis as a novel panzootic disease. Positive samples have been reported from 1938 in South Africa (Weldon et al., 2004b), 1933 in Cameroon (Soto-Azat et al., 2010), 1911 in North Korea (Fong et al., 2015), 1902 in Japan (unpublished, cited in Goka et al. 2009), 1894 in Brazil (Rodriguez et al., 2014), and 1863 in Bolivia (Burrowes & De la Riva, 2017), areas where *Bd* has not been conclusively linked to mass mortality events and declines (but

see Carvalho, Becker and Toledo (2017), who link *Bd* prevalence in Brazilian museum tadpole samples to historical declines). The geographic origins of the pathogen have thus been the subject of much speculation, with Africa (Weldon et al., 2004b), North America (James et al., 2009; Talley et al., 2015), South America (Rodriguez et al., 2014), and East Asia (Bataille et al., 2013; Goka et al., 2009) all put forward as possible original sources.

In resolving the systematics and origins of the group, O'Hanlon et al. (2018) analysed the genomes, sequenced to a high depth, of a globally representative set of 234 isolates of *Bd* covering all previously proposed lineages and encompassing infections of all three extant orders of amphibians. In addition to *Bd*GPL, the major divergent lineages identified by phylogenetic analysis were *Bd*CAPE (associated with Africa), *Bd*ASIA-1 (associated with the Korean peninsula, which includes *Bd*CH, previously regarded as a lineage enzootic to Switzerland), and *Bd*ASIA-2/*Bd*BRAZIL (enzootic to the Brazilian Atlantic forest and also isolated from invasive North American bullfrogs in Korea). This analysis revealed *Bd*ASIA-1 to be a hyperdiverse group, one which shares more diversity with the global population of *Bd* than any other lineage. This is a compelling argument for East Asia as the geographic origin of the *Bd* panzootic. The divergence and emergence of *Bd*GPL was dated to the start of the 20th century, consistent with the expansion in global trade in amphibians. Hybrid genomes were also identified, providing evidence of recent genetic exchange between lineages, further underscoring the continued risk of movement of this pathogen and potential generation of novel genotypes. Indeed, a hybrid between the putatively hypovirulent *Bd*BRAZIL and the hypervirulent *Bd*GPL has been shown to cause higher mortality than either parent lineage in infection experiments on a native Brazilian frog species *Brachycephalus ephippium* (Greenspan et al., 2018).

Ultimately, it appears that, out of ancient associations between amphibians and a diverse endemic fungal pathogen in East Asia, and through largely unrestricted exploitation and transcontinental trade, a hypervirulent pathogen has emerged to infect novel hosts globally and cause catastrophic declines in global amphibian diversity.

1.5 PATTERNS OF BD DISPERSAL THROUGH THE ENVIRONMENT

In reconstructing the arrival and spread of *Bd* in South and Central America, Lips et al. (2008) proposed multiple (likely human-caused) introductions of the fungus as a novel pathogen, and documented a subsequent wave-like spread of population declines through affected areas. They proposed that *Bd* moved in this manner across northern South America within

approximately 20 years. The rate of spread was rapid, moving between 25 and 282 km per year through Central America and along the Andean cordilleras. A similar wave-like pattern of spread was reported in populations of mountain yellow-legged frogs (*Rana mucosa*) in the United States, in this instance moving approximately 700m per year (Vredenburg et al., 2010).

The exact means of dispersal of the pathogen remain unclear, and are likely to vary depending on scale. Direct physical contact between infected and susceptible individuals is thought to play a role in transmission (Briggs et al., 2005; Piotrowski et al., 2004), though there is an absence of experimental work demonstrating this. Indirect evidence was provided by Rowley & Alford (2007), who observed that Australian frog species with higher rates of conspecific physical contact were found to be more susceptible to chytridiomycosis-driven declines. The motile zoospore stage of *Bd* is capable of independent aquatic movement of up to 2 cm (Piotrowski et al., 2004), and transmission via infected water is well documented (Berger et al., 1998; Mitchell et al., 2008; Rachowicz & Vredenburg, 2004). In a study on multiple sites in North America, Mosher, Huyvaert and Bailey (2018) suggest that *Bd* can persist in the environment for up to five years after disease-driven declines, even in the absence of other amphibian populations. Disease surveys in Queensland, Australia, have demonstrated that, as a consequence of the aquatic nature of the fungus, frog species associated with permanent waterbodies were significantly more likely to be infected than more terrestrial species and those associated with ephemeral waterbodies (Kriger & Hero, 2007). Similar findings have been reported from Brazil, where *Bd* prevalence was positively associated with perennial waterbodies and with rainfall (Ruggeri et al., 2018). This does not preclude the existence of *Bd* in other habitats, however: infected amphibians have been detected in a variety of terrestrial contexts, from fossorial species (Doherty-Bone et al., 2013; McCracken et al., 2009) to canopy-dwellers (McCracken et al., 2009). *Bd* remains detectable on foliage that infected animals have been in contact with (Kolby et al., 2015b), can survive for up to three months in sterile moist river sand (Johnson & Speare, 2005), and remain viable in water for up to seven weeks (Johnson & Speare, 2003). Thus, movement borne by water currents through riparian corridors may contribute to a regional-scale rate-of-spread of as much as 26km per year (Lips et al., 2006). qPCR has been used to detect the presence of *Bd* in rainwater in Honduras, raising the possibility of aerial dispersal, though its viability in this context is unknown (Kolby et al., 2015a).

The widespread and long-term persistence of *Bd* has prompted a search for possible non-

amphibian hosts. The nematode *Caenorhabditis elegans* can suffer mortality as a result of exposure (Shapard et al., 2012), as can crayfish, which were found to harbour infection for at least 12 weeks in laboratory experiments (McMahon et al., 2013). The same study found that crayfish presence in wetlands was associated with *Bd* infections in resident amphibians. The degree to which these findings are generalisable is in some doubt, however, as a subsequent investigation found no evidence of infection or deleterious effects of exposure to *Bd* in either nematodes or crayfish despite using more isolates and greater inoculum loads of the pathogen (Betancourt-Román et al., 2016). Liew et al. (2017) reported dose-dependent infection and associated pathology and mortality as a consequence of *Bd* exposure in zebrafish, demonstrating clearly for the first time the pathogen's ability to infect a non-amphibian vertebrate host. Another vertebrate-facilitated means of *Bd* dispersal may be via the keratin-rich toe-tissue of waterfowl. Garmyn et al. (2012) screened the toes of wild geese in Belgium and found that 15% harboured *Bd* DNA. Further, they showed *in vitro* that *Bd* is attracted to, and can proliferate on, goose toe tissue. Screening of museum specimens of aquatic birds from Bolivia detected the presence of *Bd* in samples collected as early as 1982 and congruent with periods of severe amphibian declines in the area (Burrowes & De la Riva, 2017). *Bd* can also attach to and grow on sterile feathers (Johnson & Speare, 2005). Dispersal of *Bd* across distances and landscapes beyond what would be possible through amphibian hosts and river courses alone could thus be facilitated by birds. However, the true scale of the influence of birds, and the other potential non-amphibian hosts mentioned above, in the spread and persistence of *Bd* in the environment remains unclear.

Rapid intercontinental spread of *Bd* is expected to be human-facilitated through the global trade in amphibians. The widely-farmed and traded North American bullfrog (*Rana catesbeiana*) and African clawed frog (*Xenopus laevis*) can be asymptomatic carriers of the pathogen (Schloegel et al., 2012; Weldon et al., 2004b). Genotyping of *Bd* isolates found within the *R*ana *catesbeiana* trade and invasive populations detected the human-influenced presence and movement of *Bd*GPL and *Bd*Brazil in Japan and Brazil, as well as a putative hybrid between these lineages in Brazil (Schloegel et al., 2012). Investigation of museum records has shown an association between invasions of *R*ana *catesbeiana* and the spread of *Bd* in western United States (Yap et al., 2018). Sampling of amphibians imported into the United Kingdom for the pet trade has revealed the presence of *Bd* on six genera in consignments originating from the USA and Tanzania (Wombwell et al., 2016). These

examples illustrate how the intercontinental movement of animals in trade also constitutes movement in their resident pathogens, which, if sufficiently broad in the range of hosts they can infect, can pose significant threats to wildlife.

1.6 VARIABLE PATTERNS IN HOST-BD DYNAMICS AND THEIR DRIVERS

The circumstances in which *Bd* is found are highly diverse, and its impact varies. Negative consequences, such as disease-driven mortality and extinction, are context dependent, and resolving the factors that mediate susceptibility remains challenging. In this section, the main sources and drivers of variation in host-pathogen dynamics in this system are described. Though these are sub-divided into discrete sections, they are non-mutually exclusive and interact to produce patterns of disease in natural contexts.

1.6.1 HOST SPECIES-LEVEL VARIATION

Bd is remarkable for its global reach and the breadth of species it can infect: it is present on all continents that amphibians inhabit, and has been detected on almost 700 species across 52 families (Olson & Ronnenberg, 2014). Chytridiomycosis-driven mass mortalities, population declines, and extinctions have been well-documented in species around the world: in North America (Rachowicz et al., 2006), Central America (Lips et al., 2006, 2008; Ryan et al., 2008), South America (Lips et al., 2008), Caribbean islands (Hudson et al., 2016), continental Europe (Bosch et al., 2001), and Australia (Berger et al., 1998). However, it is not universally destructive, and some species and assemblages appear to be able to persist despite exposure. American bullfrogs (*Rana catesbiana*) and African clawed frogs (*Xenopus laevis*), globally invasive species introduced via the amphibian trade, appear to tolerate infection with minimal clinical signs and may therefore act as vectors (Hanselmann et al., 2004; Weldon et al., 2004a). Extensive long-term survey data covering seven years and 36 species in the southeastern United States showed widespread occurrence of *Bd* but limited evidence of *Bd*-driven declines. In amphibian assemblages in Brazil and in East Asia, the possible ancestral origin of *Bd* (O'Hanlon et al., 2018), there is evidence of long-term coexistence with the pathogen (Bataille et al., 2013; Rodriguez et al., 2014). While some of this variation can be attributable to environmental factors or the strain of *Bd* involved, there is clear evidence for disease outcomes being mediated by species identity. Wilcox's frog (*Litoria wilcoxii*) in Queensland, Australia and the Pacific chorus frog (*Pseudacris regilla*) in California, United States, both show persistence despite manifesting high *Bd* infection prevalence in communities in which sympatric species have suffered major declines (Reeder

et al., 2012; Retallick et al., 2004). Variation in the susceptibility of species has been further demonstrated in laboratory experiments in which multiple species have been exposed to *Bd* under standardised conditions and shown differential infection probability, sub-lethal effects, and survival patterns (Bielby et al., 2015; Gahl et al., 2012; Searle et al., 2011). Gervasi et al. (2017) undertook a major study experimentally infecting under standardised conditions 20 North American amphibian species across three major families: the Ranidae ("true frogs"), the Hylidae ("tree frogs"), and the Bufonidae ("true toads"). They sought to examine the roles played by phylogenetic history, habitat use and life history traits in shaping species-level variation in response to *Bd* exposure. They found that, on the family level, true frogs, the Ranidae, showed the lowest infection intensities, with a majority of species testing negative. This was attributed to the diverse set of antimicrobial peptides produced on the skin of ranids which have been shown to be inhibitory to *Bd* growth in culture (Rollins-Smith et al., 2002). There is also evidence that ranids initiate a more robust immune response to *Bd* than do the Hylidae (Gervasi et al., 2014). The group most susceptible to *Bd*-driven mortality was the Bufonidae. It is unclear what mechanisms drive this difference, but the authors speculate that, given the rapid rates of mortality observed in their study, bufonid species may be less tolerant of proteolytic zoospore metabolites associated with early stages of infection. They also postulate that the different chemical, mechanical, and structural characteristics of the skin of bufonids may better facilitate growth of *Bd*.

The habitat with which a species was associated was also found to be predictive of infection burden. Species that occupied ephemeral habitats were found to have heavier infections when compared with those that inhabit permanent water bodies. This is perhaps surprising given *Bd*'s requirement for water and moisture for transmission, and in the light of field studies that positively associate probability of infection with species that inhabit permanent water (Kriger & Hero, 2007; Ruggeri et al., 2018). The authors speculate that this potentially unexpected relationship may be due to the more stable presence of *Bd* in permanent water bodies imposing a higher selective pressure for *Bd* resistance on species that live there, but in the absence of elaboration on the history of *Bd* presence in the populations from which their samples were drawn, it is difficult to evaluate this contention. It is worth noting that these data arise from laboratory infection experiments, and so natural ecological context and pathways of transmission are absent. A result that contrasts with field observations is therefore not necessarily unexpected, and these findings may represent variation in the

innate susceptibility of species that is unrealised in their natural habitat.

Life history traits were also found to be important in influencing responses to exposure. Species with shorter lifespans, and that mature at a younger age, had lower survival. This could be as a result of a tendency for longer-lived species to invest more in adaptive immune responses, as has been observed in, for example, wild rodents (Previtali et al., 2012).

Finally, while within-species comparisons corroborate the frequently-reported observation that larger individuals show greater survival and lower infection burdens (Garner et al., 2009; Garner, Rowcliffe and Fisher, 2011; Bielby et al., 2015, and see 1.6.7 and Chapter 2) , between-species comparisons yielded the opposite relationship. This is consistent, though, with modelling of *Bd* prevalence data from the continental United States and Australia (Bancroft et al., 2011; Murray & Skerratt, 2012), which found a positive association between species body size and probability of *Bd* detection. The latter finding could be explained by a tendency for species that mature at a larger size to have greater longevity, and thus have a longer period of time to encounter and accumulate parasites. It is less clear how this relationship arose in the infection experiments of Gervasi et al. (2017). It may simply be a consequence of the fact that larger animals have more keratinised epidermis to infect, but is not obvious why this would not also apply on an intraspecific level. The authors propose that the negative correlation between body mass and infection burden within a species may be as a result of infection inhibiting growth, but given that in this and other studies mass is measured prior to exposure to *Bd,* this suggestion is rather puzzling.

1.6.2 HOST POPULATION-LEVEL VARIATION

Variation in response to exposure to *Bd* occurs not only between communities and individual species, but also on an intraspecific level; with some populations experiencing declines and extirpations while other populations of the same species persist. Amongst populations of the Mallorcan midwife toad (*Alytes muletensis*), for example, four have been recorded as infected with *Bd* after a hypovirulent lineage of the pathogen, *Bd*CAPE, was introduced to the island, likely in the 1990s (Doddington et al., 2013; Walker et al., 2008). Two of these populations have remained stable, but the remaining two, despite both consistently showing infection rates approaching 100%, have experienced remarkably divergent trajectories. One population has declined almost to extirpation since the early 1990s, whereas the other, established in 1995, has increased in size considerably. Using mathematical models and field data, Doddington et al. (2013) attribute this divergent response to local differences in

temperature, and not epidemiological dynamics or variation in host or pathogen genotype. *Bd* was first definitively detected in the Sierra Nevada of California in 1998 (Fellers et al., 2001). Its invasion led to rapid declines and extirpations of resident frog populations (*Rana muscosa*, *R. sierrae*). In some areas, however, populations persisted, albeit at low densities after declines (Briggs et al., 2010, 2005; Lam et al., 2010). Persistence in this case may in part be due to differences in microbiome communities: populations that have been able to coexist with *Bd* have higher proportions of symbiotic skin bacteria that are inhibitory to *Bd* growth *in vitro* (Lam et al., 2010). Briggs, Knapp and Vredenburg (2010), however, argue that different disease outcomes in this system may be driven entirely by density-dependent host-pathogen dynamics, in which infection reaches higher intensities in dense frog populations. Thus, they suggest that intraspecific variation in the response of populations to *Bd* exposure can be explained without the need to invoke differences in environmental variables, host susceptibility, or pathogen phenotype.

Host population-level variation in response to the same strain of *Bd* has, however, been demonstrated on multiple occasions in controlled experiments on animals raised from eggs in standardised laboratory conditions, a phenomenon not readily explicable by abiotic factors or density-dependent host pathogen dynamics. Bradley et al. (2015) investigated intraspecific variation in sensitivity of wood frogs (*Lithobates sylvaticus*) to *Bd* through exposure experiments on animals originating from 10 different populations and raised from eggs to metamorphosis in standardised conditions. They identified a significant association between population of origin and mortality rates, and speculated that this is driven by intrinsic genetic differences between populations, though they did not investigate potential mechanisms. In their infection experiments on population-level variation in disease outcomes in Australian alpine tree frogs (*Litoria verreauxii alpina*), Bataille et al. (2015) investigated immunogenetic mechanisms, and found that this variation was partly mediated by characteristics of the major histocompatibility complex (MHC), a family of genes involved in initiating and regulating the adaptive immune response (Hughes & Yeager, 1998). Luquet et al. (2012) demonstrated the subtle influence of population genetic effects on response to exposure to *Bd* by showing that tadpoles descended from isolated European treefrog populations with comparatively lower genetic variability, while showing no detectable infection, took longer to metamorphose and died more quickly when exposed to high doses. This was potentially as a result of a trade-off between investing in resistance to a pathogen

and in growth and development. They concluded that genetic erosion reduced the capacity to invest in resistance, growth, and metamorphosis simultaneously.

1.6.3 HOST IMMUNE RESPONSES

An increasing body of evidence is highlighting the role of host immune defences in influencing susceptibility to *Bd* (Bataille et al., 2015; Richmond et al., 2009). Like all jawed vertebrates, the mechanisms by which amphibians defend themselves from pathogens can be divided into innate and adaptive immune systems (although it should be recognised that this dichotomy understates the degree to which the two systems are integrated, Flajnik & Du Pasquier 2004). The innate system can be regarded as the first line of defence that responds rapidly in a non-specific way to the invasion of pathogens. These generic responses do not depend on previous exposure (i.e., there is no memory component) and include chemical and physical barriers to foreign substances, inflammation, destruction of infected cells, removal of foreign material, and activation of the adaptive system. Amphibian skin can produce a suite of bioactive substances that defend against predators and pathogens. Amongst these are antimicrobial peptides (AMPs), which may constitute a critical component of amphibian innate immunity (Conlon, 2011; Nicolas & Mor, 1995). Amphibian AMPs are small (10-48 amino acid residues), basic peptides that can target microorganisms and disrupt their cellular membranes (Conlon, 2011; Nicolas & Mor, 1995). A growing number of AMPs produced by multiple species have been shown to be inhibitory to *Bd* growth *in vitro* (Rollins-Smith, 2009), and the capacity to produce anti-*Bd* AMPs has been associated with patterns of susceptibility in some wild populations (Woodhams et al., 2006), and in controlled laboratory experiments (Woodhams et al., 2007). However, these form a restricted set of examples, and the importance of AMPs in defending against this pathogen remains unclear; species that produce AMPs inhibitory to *Bd* growth *in vitro* can still be susceptible in the wild (Rollins-Smith et al., 2006). Many amphibian species do not produce these peptides at all, and a clear correlation between *Bd* resistance and AMP production on a broader scale has not been demonstrated (Conlon, 2011). Host factors such as AMPs do not exist in isolation, and the contribution of the diverse microbial communities that inhabit the amphibian skin may be significant in defence against pathogens. When the anti-*Bd* activity of the ecosystem of the skin mucus (i.e. including both host and microbial factors) was assayed for multiple amphibian species, it was found to be predictive of infection risk in wild populations and survival in laboratory experiments (Woodhams et al., 2014).

Toll-like receptors (TLRs) form a family of membrane-bound proteins expressed on a number of important immune cells, including monocytes, macrophages, dendritic cells, and B- and T-cells. They confer the capacity to recognise pathogen-associated molecular patterns (PAMPs), molecular signatures conserved across a broad range of microbial pathogens including fungi (Kawai & Akira, 2007; Roeder et al., 2004). TLR-enabled recognition of PAMPs by immune cells such as macrophages and dendritic cells initiates an intracellular signalling cascade that leads to the production of cytokines and other mediators of inflammation, stimulating migration of these antigen-presenting cells to immunostimulatory centers (the spleen in amphibians) where they interact with and activate B- and T-cells (Kawai & Akira, 2007; Richmond et al., 2009). TLRs therefore play an important role in initiating and linking innate and adaptive immune responses, and, given their known importance in recognition of fungi in other vertebrates (Luther & Ebel, 2006; Roeder et al., 2004), may be of particular importance in amphibian defences against *Bd*.

Sequencing of transcriptomes of the Panama Golden Frog, a species highly susceptible to *Bd*, revealed increased expression of seven TLR genes in experimentally-infected individuals, alongside increased expression of multiple immunogenetic markers associated with both innate and adaptive pathways (Ellison et al., 2014). However, this was in the context of an ineffective immune response, suggesting that susceptibility to chytridiomycosis exists despite a robust response of elements of both innate and adaptive immunity. Because of reduced expression of B- and T-cell genes in the spleen of infected individuals, immunosuppression by the pathogen has been put forward as a possible mechanism for susceptibility in this example (Ellison et al., 2014). These results appear to contradict previous transcriptome studies that found no activation and even decreased expression of immune genes in susceptible species, although each study only provides data for one TLR gene (Rosenblum et al., 2012, 2009). While these contrasting findings may reflect genuine variation between species in the initiation of a robust immune response, they may also be a result of different methodological approaches and the greater sensitivity of RNA-Seq analysis used in the more recent work over the microarray-based approach pursued by previous studies (Ellison et al., 2014).

The adaptive—or “acquired”—immune system consists of a more specialised set of responses tailored towards specific non-self antigens. Initial pathogen exposure leads to the generation of immunological memory, optimising the system for rapid clearance of infection upon subsequent encounters of these particular antigens. The genes of the major

histocompatibility complex (MHC) encode a family of cell-surface receptors central to the adaptive immune response. Their role is to bind non-self-derived protein fragments with a high degree of specificity and transport them to the cell surface for recognition by T-cells (Hughes & Yeager, 1998). The antigen recognition components of the MHC are divided into two classes. Class Ⅰ molecules are expressed on all nucleated somatic cells, and bind foreign peptides within the cytoplasm (e.g. from viruses) for presentation to killer T-cells. Class Ⅱ molecules are primarily expressed on antigen-presenting cells (such as macrophages, dendritic cells, and B-cells) and bind extracellular peptides derived from bacteria, parasites, and fungi for presentation to helper T-cells (Hughes & Yeager, 1998; Richmond et al., 2009). MHC molecules govern key interactions in the adaptive immune response, with the range of foreign peptides recognised by the system defined by the repertoire of MHC genes. Thus, the characteristics and variability of MHC genes are important components of immunocompetence (Hughes & Yeager, 1998).

Patterns of MHC polymorphism in a population can be governed by pathogen-driven selection in a number of ways. Heterozygotes can have a selective advantage simply by possessing a broader repertoire of receptors, which can lead to higher levels of polymorphism at MHC loci than observed for selectively neutral loci (Doherty & Zinkernagel, 1975). Alternatively, if a particular allele confers resistance to a common pathogen, directional selection can increase the frequency of that allele (Teacher et al., 2009). However, increasing frequency of a single allele or genotype can drive frequency-dependent selection, in which adaptation of the pathogen to common host genotypes gives rarer host genotypes a selective advantage, leading to an increase in their frequency until they too become common and come under the same selection (Takahata & Nei, 1990). Finally, balanced polymorphism can occur when pathogen characteristics vary in time and space (Garrigan & Hedrick, 2003). Any of the above mechanisms are likely to have played a role in defining patterns of MHC diversity in wild populations (Bernatchez & Landry, 2003).

An increasing body of evidence suggests an association between MHC characteristics and susceptibility to *Bd*. Heterozygosity of MHC class Ⅱ loci has been linked to resistance to *Bd* in the lowland leopard frog both in laboratory experiments and resilient wild populations (Savage & Zamudio, 2011), but was not found to be protective in experimental infection of wild alpine treefrogs (Bataille et al., 2015). Evidence both from global sampling of amphibian populations of differing resistance and exposure histories and laboratory infection

experiments has indicated that *Bd*-resistant populations and individuals share particular MHC class Ⅱ alleles (May et al., 2011; Savage & Zamudio, 2011), or characteristics of MHC class Ⅱ peptide-binding pockets (Bataille et al., 2015). In wild populations of the lowland leopard frog, MHC alleles and supertypes that are associated with resistance to *Bd* show signs of positive selection (high non-synonymous relative to synonymous substitution rates) whereas those that are not associated with resistance do not, while a comparison of MHC variability with neutral markers (microsatellites) shows evidence of directional selection for an allele associated with survival in a laboratory infection experiment (Savage & Zamudio, 2016).

Transcriptome sequencing to identify a change in expression of MHC genes in response to *Bd* exposure has yielded varying results: up-regulation of MHC genes in infected individuals has been observed in the highly susceptible panama golden frog (Ellison et al., 2014) and in the context of a lack of response, or even decreased expression, of other aspects of immune function in the susceptible yellow legged mountain frog (Rosenblum et al., 2012). However, transcriptomes of infected common frogs and *Xenopus tropicalis* showed no activation of MHC genes (Price et al., 2015; Rosenblum et al., 2009). The nature of these studies restricts interpretation of transcription to single points in time, both in terms of infection stage and life history stage, and are not guaranteed to detect substantial changes in expression of all genes that shape host and population response to a pathogen. Nevertheless, these data suggest that the MHC—and thus adaptive immunity—can play a role in some host species' defences against *Bd*, that *Bd*-driven selection can shape patterns of MHC variability, and that some populations are adapting to this pathogen through positive selection for MHC characteristics that confer resistance.

1.6.4 NEUTRAL GENETIC DIVERSITY

Genetic diversity is a measure of fundamental importance to the viability of wild populations of animals: low levels of variability can increase the risk of inbreeding depression through the increased occurrence of deleterious recessive alleles in a homozygous state, and the decreased instance of beneficial heterozygote overdominant states. Low levels of genetic variability within a population also render it more vulnerable to novel threats such as environmental change or emergent pathogens, as a smaller repertoire of alleles leads to a decreased probability of the existence of alleles adaptive to new pressures. The degree to which heterozygosity at neutral markers is reflective of genome-wide diversity in general,

and at loci that have a bearing on fitness specifically, is context-dependent. For heterozygosity at a neutral locus to be in any way informative of the state of a locus affecting fitness, the loci in question must be in either linkage or identity disequilibrium. Linkage disequilibrium refers to the non-random association of alleles at two loci in gametes (Szulkin et al., 2010). This can arise through genetic drift, immigration, or selection, and does not require that the loci are in close physical proximity (Bodmer & Felsenstein, 1967; Briscoe et al., 1994; Ohta, 1982). Identity disequilibrium occurs when there is a non-random association between diploid genotypes across loci in zygotes, and heterozygosity at neutral loci is therefore reflective of general heterozygosity across the genome (Bennett & Binet, 1956; Szulkin et al., 2010). This can arise through variation in inbreeding rates within a population such that relatively inbred individuals are more homozygous both at loci under selection and neutral markers (Chapman et al., 2009). In small or admixed populations, identity disequilibrium can arise as a consequence of linkage disequilibrium, as alleles linked at two loci in the gamete phase will, under random mating, lead to a disproportionate number of double-heterozygotes or double-homozygotes (Szulkin et al., 2010; Yang, 2000). Heterozygosity fitness correlations (HFCs) have been studied for decades, and while associations are often weak, they have been identified across multiple taxa and systems (Chapman et al., 2009; Reed & Frankham, 2003; Szulkin et al., 2010). Clear HFCs have been documented in amphibians, who may be especially vulnerable to a loss of genetic diversity as a result of their low dispersal capability, philopatry to discrete breeding sites in increasingly fragmented habitats, and tendency towards low effective population sizes (reviewed in Cushman, 2006, and Allentoft and O'Brien, 2010).

A small number of studies have examined the role of neutral genetic diversity in predicting susceptibility to *Bd.* In laboratory experiments, Luquet et al. (2012) found that individuals derived from genetically eroded populations were more likely to suffer sub-lethal deleterious effects in response to exposure (see section 1.6.2). Field surveys linking *Bd* prevalence with host genetic variability have yielded mixed findings, with positive (Addis et al., 2015; Horner et al., 2017), negative (Savage et al., 2015), and neutral (Wagner et al., 2017) associations all reported (see Chapter 2). Ultimately, however, the uncharacterised demographic, coevolutionary and epidemiological history of each unique system make the role played by host heterozygosity *per se* in mediating response to a pathogen difficult to discern from contemporary field-based data. To disentangle these issues, there is a clear

need for laboratory-based experimental work examining the responses to *Bd* exposure in individuals derived from populations with no history of exposure to the pathogen.

1.6.5 HOST MICROBIOME

It is increasingly recognised that microbial life plays a crucial role in shaping the phenotype of multicellular organisms. The development, nutrition, behaviour, and health of a host is dependent on various complex interactions with its resident microbiome (Hooper et al., 2012b; McFall-Ngai et al., 2013; Ottman et al., 2012). The amphibian skin, the site of *Bd* infection, harbours a diverse assemblage of bacterial and fungal taxa (Kueneman et al., 2016; Woodhams et al., 2014). Some of these produce antifungal metabolites and volatile compounds that have been shown to be inhibitory to *Bd* growth *in vitro* (Woodhams et al., 2018). For example, the bacterium *Janthinobacterium lividum* isolated from the skin of the red-backed salamander *Plethodon cinereus* produces the metabolite violacein at concentrations lethal to *Bd* (Brucker et al., 2008). Artificial application of *J. lividum* to red-backed salamanders resulted in increased levels of violacein on the skin of individuals, which was associated with increased survival subsequent to *Bd* exposure (Becker et al., 2009). The composition of the bacterial skin community has been shown to be predictive of *Bd* infection outcomes in laboratory studies (Becker et al., 2015; Walke et al., 2015) and population persistence in the wild (Lam et al., 2010). In field studies, bacterial community structure has been associated with *Bd* presence and dynamics (Bates et al., 2018; Bell et al., 2018), though the effect of *Bd* on the microbiome can be greater than the effect of the microbiome on *Bd* (Jani & Briggs, 2014, 2018).

In addition to the microbial community, the environment of the skin of the amphibian consists of host factors such as mucosal antibodies and antimicrobial peptides, and these can act in concert to influence the activity of pathogens. When the anti-*Bd* activity of the ecosystem of the skin mucus (i.e. including both host and microbial factors) was assayed for multiple amphibian species, it was found to be predictive of infection risk in wild populations and survival in laboratory experiments (Woodhams et al., 2014).

Taken together, these findings suggest that the utilisation of probiotics may represent a promising strategy to mitigate the effects of *Bd* in wild populations. Such bioaugmentation is used extensively to control disease in agricultural contexts, for example poultry rearing and aquaculture (Newaj-Fyzul et al., 2014; Smith, 2014). A preliminary field trial applying an anti-fungal bacteria, *J. lividum,* to individuals in a wild yellow legged mountain frog population in

the Sierra Nevada showed an association between inoculation with the bacteria and reduced infection rate (Vredenburg et al., 2011). The degree to which any bioaugmentation strategy can be generalised remains doubtful, however. The interactions between hosts, microbial communities, and pathogens are complex and context dependent; and the viability of any putative probiotic is likely to vary according to host-specific factors, temperature, pH, as well as existing microbial community structure (Belden & Harris, 2007; Daskin & Alford, 2012).

1.6.6 HOST DEVELOPMENTAL STAGE

The extent and consequences of disease responses to pathogens can vary depending on the developmental stage of the host. Such ontogenic variation has been characterised across a variety of taxa, including plants (Ficke et al., 2002), insects (Bull et al., 2012), fish (Kelly et al., 2010), birds (Killpack & Karasov, 2012), and mammals (Burns-Guydish et al., 2005). The capacity for *Bd* to infect and cause pathologies in the larval stage of amphibians is limited by the fact that the keratinised cells which it infects are restricted to mouthparts (Berger et al., 1998). Tadpoles often therefore show low susceptibility in species for which the effects of infection in post-metamorphic animals can be severe (Berger et al., 1998; Fellers et al., 2001; Rachowicz & Vredenburg, 2004). Nevertheless, sub-lethal deleterious effects such as reduced foraging activity, delayed metamorphosis, and reduced mass at metamorphosis have been documented in tadpoles exposed to *Bd* (Garner et al., 2009; Parris & Cornelius, 2004; Venesky et al., 2009). This serves to underscore that *Bd* can have an important effect on the viability of amphibians even in the absence of mortality and detectable infection (Garner et al., 2009; Luquet et al., 2012). The existence of a life stage that can become infected but remain healthy may pose a threat to populations if aggregating groups of tadpoles become a source of infectious zoospores for more vulnerable post-metamorphic life stages (Bosch et al., 2001; Rachowicz & Vredenburg, 2004).

Multiple laboratory studies have documented the elevated vulnerability of newly-metamorphosed individuals, or in the case of a direct-developing frog, juvenile froglets (Abu Bakar et al., 2016; Langhammer et al., 2014; Ortiz-Santaliestra et al., 2013; Rachowicz & Vredenburg, 2004). In the species studied, susceptibility then declined with development into adulthood. Ortiz-Santaliestra et al. (2013) speculated that as the keratinised outer layer of skin became thicker with development, the germ tube of the *Bd* zoospore had greater difficulty penetrating through it to the layer of living cells beneath. A greater capacity to resist or tolerate infection with increasing age could also be associated with maturation of

immunocompetence. Abu Bakar et al. (2016) demonstrated that the capacity to mount an immune response as detected using a phytohaemagglutinin assay increased with developmental stage as susceptibility to chytridiomycosis decreased in post-metamorphic green and golden bell frogs (*Litoria aurea*). Finally, decreased susceptibility with development could simply be a consequence of the benefits of increased size. Within a species, increased size tends to be associated with decreased susceptibility to chytridiomycosis (Garner et al., 2009; Garner, Rowcliffe and Fisher, 2011; Bielby et al., 2015, and see section 1.6.7 and Chapter 3), potentially attributable to allometric scaling of ion loss and mass-specific metabolic rate in infected individuals (Wu et al., 2018). Thus, though the effects likely vary depending on context, a generalised picture can be built of the importance of developmental stage-related differences in amphibian responses to exposure to *Bd*. The larval stage can serve as a tolerant reservoir of *Bd* while suffering sub-lethal deleterious effects. Susceptibility to chytridiomycosis then peaks in newly-metamorphosed individuals as keratinisation of the skin is completed. As individuals further develop, increased skin thickness, the maturation of immunocompetence, and increased size may all contribute in reducing the negative consequences of exposure to the pathogen.

1.6.7 HOST SIZE

For animals with complex life histories such as amphibians, body size has an important bearing on fitness and survival, especially at crucial life history stages such as soon after metamorphosis. In anurans, being larger at this stage tends to confer an advantage in fitness-related traits such as jumping capacity (Richter-Boix et al., 2006), foraging ability (Newman, 1999), ability to escape from predators (Ward-Fear et al., 2010), and overall survival (Altwegg & Reyer, 2003; Cabrera-Guzmán et al., 2013).

Given that exposure to a pathogen will generally incur a cost to the host, whether through the mounting of an immune response, the nutrient demands and direct damage associated with infection, or the impacts of disease itself (Viney et al., 2005), it might also be expected that hosts with better body condition, or greater mass, would be better equipped to incur these costs. There is indeed evidence that greater mass can be associated with a greater capacity to defend against pathogens and reallocate physiological resources into an immune response in, for example, mammals (Wagland et al., 1984) and fish (Overturf et al., 2010), and that superior body condition can facilitate investment in immune resources in birds (Møller et al., 1998). In mammals that have evolved sexual size dimorphism, sex-biased

parasitism has also been observed, with the larger sex showing heavier parasite burdens (Harrison et al., 2010; Moore & Wilson, 2002). This may arise through a trade-off between investing in growth and investing in immunological resources or the simple fact that larger animals constitute larger targets for parasites.

In the amphibian-*Bd* system, laboratory experiments consistently show that larger individuals experience greater survival when exposed to the pathogen (Bielby et al., 2015; Burrow et al., 2017; Carey et al., 2006; Garner et al., 2011, 2009; Searle et al., 2011; Wu et al., 2018). In addition to the general increase in vigour that greater size can confer, this relationship may arise through smaller individuals being especially more vulnerable to the deleterious effects of a skin disease. In post-metamorphic individuals, *Bd* infects the superficial layers of the epidermis, compromising the osmoregulatory function of the skin leading to electrolyte imbalance and associated pathologies (Berger et al., 2005a; Voyles et al., 2009). Given that skin surface area and metabolic rate scale allometrically with mass in amphibians (Klein et al., 2016; White et al., 2006), smaller animals may be especially vulnerable to the costs associated with disruption caused by cutaneous *Bd* infection. This phenomenon has been demonstrated in experimental *Bd* exposures of green tree frogs (*Litoria caerulea*), in which smaller infected animals exhibited higher rates of energetically costly skin sloughing, and suffered greater rates of ion loss than larger counterparts (Wu et al., 2018).

Gervasi et al. (2017) conducted a large standardised infection experiment across 20 North American anuran species and, while their findings supported a positive relationship between body mass and survival on an intraspecific level, they interestingly report the opposite relationship on an interspecific level. In this, there may be some alignment with the findings of Bancroft et al. (2011) and Murray and Skerratt (2012) who report a positive association between species-level measures of body size and the probability of having a positive *Bd* record in the United States and Australia, respectively. However, these data refer simply to positive detections of infection in the field, and could be explained by larger species being more conspicuous for sampling for *Bd* or being longer lived, and thus having a longer period of time over which to accumulate parasites. In any case, as they do not reflect mortality or population declines, these findings are not necessarily incongruent with laboratory experiments showing a positive relationship between mass and survival. Though Lips, Reeve and Witters (2003) do find an association between species body size and increased risk of population decline in Central America, this is not explicitly linked with *Bd* in their study.

There is thus a tendency for higher mass to be protective against *Bd* mortality within a species. It does not appear to be the case, however, that larger species are less vulnerable and there are indeed indications that the opposite may be true. Studies that explicitly focus on size variation both within and across species, and the role this plays in mediating response to *Bd* exposure, are needed to further clarify this fundamental component of amphibian-*Bd* dynamics.

1.6.8 TEMPERATURE

The importance of temperature in mediating the effect of a pathogen on its host depends on the interplay between the optimal temperature ranges for pathogen growth and host immune function, as well as host ecology, behaviour, and life history. Research on the physiology of *Bd in vitro* has identified an optimum temperature range for growth at 17-25°C (Piotrowski et al., 2004; Woodhams et al., 2008). Amphibian immune systems also have thermal optima, and tend to be suppressed at lower temperatures (Maniero & Carey, 1997). Thus, in *in vivo* infection experiments across multiple amphibian taxa, *Bd* causes higher mortality in colder temperatures (11-17°C) outside its optimal growth range *in vitro* than at warmer temperatures (18-27°C) mostly within its optimal growth range (Andre et al., 2008; Berger et al., 2004; Bustamante et al., 2010; Murphy et al., 2011; Sonn et al., 2017). Even mild pulses of heat above the thermal optimum for *Bd* limit its growth both *in vitro* and *in vivo* (Greenspan et al., 2017). These experimental observations broadly corroborate field observations. The effects and prevalence of *Bd* tend to higher at winter and cooler high elevation sites (Gründler et al., 2012; Lips, 2016; Sapsford et al., 2013; Skerratt et al., 2007; Whitfield et al., 2016). Even short-term exposure to warm microhabitats, such as sun-warmed rocks, can aid host persistence (Daskin et al., 2011). Field studies from some temperate northern hemisphere regions are, however, less clear cut, with a lack of association between temperature and *Bd* infection intensity reported (Knapp et al., 2011), and indeed a positive association shown between temperature and *Bd* prevalence in at least two systems (Muths et al., 2008; Spitzen-van der Sluijs et al., 2017). A more nuanced interpretation is presented by the thermal mismatch hypothesis (Cohen et al., 2017). This hypothesis posits that, given that parasites tend to have broader thermal performance breadths than their hosts, temperature shifts away from a host's thermal optimum could result in disease outbreaks, with parasite growth greatest at the point it most outperforms its host, not necessarily when it grows best in isolation. Thus, warm-adapted hosts are most

at risk at relatively cool temperatures, while cool-adapted hosts are most at risk at relatively warm temperatures. Laboratory experiments and field data of declines in Latin American amphibian species are consistent with the thermal mismatch hypothesis, with the greatest susceptibility to *Bd* in warm- and cool-adapted hosts occurring at relatively cool and warm temperatures, respectively (Cohen et al., 2019, 2017). Hosts adapted to cold environments may therefore become increasingly under threat from chytridiomycosis in a warming climate.

1.6.9 BD STRAIN

Early research exploring the potential sources of variation in disease outcomes in the amphibian-*Bd* system focussed on parameters such as temperature, pathogen dose, host ontogeny, and host species (Berger et al., 2004; Carey et al., 2006; Rachowicz & Vredenburg, 2004; Woodhams et al., 2006). The potential importance of variation in pathogen genotype was largely overlooked, possibly as a result of its low global genetic diversity (Morehouse et al., 2003; Morgan et al., 2007). However, Berger et al. (2005) and Retallick and Miera (2007) showed that different *Bd* strains collected within the same region would produce different patterns of mortality in standardised laboratory infection experiments, indicating potential phenotypic variation within *Bd.* Fisher et al. (2009) examined a broader set of isolates from Europe, Australia and the USA and showed that genotypic differentiation amongst isolates was associated with phenotypic and proteomic variation, and ultimately with differential host survival in infection experiments. The importance of lineage variation within *Bd* was further underscored with the discovery that one clade of *Bd*, dubbed the global panzootic lineage (*Bd*GPL), was the primary driver of amphibian population declines globally, and was characterised by hypervirulence in comparison to divergent lineages (Farrer et al., 2011). This variation may have important implications for the arrival in habitats of *Bd* as a novel pathogen. For example, the IUCN red-listed Mallorcan midwife toad (*Alytes muletensis*) was threatened by the introduction, probably in the early 1990s, of *Bd* to Mallorca (Walker et al., 2008). While declines were recorded in one of four Mallorcan *A. muletensis* populations infected with *Bd,* the remainder persisted apparently unaffected. This is probably attributable to the fact that the lineage of *Bd* introduced, *Bd*CAPE, is hypovirulent in infection experiments on *A. muletensis*, whereas *Bd*GPL causes significant mortalities (Doddington et al., 2013). There is variation in disease outcomes even between strains of *Bd*GPL. An isolate collected from a declining population of Cascades frogs (*Rana cascadae*) in

California caused higher mortalities in an infection experiment, and showed higher immmunotoxicity and optical density *in vitro*, compared with one isolated from a more stable population of the same species (Piovia-Scott et al., 2015). It remains unclear precisely what aspects of variation in *Bd* genotype are responsible for variation in virulence and disease outcomes, or indeed the traits that enable the unusual pathogenicity of this chytrid genus as a whole. The majority of *Bd* isolates show chromosomal copy number variation, a feature associated with pathogenic fungi and a potentially important source of genomic variation and adaptive potential (Farrer et al., 2013). In comparison to non-pathogenic chytrids, both *Bd* and *Bsal* show an expansion of protease and chitin-binding gene families, likely linked to pathogenicity, some of which are highly expressed in *in vivo* infection (Farrer et al., 2013, 2017). There is also evidence that these families are under directional selection, especially within *Bd*GPL (Farrer et al., 2013). The significance of many features of the unusually dynamic genome of *Bd* is as yet unknown, and further work is needed in characterising the diversity of such features amongst lineages and in linking them to variation in virulence and pathogenicity.

1.6.10 DOSE

Beginning with the exposure trials carried out by Berger et al. (1998) that helped firmly establish chytridiomycosis as a cause of mortality and population declines in anurans, laboratory experiments have been a crucial asset in understanding host-pathogen dynamics in the amphibian-*Bd* system. Carey et al. (2006) showed that increased dosage of active *Bd* zoospores, and duration of exposure, were associated with decreased survival times. While what constitutes a “high” or “low” has varied between experiments, multiple studies have supported the claim that amphibians respond to *Bd* exposure in a dose-dependent manner, showing increased infection probability, infection burden, and mortality with increased levels of exposure (Bielby et al., 2015; Fisher et al., 2009a; Garner et al., 2011, 2009), though this can vary between species and does not seem to prevail in all contexts (Gervasi et al., 2013).

1.7 STUDY SPECIES: THE COMMON TOAD (BUFO BUFO)

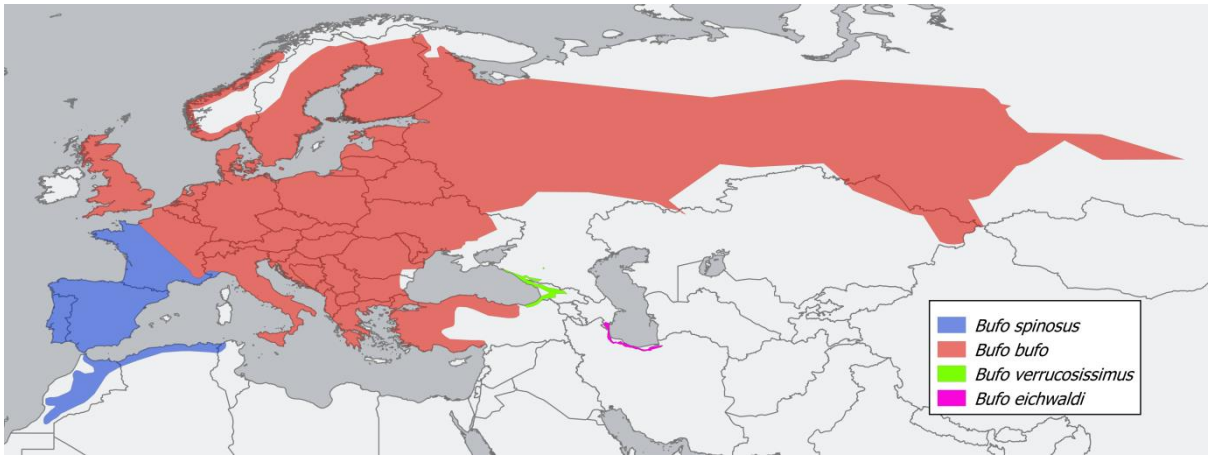

Figure 1.2 Approximate distribution of the members of the *Bufo bufo* species complex. Data modified from Agasyan et al. (2009) using information on *Bufo spinosus* distribution detailed in Arntzen et al. (2017).

The European common toad, *Bufo bufo,* comprises a four-species complex distributed across much of the western Palearctic (Arntzen et al., 2013b). The systematics of the group continue to be resolved: *Bufo spinosis* forms a genetically distinct unit ranging from northern Africa northwards into the Iberian Peninsula to France. There, a boundary running diagonally from north western France to north western Italy exists between *B. spinosus* and *Bufo bufo* (*sensu stricto*), with molecular and morphological evidence in some areas of a narrow hybrid zone between the ranges of the two species (Arntzen et al., 2017, 2016; Trujillo & Arntzen, 2017). The range of *B. bufo* (*sensu stricto*) extends from this contact zone in France to western Scotland in the west, the Arctic Circle in the north, and Lake Baikal in southern Siberia in the east (Arntzen et al., 2013b; Garcia-Porta et al., 2012; Recuero et al., 2012). *Bufo verrucosissimus*, a species more recently diverged from *B. bufo*, is found in the western Caucasus and Anatolia. There is sympatry between the two lineages at Lake Abant in northwest Anatolia, but no evidence as yet of hybridisation in the wild (Arntzen et al., 2013b). Finally, the geographically isolated Talysh and Elburz mountains in south eastern Azerbaijan and northern Iran host the most divergent lineage in the group, *Bufo eichwaldi* (Arntzen et al., 2013b; Litvinchuk et al., 2008, 2012).

Figure 1.3 Common toad (*bufo bufo*) pair in amplexus. Image: Bernie Kohl, Creative Commons CC0 1.0 Universal Public Domain Dedication

The common toad (*sensu lato*) constitutes the largest European toad species, growing up to 15cm in length (Arnold, 2002). It has warty skin, with colour varying considerably between many different shades of brown, and has prominent paratoids, the gland responsible for production of anti-predatory skin secretions, positioned on the dorsal side of the head (Arnold, 2002; Arntzen et al., 2013a). Morphological characteristics vary geographically: *B. spinosus*, distributed from North Africa to France, tend towards a larger body size, more warty skin, and paratoids positioned at a more divergent angle than more northern European forms of *B. bufo*, though there is some overlap in these traits between species (Arntzen et al., 2017, 2013a, 2016; Trujillo & Arntzen, 2017).

Though often low in density, *B. bufo* is a widespread species, covering a variety of habitats in both urban and rural contexts (Arnold, 2002; Hitchings & Beebee, 1998). Breeding takes place in permanent ponds and lakes (Sinsch, 1990), and there is strong breeding site fidelity: Reading et al. (1991) found that between 79% and 96% of adults in English and Swedish populations that survive from one breeding season to the next return to the same breeding site. Typically for anurans, *B. bufo* have low vagility and very small home ranges, most remaining within between 400 m and 1.5 km of their breeding sites during summer (Glandt, 1986; Sinsch, 1990), though some have been recorded beyond this, as far as 3 km (Heusser,

1969). Reproductive maturity can be reached as early as three years for males and four years for females, though this varies geographically (Cvetković et al., 2009; Hemelaar, 1988; Reading, 1991), and these toads can survive for as long as 15 years in the wild (Cvetković et al., 2009). Reproductive individuals begin to migrate towards their breeding pond in early autumn before hibernating 50-500 m away from the site (Sinsch, 1990). Migration is completed in early spring, often involving large numbers of toads over a short period of time, an especially conspicuous phenomenon when migration routes intersect with roads (Arnold, 2002; Petrovan & Schmidt, 2016). Males, who can mate multiple times, arrive at the pond earliest and remain there for an explosive breeding period that lasts just a few weeks, while females, who only spawn once, spend a shorter period of time at the pond (Davies & Halliday, 1977). Female *B. bufo* are larger than males, larger females tend to lay more eggs, and a larger male-to-female size ratio is associated with higher fertilisation success (Davies & Halliday, 1977). Females lay eggs in dual jelly-encased strings, which are wound around pond vegetation and can be up to 5 m in length, containing up to 8,000 eggs (Arnold, 2002). The eggs are fertilised as they emerge by a male tightly gripping the female in amplexus (Davies & Halliday, 1979). At any single point in time there is a heavily male-biased sex ratio at a breeding pond, and the resulting competition for breeding opportunities often results in mating balls consisting of several males in amplexus with a single female, a situation which can be fatal for the female (Davies & Halliday, 1977, 1979). Within 2-3 weeks, eggs hatch into free-swimming tadpoles, which congregate in large feeding groups before metamorphosing into toadlets within about 4-5 weeks (Arnold, 2002; Goater, 1994; Hemelaar, 1988).

The conservation status of this widespread species is currently classified as “least concern” by the IUCN (Agasyan et al., 2009). However, long term citizen science datasets collected by volunteers moving toads across roads adjacent to hundreds of breeding sites in Great Britain and Switzerland indicate a continuous decline in *B. bufo* population sizes in both countries since the 1980s (Petrovan & Schmidt, 2016), and a similar approach has documented significant declines in Italy (Bonardi et al., 2011). These declines may be partly attributable to an increased threat posed by road traffic: mortality on roads of migrating adult toads has been linked with reductions in population size in the species (Cooke & Sparks, 2004), and the period between 1985 and 2016 has seen a 68% rise in vehicle miles on the roads of Great Britain (Transport Statistics Great Britain: 2017, https://www.gov.uk/government/statistics/transport-statistics-great-britain-2017 [accessed

06/03/2019]). Additionally, amphibians are sensitive to habitat destruction, degradation (for example through agrochemical pollution), and fragmentation (Beebee & Griffiths, 2005; Ficetola & De Bernardi, 2004; Hitchings & Beebee, 1998). There is also evidence that a warmer climate is associated with a reduction in body condition of breeding females in *B. bufo*, resulting in decreased fecundity and survival (Reading, 2007). The role played by disease is unclear: while *Bd* has been detected on the skin of *B. Bufo* in a number of localities across Europe including Great Britain (http://www.bd-maps.net/ [Accessed 06/03/2019]), and the pathogen has been found to be associated with unusual mortalities in Spanish *B. spinosus* populations (Bosch & Martínez-Solano, 2006), it has not been conclusively linked with any population declines in *B. bufo*.

Partly as a result of its intermediate susceptibility to the pathogen, *B. bufo* has been used as a model species in laboratory infection experiments examining key questions surrounding the disease and its dynamics. For example, *B. bufo* tadpoles and metamorphs have been used to demonstrate phenotypic variation between different lineages of *Bd* and highlight the hypervirulence of the Global Panzootic Lineage (GPL) (Farrer et al., 2011; Fisher et al., 2009b; O'Hanlon et al., 2018). In investigating the possible influence of climate change on host and pathogen responses, Garner et al. (2011) used *B. bufo to* show that, while a warmer overwintering temperature was associated with an increased infection probability, it was also associated with a decreased infection burden, and overwintering temperature regime did not have a significant effect on eventual mortality. *B. bufo* has also been used to demonstrate that the consequences of exposure to *Bd* are not merely restricted to infection and direct mortality, but can manifest in lower mass at metamorphosis even in the absence of detectable infection, a possible result of the reallocation of energetic resources necessary in mounting a successful immune response against the pathogen (Garner et al., 2009). A consistent finding, however, in research on *Bd* and its effects on *B. bufo* has been the importance of body size in determining the consequences to the host of exposure. Lower post-metamorphic mass is associated with decreased survival probability as a result of *Bd* exposure, more so than dose of *Bd* administered and even infection status itself (Bielby et al., 2015; Farrer et al., 2011; Garner et al., 2011, 2009).

1.8 AIMS AND OBJECTIVES

The host-pathogen dynamics of chytridiomycosis, an emerging infectious disease causing dramatic declines and extinctions of amphibian species and populations worldwide, are poorly understood. There is a notable variation in responses to *Bd*, both among species and between

populations of a single species, and a knowledge gap in understanding predictors of risk in previously unexposed populations. This aim of this thesis is to examine how natural amphibian populations vary in their genetic characteristics and responses to disease and to identify predictors, both genetic and phenotypic, of *Bd*-driven mortality. To investigate these, the following objectives were set:

1. Document patterns of genetic variability in a natural system of amphibian populations and investigate the role of seawater in population differentiation and reduction of genetic variability through isolation (Chapter 2).
2. Examine how population of origin influences survival time in response to *Bd* exposure, and whether these responses are associated with population-level allelic diversity (Chapter 3).
3. Test the hypotheses that individual heterozygosity and body size would be positively associated with survival time in response to *Bd* exposure (Chapter 3).
4. Examine the increased power of a dataset of SNP markers to discern genetic structure in individuals sampled from different populations (Chapter 4).
5. Assess the potential of a SNP dataset to identify loci influential in local adaptation and disease response (Chapter 4)

CHAPTER 2 PATTERNS OF GENETIC VARIABILITY IN NATURALLY FRAGMENTED COMMON TOAD POPULATIONS IN WESTERN SCOTLAND

2.1 INTRODUCTION

Population fragmentation and isolation are important processes influencing the structure and viability of wild populations. Features of habitat and geography can mediate and prevent dispersal, creating populations largely or entirely isolated from migration. The resultant lack of gene flow can contribute to population differentiation, and the loss of allelic diversity in isolated populations due to genetic drift (Keyghobadi, 2007; Manel & Holderegger, 2013). Spatial genetic structure attributable to population fragmentation has been investigated on a number of scales across a variety of taxa. For example, Hartl et al. (2005) documented significant differentiation among populations of red deer (*Cervus elaphus*) attributable to geographic barriers to dispersal even at a small geographic scale. Similarly, Wang, Glor and Losos (2013) demonstrated that geographical isolation was the dominant force in creating genetic divergence in 17 species of *Anolis* lizards in the Greater Antilles.

Genetic erosion associated with isolation of populations can have negative impacts on the fitness of individuals as well as the long-term viability of populations (Reed & Frankham, 2003; Willi et al., 2006). In the absence of incoming gene flow and novel mutations in a finite population, alleles disappear through random mating, differential breeding success and mortality. This loss of allelic diversity means that individuals are more likely to be autozygous at any given locus. Decreased heterozygosity can have negative impacts on phenotypic traits linked to fitness, as recessive deleterious alleles are more likely to occur in a homozygous state. Heterozygosity itself can also be beneficial at particular loci at which, for a given two alleles, the heterozygote state confers an advantage over either homozygote state, a phenomenon known as overdominance. Heterozygosity fitness correlations (HFCs) have been studied for decades, and while associations are often weak, they have been identified across multiple taxa and systems (Chapman et al., 2009; Reed & Frankham, 2003; Szulkin et al., 2010).

Due to several distinctive aspects of their biology, amphibians have received particular attention in investigations of these processes (Allentoft & O'Brien, 2010; Cushman, 2006). For example, philopatry to breeding sites like ponds that have patchy, non-continuous distributions can produce a strong spatial genetic structure. Low dispersal capacity limits

migration and further promotes the development of demes (Ficetola et al., 2007; Kraaijeveld-Smit et al., 2005). Amphibians can also often have small effective population sizes, leading to an increased risk of genetic drift (Beebee & Griffiths, 2005; Rowe & Beebee, 2004), which can be compounded by differential mortality on the clutch level (Dubois, 2004). These traits may act to make amphibians especially vulnerable to loss of genetic diversity, and amplify the threats currently posed to wild populations (Allentoft & O'Brien, 2010). Amphibians constitute the most vulnerable group of vertebrates on the planet with 41% of species now classified as threatened under IUCN Red List criteria (IUCN, 2018). Declines have been driven by a suite of threats, including habitat destruction, pollution, climate change and infectious diseases such as chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Beebee & Griffiths, 2005; Fisher et al., 2009b).

The threat posed by *Bd* is of particular concern as it extends beyond the reach of areas directly impacted by humans, spreading as an emerging infectious disease into otherwise pristine habitat, decimating populations naïve to the pathogen (Farrer et al., 2011; Fisher et al., 2009b; Lips et al., 2008). The patterns of genetic variation in amphibians in fragmented natural habitats, and the degree to which this influences phenotypic traits such as response to exposure to novel pathogens, is therefore of particular relevance to conservation biology. This chapter uses microsatellite genetic markers to characterise patterns of genetic variability in a set of strongly spatially structured common toad (*Bufo bufo*) populations. *B. bufo* is the central member of a four-species complex distributed across much of the western Palearctic (Arntzen et al., 2013b). It displays a high level of philopatry to the permanent ponds and lakes used as breeding sites, with at least 79% of breeding adults returning to the same location from one breeding season to the next (Reading et al., 1991). The species exhibits a low dispersal capacity, generally remaining within between 400 m and 1.5 km of their breeding sites during summer (Glandt, 1986; Sinsch, 1990). Effective population sizes are often low, with strong population differentiation (Brede & Beebee, 2004; Flavenot et al., 2014; Roth & Jehle, 2016; Scribner et al., 1997). *B. bufo* can be found on islands isolated by sea, and given that the species is regarded as having a low salinity tolerance (Ferreira & Jesus, 1973), it might be expected that populations situated on such islands would show greater differentiation and a loss of genetic variability as a result of reduced gene flow.

Seas and oceans can play an important role in shaping the distribution and diversification of amphibians, and populations inhabiting oceanic islands provide compelling contexts in which

to examine the genetic consequences of population isolation. For example, an extensive study sampling 24 island populations of black spotted frogs in the Zhoushan Archipelago, situated in the East China Sea, and three neighbouring populations on mainland China, showed that mainland populations harboured higher levels of genetic diversity, and that, subsequent to Holocene sea level rises, seawater has effectively isolated island populations from gene flow (Wang et al., 2014). Similarly, populations of Natterjack toad (*Bufo calamita*) inhabiting seven islands of the Bohuslän archipelago on the western coast of Sweden show limited dispersal ability over sea. Rogell et al. (2010) reported a well-defined genetic structure and considerable variation in levels of genetic diversity between populations. Thus far, however, studies on such systems have failed to demonstrate this expected pattern for *B. bufo* (Roth & Jehle, 2016; Seppä & Laurila, 1999).

The area investigated in this chapter is situated in the Inner Hebrides, an archipelago off the western coast of Scotland. This chain consists of over 150 named islands adjacent to a complex mountainous coastline characterised by a series of sounds and inlets (Boyd, 1983). The geological history of the area is heavily influenced by the recessions and readvances of glaciers during the quaternary Ice Age (Boyd, 1983). For this reason, the area has attracted much scientific interest, especially in describing the patterns of migration and differentiation of the resident flora and fauna. Some islands host animal and plant populations that are profoundly genetically distinct from their mainland counterparts, and may have experienced limited gene-flow since post-glacial colonisation (Berry, 1983). For example, the common shrew (*Sorex araneus*) is found on many of the islands, having probably colonised them at the end of the last period of glaciation with minimal subsequent migration (White & Searle, 2007). Microsatellite genotyping of shrews sampled on 13 islands of the Inner Hebrides and neighbouring Clyde Islands and six regions on the adjacent mainland showed that island populations were significantly genetically divergent from each other, and exhibited lower levels of genetic diversity than those on the mainland (White & Searle, 2007). Sea has inhibited dispersal in the region even for flying insects. *Bombus muscorum*, a bumblebee, shows differentiation and reduction in genetic diversity associated with isolation by sea in the Inner and Outer Hebrides (Darvill et al., 2006). In a reintroduced population of Red Deer (*Cervus elaphus*) on the island of Rum loss of genetic variation associated with isolation may be mitigated through selection in favour of heterozygotes, or temporally fluctuating directional selection (Pemberton et al., 1996).

This chapter focusses on a set of *B. bufo* populations situated around Skye, the largest island

of the Inner Hebrides. The breeding ponds sampled are situated on both mainland western Scotland and neighbouring isolated island populations, separated by seawater since approximately 9500 years BP (Lambeck, 1993). My aim is to document patterns of genetic variability in this system, and investigate the role of seawater in population differentiation and reduction of genetic variability through isolation. This characterisation will serve as a basis for further studies on the role of population identity and genetic architecture in mediating response to exposure to *Bd*.

2.2 MATERIALS AND METHODS

2.2.1 STUDY SITES AND SAMPLE COLLECTION

Samples were taken from 11 toad breeding populations over an area of approximately 60 x 73 km around the Isle of Skye, western Scotland (Figure 2.3). Two sites (MAK, MAT) were situated on the mainland, and two sites (SKB, SKP) considered to be in effect mainland sites, were situated on Skye, which is connected to the mainland by the Skye Bridge, approximately 500 m long and constructed between 1992 and 1995. The remaining seven sites (CRO, PAB, RAB, RAO, ROI, ROT, and SCD) were located across four islands completely isolated by sea situated between Skye and the Applecross peninsula. Two widely separated sites were sampled on each of the larger islands, Raasay and Rona. The isolated islands range in size from 1.3 km^2 (Pabay) to 53.4 km^2 (Raasay) and have been separated by sea since the last period of glacial activity in the area ended approximately 9500 years BP (Lambeck, 1993). Tadpoles were collected between 2013 and 2015 at ten populations by Scottish Natural Heritage staff and stored in 1.5 ml 100% ethanol-filled Eppendorf tubes. Shorelines were evenly sampled with sample points separated by a minimum of 10m to reduce the risk of multiple collections from a single clutch. Genetic samples for one island population, Crowlin, came from metamorphs raised in captivity from spawn collected in 2016 (see Chapter 3).

2.2.2 DNA EXTRACTION AND PCR PROTOCOLS

Individuals were genotyped at eight previously-characterised *B. bufo* microsatellite loci (*Bbuf*µ11, *Bbuf*µ15, *Bbuf*µ24, *Bbuf*µ39, *Bbuf*µ46, *Bbuf*µ54, *Bbuf*µ62, and *Bbuf*µ65, Brede et al. (2001)). DNA was extracted from the tails of ethanol preserved tadpoles using a Qiagen DNEasy extraction kit following the manufacturers protocol (Qiagen, UK). Concentration of extracted DNA was quantified using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA) and then standardised to approximately 10 ng/µl. Microsatellite loci were

amplified by PCR using the protocols described in Brede et al. (2001), with the exception of *Bbuf*11, which was found to yield more product at an annealing temperature of 56°C as compared with the recommended temperature of 44°C (see Table 2.1 for details on the primers used in this study). All PCR reactions were carried out in a Veriti^{TM} 96-well thermal cycler (Applied Biosystems^{TM}, UK). The total reaction volume was 10 µl, and contained 10 ng of genomic DNA, 5 pmol of each primer, 0.5 U Taq polymerase (BIOTAQ^{TM}, Bioline Reagents Ltd, UK), 0.15 mM of each dNTP, 2 mM MgCl2, and 1 µl of 10x NH4 reaction buffer. A "touchdown" PCR reaction, which maximises yield of the target sequence, was performed on all loci. Nonspecific priming by one or both of the primers can lead to amplification of small non-target sequences, resulting in the appearance of multiple spurious bands when the PCR products are viewed on an agarose gel. Increasing the annealing temperature towards the melting point of the primer-template complex decreases spurious binding, thus increasing the specificity of the reaction. However, this comes at the cost of a decreased yield of PCR product. A trade-off therefore exists between increased yield at lower temperatures and increased specificity at higher temperatures (Hecker & Roux, 1996). A touchdown PCR technique circumvents this trade-off by setting the annealing temperature of the initial cycles of the reaction at or above the melting point of the primer-template complex, and then decreasing the annealing temperature incrementally for each subsequent cycle (Don et al., 1991). Initial priming is thus highly specific in early cycles, allowing the target product to outcompete any nonspecific product once the annealing temperature is dropped to a point at which nonspecific priming would normally occur. The reaction then continues for multiple cycles at the touchdown annealing temperature, yielding a high quantity of the target product (Don et al., 1991; Hecker & Roux, 1996). PCRs commenced with a 4 minute denaturation period at 94 °C, followed by a series of annealing steps, each of which consisted of two cycles of 1 minute at a primer-specific annealing temperature, and extension for a primer-specific time period (1 or two minutes) and temperature (see Table 2.1). The annealing temperature in each step decreased in 2 °C increments, until a fifth and final primer-specific annealing temperature, which proceeded for 22 cycles. Each reaction ended with a 10-minute elongation period at the primer-specific extension temperature. Primers were labelled with fluorochromes and amplified DNA fragments were separated by capillary electrophoresis using an ABI 3130 Genetic Analyser (Applied Biosystems). Fragments were sized using Peak Scanner Software v1.0 (Applied Biosystems).

Table 2.1 Details of *Bufo bufo* microsatellite primers used in Chapter 2 and Chapter 3. Entries in bold face denote the loci used in this chapter. The remainder were added for analysis in Chapter 3. Primers and protocols originally described by Brede et al. (2001)

Locus	Primer sequence (5'–3')	Final annealing temperature (°C)	Extension temperature (°C)/time (mins)	Allele size range	Fluorescent dye used
***Bbuf*µ11**	**F: GTCACATGGATAATAAATGAGACC**	**56**	**60 (2)**	**116-144**	**ATTO550**
	R: TCTAATATTGATGACCAGACAACC				
*Bbuf*µ13	F: AGCCTACATTGCTCAATCCGATAC	52	70 (1)	163-197	HEX
	R: GTGGCGTGGAGGGTCATAAAATC				
***Bbuf*µ15**	**F: TCAATATAGGAGTCCCAGAATGTC**	**50**	**70 (1)**	**160-170**	**HEX**
	R: AATCCCCTAGCGTACACAAGATAC				
***Bbuf*µ24**	**F: TTTGGAGAGGGGAAAACTTCACAC**	**56**	**60 (2)**	**138-150**	**FAM**
	R: CGGATTCTGTTGGGGGTGCTC				
***Bbuf*µ39**	**F: GATCCCCATCCACTGGTCA**	**45**	**70 (1)**	**192-198**	**HEX**
	R: AAAAATGTCTCTTTCTCCCCTCTC				
***Bbuf*µ46**	**F: GATTTCCTGCCGTGAGCCCAGTG**	**63**	**70 (2)**	**134-162**	**ATTO550**
	R: CGCCCGCCAAACCTTCCTGAAC				
*Bbuf*µ49	F: GATCTGGGCAGTGTTGGATTG	52	70 (1)	175-213	ATTO550
	R: ATTCCGTCTGCTAAATGTCTCTTG				
***Bbuf*µ54**	**F: CATTGCGCTGCTGTCAGATTACAC**	**53**	**70 (2)**	**159-187**	**FAM**
	R: TTAGGGATTGCCGTCCAGTTGTC				
***Bbuf*µ62**	**F: GCACATTCCTGTGTCCGTGTATAG**	**50**	**70 (1)**	**184-204**	**HEX**
	R: ATTCCGAAAACGAAAAGAAAAGAG				
*Bbuf*µ63	F: TCGGGGCACCATCAAGTGTCAC	56	70 (2)	93-109	HEX
	R: ATCATCATGGTTAGCGGCTCTTG				
***Bbuf*µ65**	**F: GGATCTAAGCGCTGTGAGAGTGA**	**52**	**70 (1)**	**160-212**	**FAM**
	R: CGGTCCGTGTTACCACTGATGC				

2.2.3 POPULATION GENETIC ANALYSIS

Tests for linkage disequilibrium between pairs of loci, observed (H_o) and expected (H_e) heterozygosities, and pairwise F_{ST} values between populations were calculated using the software GENEPOP 4.4 (Rousset, 2008). Allelic richness values for each population were calculated using FSTAT (Goudet, 1995). Following Rousset (1997), isolation by distance was tested for using Mantel tests (10000 permutations) comparing linearised F_{ST} values (F_{ST}/(1-F_{ST})) with log-transformed pairwise geographic distances. These calculations were carried out using the *R* package VEGAN (Oksanen et al., 2018). A Kruskal-Wallis test was carried out in *R* version 3.5.0 (*R* Core Team, 2018) to compare F_{ST} values calculated for populations separated by sea and those separated by land. Spearman rank correlations between the mean of a population's F_{ST} values and both its allelic richness and expected heterozygosity were also calculated using *R*. STRUCTURE 2.3.4 (Pritchard et al., 2000) was used to identify the most likely number of genetic clusters (*K*) within the dataset. STRUCTURE uses a Bayesian iterative algorithm to assign to each sample probabilities of membership to a pre-defined number of clusters, each of which is characterised by patterns of allele frequencies at each

locus. Largely following Porras-Hurtado et al. (2013), 20 independent runs were performed for each value of *K* from 1 to 13, with 200 000 Markov chain Monte Carlo iterations after a burn-in of 200 000 iterations. The best-supported value of *K* was determined using Δ*K*, related to the rate of change in log probability between successive *K* values (Evanno et al., 2005), this analysis was carried out using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Replicates for each level of *K* were aligned using CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and graphical output was produced using DISTRUCT 1.1 (Rosenberg, 2004).

2.3 RESULTS

154 individuals across 11 populations were genotyped (range 9-31 individuals per population) with a PCR success rate of 91%. No loci pairs were found to be in linkage disequilibrium. For each population, the mean number of alleles per locus ranged from 5.38 for MAK, situated on the mainland, to 1.63 for PAB, situated on a small, isolated island (Table 2.2). Both mainland populations and one Skye population (SKB) showed higher levels of allelic richness (3.14-3.73) than all but one of the island populations (SCD). All loci were polymorphic on mainland and Skye populations, whereas four of the seven remaining island populations had at least one monomorphic locus, with four out of eight loci monomorphic on Pabay (Table 2.2). On Crowlin, four of eight loci deviated significantly from Hardy-Weinberg equilibrium, likely due to the sampling of siblings. Isolation by seawater was associated with marked population differentiation. F_{ST} values for populations separated by land (including the Skye Bridge) ranged from -0.1 to 0.13, whereas those between populations separated by sea ranged from 0.07 to 0.5 (Table 2.3), and these two groups of comparisons differed significantly (Kruskal-Wallis, p <0.001). The allelic richness of a population was strongly negatively associated with its degree of isolation, as defined by the mean of the pairwise F_{ST} values calculated between it and all other populations (Spearman rank correlation coefficient -0.84, p = 0.001, Figure 2.1). Similarly, the expected mean heterozygosity of a population showed a non-significant tendency towards a negative correlation with mean F_{ST} (Spearman rank correlation coefficient -0.58, p = 0.062, Figure 2.1). A weak, but significant, isolation-by-distance effect was found (Mantel test, r = 0.23, p = 0.04).

Table 2.2 Genetic variability parameters for 11 *Bufo bufo* populations characterised at 8 microsatellite loci.

Population type	Location	Site	*n*	A/L	AR	H_o	H_e	HW Disequilibria	ML	PA
Mainland	Loch Iain Oig, Kyle of Lochalsh	MAK	10	5.38	3.73	0.54	0.65	*Bbufμ*54	0	7
Mainland	Toscaig, nr Applecross	MAT	10	4.50	3.14	0.51	0.51		0	2
Mainland (Skye)	Lochan Dubh, Broadford, Skye	SKB	11	3.88	3.33	0.36	0.41	*Bbufμ*39, 54, 65	0	2
Mainland (Skye)	Loch a Mhuilinn, Portree	SKP	20	4.25	2.73	0.49	0.51	*Bbufμ*11, 65	0	2
Island	Pabay	PAB	19	1.63	1.57	0.26	0.20		4	0
Island	Loch Beag, Raasay	RAB	9	3.38	2.72	0.49	0.50		1	1
Island	Oskaig, Raasay	RAO	10	4.00	2.86	0.45	0.47	*Bbufμ*54	1	2
Island	Loch na h Iolaire, Rona	ROI	11	3.25	2.49	0.34	0.39		0	1
Island	Township reservoir, Rona	ROT	13	2.88	2.33	0.37	0.36	*Bbufμ*46, 65	3	1
Island	East of Loch Dubh, Scalpay	SCD	10	4.50	3.30	0.56	0.58	*Bbufμ*11	0	3
Island	Crowlin	CRO	31	4.73	2.90	0.56	0.61	*Bbufμ*11, 65, 15, 46	0	6

n, number of individuals sampled; A/L, mean number of alleles per locus; AR, allelic richness; H_o, observed heterozygosity; H_e, expected hetrozygosity; ML, number of monomorphic loci; PA, number of private alleles.

Table 2.3 Pairwise F_{ST} values for 11 *Bufo bufo* populations. Continuous box borders denote populations separated by land, the broken border denotes populations separated by land and the Skye Bridge, the remaining values show populations separated entirely by sea.

	MAK	MAT	SKB	SKP	PAB	RAB	RAO	ROI	ROT	SCD
MAT	0.04									
SKB	-0.01	0.05								
SKP	0.10	0.13	0.06							
PAB	0.32	0.38	0.34	0.32						
RAB	0.11	0.20	0.09	0.17	0.35					
RAO	0.15	0.30	0.18	0.23	0.37	0.07				
ROI	0.24	0.27	0.21	0.28	0.49	0.29	0.32			
ROT	0.30	0.36	0.30	0.37	0.50	0.33	0.32	0.05		
SCD	0.07	0.13	0.09	0.18	0.21	0.13	0.15	0.25	0.27	
CRO	0.13	0.22	0.11	0.20	0.40	0.17	0.24	0.28	0.32	0.20

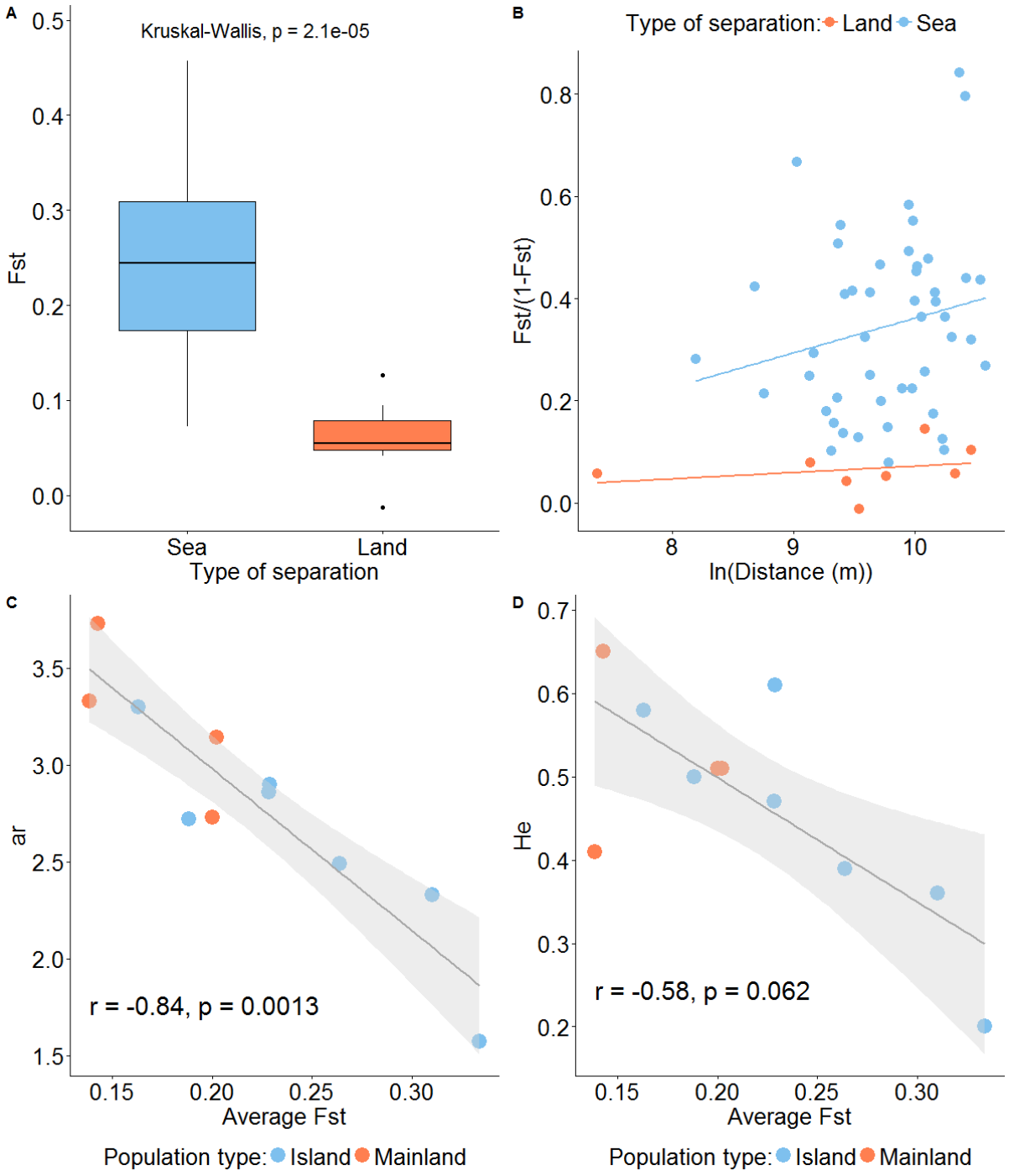

Figure 2.1 Patterns of population differentiation (**A**) Boxplots comparing pairwise F_{ST} values between populations separated by sea and those separated by land. (**B**) Association between log-transformed geographic distance separating populations and their respective linearised F_{ST} values (F_{ST}/(1- F_{ST})). Spearman rank correlation between F_{ST} values averaged for each population and (**C**) allelic richness and (**D**) expected mean heterozygosity at each population

The log probability of numbers of clusters according to the STRUCTURE analysis increased from K = 1 through K = 7, with a modal value of ΔK at K = 4 (Figure 2.2). The four genetic clusters identified reflected their geographic context (Figure 2.3). Individuals sampled at all mainland

and Skye locations and the islands of Raasay and Scalpay were predominantly assigned to a single cluster, whereas each of the remaining three islands represented a distinct genetic unit (Figure 2.3).

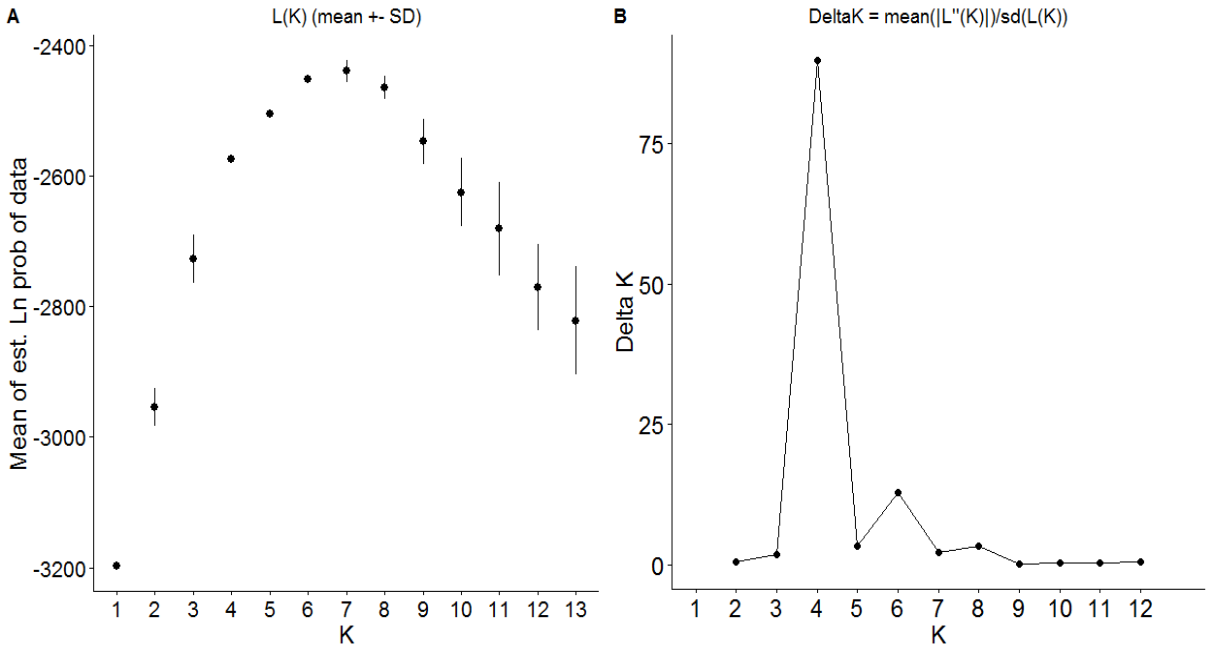

Figure 2.2 Estimated probability of data for each *K* value in STRUCTURE analysis. (**A**) Plot of mean likelihood, L(K), and variance across 20 replicates of each *K* value. (**B**) Δ*K* calculated using the Evanno, Regnaut and Goudet (2005) method. The modal value (*K*=4) is taken as the most probable number of clusters**.**

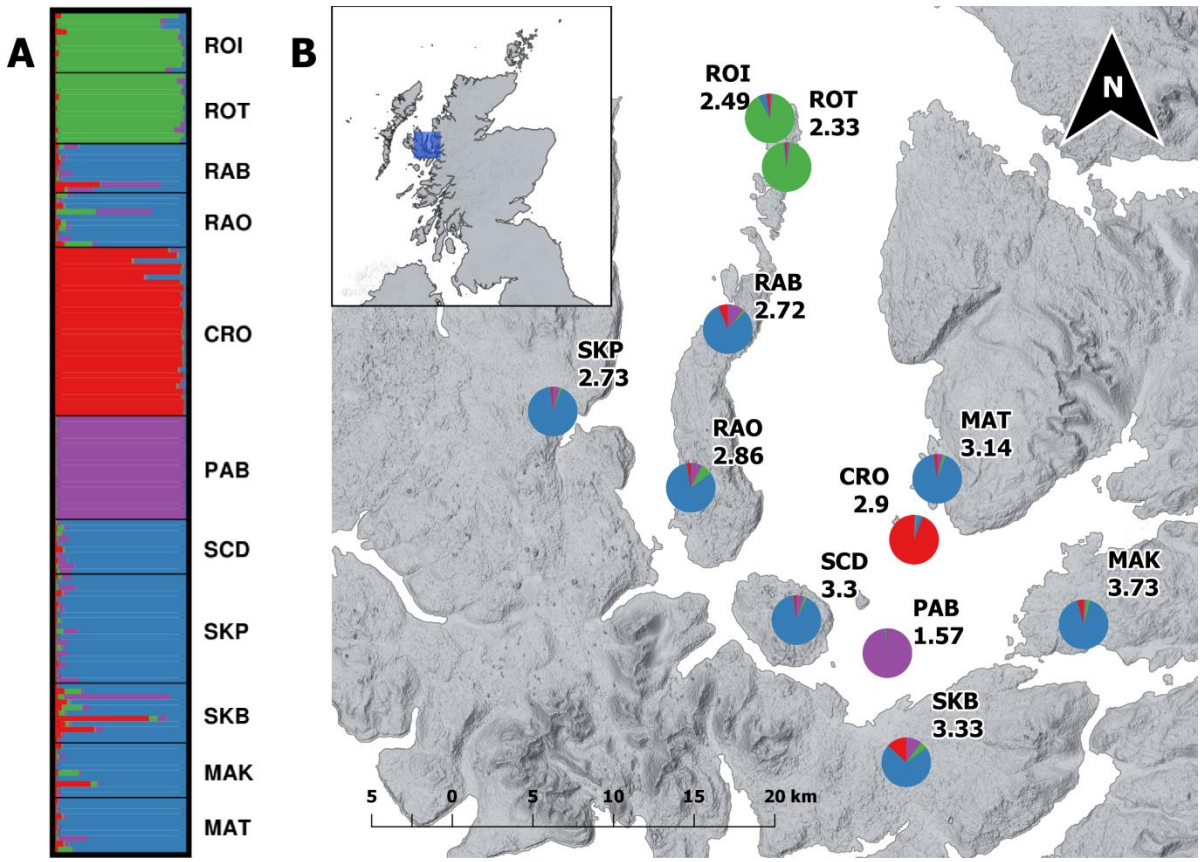

Figure 2.3 Genetic structuring and geographical distribution of sampled populations (**A**) Bar plot showing assignment of all sampled individuals to the 4 genetic clusters determined by STRUCTURE analysis. Each horizontal line denotes an individual, with the size of each colour bar corresponding to the probability of membership of each of four clusters. (**B**) The locations of *Bufo bufo* breeding populations sampled in this study. A three-letter site code and allelic richness value is shown for each pond. Each circle is coloured according to the proportion of assignments into each of the four clusters for each population. Inset shows the location of the study area in Scotland.

2.4 DISCUSSION

This study characterised patterns of genetic variability in a strongly spatially structured set of *B. bufo* populations on the north-western fringe of the species distribution. The principal findings are that seawater serves to isolate demes, producing high levels of population differentiation, and that loss of genetic diversity parallels degree of isolation.

Given the low vagility of the species, the overall tendency for isolation by geographic distance found here is unsurprising. F_{ST} values were significantly higher for populations separated from each other by sea, with eight such estimates exceeding all previously reported values for this species (Brede & Beebee, 2004; Luquet et al., 2015a; Martinez-Solano & Gonzalez, 2008; Roth & Jehle, 2016; Seppä & Laurila, 1999; Wilkinson et al., 2007). The presence of monomorphic loci on the majority of island populations, coupled with their absence from mainland populations, also suggests that isolation by seawater has promoted

genetic drift. Generally, isolation was associated with decreased genetic diversity, as the mean of a population's pairwise F_{ST} estimations was significantly negatively correlated with its level of allelic richness. STRUCTURE analysis further emphasised the profound geographic influence on the partitioning of the four genetic units characterised in this system. Individuals sampled at all mainland sites and sites on the two islands closest to the mainland were predominantly assigned to a single cluster. Each of the remaining three genetic clusters was distinctly associated with each of the remaining three islands furthest from the mainland.

The *B. bufo* populations inhabiting the mainland and islands of this pocket of the Inner Hebrides thus follow the pattern shown by multiple plants and animals of the area; one shaped by a complex history of colonisation and eventual isolation by seawater. While humans have probably been influential in shaping the distribution of mammalian fauna in these islands (Berry, 1983), their role in mediating dispersal of the common toad is less clear. Similar to that of the common shrew (*Sorex araneus*) (White & Searle, 2008), the colonisation of islands in this area by common toads bears the hallmarks of natural dispersal from the mainland, possibly over land or ice bridges present following the last period of glaciation in the area. As shown here, distance from the mainland is associated with membership of distinct genetic clusters. Proximity to the mainland also dictates presence: despite human habitation, the common toad is absent from the more distant isles of the Inner Hebrides (e.g. Coll, Tiree, and Rùm), and entirely from the Outer Hebrides.

The power of seawater in promoting population divergence does however contrast somewhat with previous examinations of *B. bufo* inhabiting sea islands. In characterising a set of mainland and island populations on the Norwegian coast, Roth and Jehle (2016) found little difference between F_{ST} values comparing populations separated by land and those separated by sea. This observation was in the context of substantial differentiation overall, however, and may be attributable to limited dispersal through a terrestrial habitat characterised by mountains and fjords. Seppä and Laurila (1999) reported extremely low F_{ST} values between *B. bufo* populations situated across various islands in an archipelago of the northern Baltic Sea. They attributed this apparently unrestricted gene flow between islands to a tolerance in migrating juveniles of the low-salinity brackish water that characterised their study area. By contrast, the populations investigated in this study were separated by comparatively high-salinity seawater (Manders et al., 2010), likely exceeding the low salinity tolerance of *B. bufo* (Bernabò et al., 2013; Ferreira & Jesus, 1973), and therefore providing a

far more effective barrier to dispersal in this system.

Overall, *B. bufo* populations situated in the area of Skye, north-western Scotland, are characterised by variation in genetic diversity and marked differentiation facilitated by isolation by seawater. The genetic consequences of a fragmented habitat can impact phenotypic traits associated with fitness, and the patterns reported here serve as an opportunity to explore these impacts in the context of the emerging infectious disease, chytridiomycosis.

CHAPTER 3 BODY SIZE AND POPULATION OF ORIGIN, BUT NOT HETEROZYGOSITY, PREDICT SURVIVAL TO BD EXPOSURE IN THE COMMON TOAD

3.1 INTRODUCTION

Amphibians represent a particularly prominent illustration of the rapid and severe loss of biodiversity—regarded by some as this planet's sixth mass extinction—currently underway (Barnosky et al., 2011; Ceballos et al., 2015; Dirzo & Raven, 2003; Stuart et al., 2004). They constitute the most vulnerable major grouping amongst vertebrates: 40% of amphibian species for which there is sufficient data to make an assessment are classed as threatened, as compared with 25% of mammalian species and 13% of avian species (IUCN, 2018). Since the 1980s, declines in amphibian populations have been rapid and severe (Stuart et al., 2004) and have occurred in all biogeographic regions inhabited by amphibians, exceeding what would be expected from natural population fluctuations (Ceballos et al., 2015; Houlahan et al., 2000; Pounds et al., 1997; Wake & Vredenburg, 2008). While over-exploitation and habitat loss have played a major role in many of these losses, almost half (47%) of the species defined as "rapidly declining" do not have these pressures, with many declines and extinctions occurring in protected and apparently pristine areas (Hero et al., 2004; Laurance et al., 1996; Pounds et al., 1997; Stuart et al., 2004). Chytridiomycosis, an emerging infectious disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter "*Bd*"), is increasingly recognised as a primary driver of these "enigmatic declines", as well as being the most devastating vertebrate disease ever recorded (Fisher et al. 2009; Skerratt et al. 2007; Becker & Zamudio 2011). *Bd* is an aquatic member of the phylum Chytridiomycota, a widespread and diverse group of fungi normally found as primary degraders and saprobes in soil and water (Berger et al., 1998; Longcore et al., 1999). Together with the recently-described *Batrachochytrium salamandrivorans*, now known to be responsible for declines of salamanders in Europe, it forms a genus unique amongst the Chytridiomycota for its pathogenicity to a vertebrate host (Berger et al., 1998; Martel et al., 2013). Pathogenesis remains somewhat obscure (Kilpatrick et al., 2010), but in metamorphosed individuals arises from infection and disruption of the epidermis, compromising the critical role the amphibian skin plays in maintaining electrolyte balance (Baitchman & Pessier, 2013; Voyles et al., 2007, 2009; Wu et al., 2018).

The chytridiomycosis panzootic has been associated with a particular hypervirulent lineage

of *Bd,* known as the Global Panzootic Lineage (hereafter "*Bd*GPL", Farrer et al., 2011). *Bd* has been detected on over 500 species across all continents barring Antarctica (Olson & Ronnenberg, 2014). The geographic origin of the pathogen has been the subject of much speculation, with Africa (Weldon et al., 2004b), North America (James et al., 2009; Talley et al., 2015), South America (Rodriguez et al., 2014), and East Asia (Bataille et al., 2013; Goka et al., 2009) all put forward as possible original sources. O'Hanlon et al. (2018), however, recently analysed the genomes, sequenced to a high depth, of a globally representative set of 234 isolates of *Bd*. They identified East Asia as a centre of high *Bd* diversity and probable geographic origin of the pathogen. Its geographic spread has likely been facilitated by the global trade in amphibians (O'Hanlon et al., 2018; Schloegel et al., 2012; Wombwell et al., 2016) and it has been implicated in the declines of over 200 species (Skerratt et al., 2007). There is variation, however, in how species respond to exposure to the pathogen, with some persisting despite its presence. For example, the Pacific chorus frog (*Pseudacris regilla*) in California, European water frogs (*Pelophylax esculentus* and *Pelophylax lessonae*) in Switzerland, and *Litoria wilcoxii* in Australia have remained unaffected in areas where sympatric species have suffered *Bd*-driven declines (Reeder et al., 2012; Retallick et al., 2004; Woodhams et al., 2012).

There can also be variation in response between populations of the same species. For example, in the Sierra Nevada of California, *Bd* has driven the rapid extinction of many populations of the mountain yellow-legged frog, *Rana muscosa.* However, some populations have persisted despite presence of the pathogen, suggesting survival and reproduction of infected individuals (Briggs et al., 2005). A hypovirulent lineage of *Bd*, *Bd*CAPE, is believed to have arrived on the island of Mallorca in the 1980s, infecting four populations of the endangered Mallorcan Midwife Toad (*Alytes muletensis*). The responses of these populations to the pathogen has varied: two sites at which infection prevalence was consistently close to 100% showed divergent population trends, with one declining almost to extirpation while the other increased in size. These contrasting outcomes were attributed to local environment effects and not host-pathogen dynamics or variation in host or pathogen genetics (Doddington et al., 2013).

Population-level variation in response to *Bd* has, however, been demonstrated on multiple occasions in controlled experiments on animals raised from eggs in standardised laboratory conditions, a phenomenon not readily explicable by abiotic factors. Bradley et al. (2015) investigated intraspecific variation in sensitivity of wood frogs (*Lithobates sylvaticus*) to *Bd*

through exposure experiments on animals originating from 10 different populations and raised from eggs to metamorphosis. They identified a significant association between population of origin and mortality rates, and speculate that this is driven by genetic differences between populations. Bataille et al. (2015) characterised population-level variation in *Bd*-driven mortality in laboratory experiments for Australian Alpine Tree Frogs (*Litoria verreauxii alpina*), and linked this to host major histocompatibility complex (MHC) characteristics. Luquet et al. (2012) demonstrated the subtle influence of population genetic effects on response to exposure to *Bd* by showing that tadpoles descended from isolated European treefrog populations with comparatively lower genetic variability, while showing no detectable infection, took longer to metamorphose and died more quickly when exposed to high doses. They concluded that genetic erosion reduced the capacity to invest in resistance, growth, and metamorphosis simultaneously.

Genetic erosion can come about in isolated populations through increased inbreeding and the fixation of deleterious alleles, and can have negative consequences for the fitness of individuals as well as the viability of populations (Couvet, 2002; Hale & Briskie, 2007; Whiteman et al., 2006). Genetic markers offer the opportunity to examine the role that genetic variability (e.g. heterozygosity) plays in determining fitness-related traits (Chapman et al., 2009; Reed & Frankham, 2003; Szulkin et al., 2010). However, the degree to which heterozygosity at typical genetic markers is reflective of genome-wide diversity in general, and at loci that have a bearing on fitness specifically, is context-dependent. Therefore, heterozygosity-fitness correlations as measured with microsatellites are overall weakly positive, at varying magnitudes across a wide variety of natural populations (Chapman et al., 2009; Coltman & Slate, 2003; Szulkin et al., 2010). While a quantitative review of studies in this field has identified a publication bias towards the reporting of significant results, the effect of this was not thought to change the general finding of weakly positive results (Chapman et al., 2009). A reduction in genetic diversity, and consequent impact on traits related to fitness, in fragmented and island populations has been demonstrated in multiple taxa (Eldridge et al., 1999; Keyghobadi, 2007; Westemeier et al., 1998).

Traits underpinning the capacity to mount defences against pathogens, or otherwise minimise the deleterious consequences of exposure, are of critical importance to fitness. These too can be influenced by genetic diversity. In laboratory experiments on leaf-cutting ants (*Acromyrmex echinatior*), more genetically diverse populations showed greater resistance to a fungal parasite (Hughes & Boomsma, 2004). In *Drosphila melanogaster*,

inbreeding and loss of genetic diversity led to decreased resistance to a bacterial pathogen (Spielman et al., 2004). And in fish, inbred guppies (*Poecilia reticulata*) had higher parasite loads and lower capacity to clear infection. Such relationships have been shown in wild populations. In birds, New Zealand robins (*Petroica australis*) originating from a less genetically diverse reintroduced island population were found to have lower immunocompetence when compared with the larger source population (Hale & Briskie, 2007), and host population diversity correlated negatively with parasite burdens and positively with average immune responses in the Galápagos hawk (Whiteman et al., 2006). In mammals, microsatellite heterozygosity is associated with lower susceptibility to parasitism in Soay sheep (*Ovis aries,* Coltman et al., 1999), harbour seals (*Phoca vitulina*, Rijks et al., 2008), and California sea lions (Acevedo-Whitehouse et al., 2003).

In the amphibian-*Bd* system, the role played by host genetic variability in influencing response to the pathogen remains unclear. Significant associations between heterozygosity and infection or survival have been recorded, although not always in the expected direction. In the case of immune genes, Savage and Zamudio (2011) found that MHC heterozygosity was predictive of survival in experimental infections of lowland leopard frogs (*Lithobates yavapaiensis*). However, Bataille et al. (2015) did not find MHC heterozygosity to be predictive of survival in their infection experiment on alpine tree frogs (*Litoria verreauxii alpina*), instead demonstrating an association between survival and specific MHC alleles. Using putatively neutral microsatellite loci, Savage, Becker and Zamudio (2015) found a negative association between heterozygosity and *Bd* prevalence in field-sampled *Lithobates yavapaiensis.* However, Wagner et al. (2017) found no such association in a survey of 19 populations of yellow-bellied toad (*Bombina variegata*). Furthermore, Addis et al. (2015) and Horner et al. (2017) find that probability of infection is increased in more heterozygous field-sampled individuals of boreal toads (*Bufo boreas*) and ornate chorus frogs (*Pseudacris ornata*), respectively. Contrasting findings from separate field systems is not unexpected, and the potentially surprising finding of a positive association between *Bd* prevalence and heterozygosity could come about through multiple processes. Addis et al. (2015) speculate that, in their study, increased *Bd* prevalence and increased heterozygosity share the same cause: dispersal. Populations with greater levels of immigration are expected to have higher levels of heterozygosity but also higher probability of the arrival of infected individuals. Horner et al. (2017) offer two possible explanations for the same observed pattern. First, increased heterozygosity may be associated with greater effective population size, and

larger populations may better facilitate pathogen spread through density-dependant outbreak dynamics. Second, unrecorded previous outbreaks may have occurred in some populations, leading to selection for more resistant or tolerant genotypes, resulting in both lower heterozygosity and decreased incidence of *Bd*. Ultimately, however, the uncharacterised demographic, coevolutionary and epidemiological history of each unique system make the role played by host heterozygosity *per se* in mediating response to a pathogen difficult to discern from contemporary field-based data. To disentangle these issues, there is a clear need for laboratory-based experimental work examining the responses to *Bd* exposure in individuals derived from populations with no history of exposure to the pathogen.

Furthermore, aside from genetic variation, phenotypic properties such as body size may play an important role in indirectly mediating response to pathogen exposure. Given that such exposure will generally incur a cost to the host (Viney et al., 2005), it might be expected that hosts with better body condition, or greater mass, would be better equipped to incur these costs. While there is evidence that greater mass can be associated with a greater capacity to defend against pathogens and reallocate physiological resources into an immune response (Overturf et al., 2010; Wagland et al., 1984) and that superior body condition can facilitate investment in immune resources (Møller et al., 1998), the correlation between body size and resistance to a pathogen can often be negative (for example in mammals (Moore and Wilson, 2002; Harrison, Scantlebury and Montgomery, 2010)). For animals with complex life histories such as amphibians, body size at critical life stages can indeed have a major bearing on components of fitness. Greater mass at metamorphosis has been shown to be associated with increased viability and survival (Altwegg & Reyer, 2003; Beck & Condgon, 1999; Cabrera-Guzmán et al., 2013) and, in contrast to mammals, led to reduced effects of pathogen exposure (Bielby et al., 2015; Garner et al., 2011, 2009). Growth as well as body size has further been shown to be linked to multi-locus heterozygosity (Lesbarrères et al., 2007; Rowe et al., 1999).

Controlled experiments represent a valuable tool in disentangling the suite of mechanisms that drive variability in the effects a pathogen has on its host. In an extensive review of experimental studies in the amphibian-*Bd* system, Blaustein et al. (2018) make a number of recommendations. They identify a set of transparent protocols, and emphasise the importance of raising experimental individuals from eggs in standardised conditions. They also call for more studies that examine differences in population-level susceptibility. Using

controlled laboratory exposures meeting the recommendations of Blaustein et al. (2018), this study examines population- and individual-level variation in host response to *Bd* in the common toad (*Bufo bufo*). *Bd* has been detected on the skin of *B. bufo* in a number of localities across Europe including Great Britain (http://www.bd-maps.net/ [Accessed 06/03/2019]). The pathogen has been found to be associated with unusual mortalities in Spanish *Bufo spinosus* populations (Bosch & Martínez-Solano, 2006), but has not been conclusively linked with any population declines in *B. bufo*.

Partly as a result of its intermediate susceptibility to the pathogen, *B. bufo* has been used as a model species in laboratory infection experiments examining key questions surrounding the disease and its dynamics. For example, *B. bufo* tadpoles and metamorphs were used to demonstrate phenotypic variation between different isolates of *Bd* and highlight the hypervirulence of the *Bd*GPL (Farrer et al., 2011; Fisher et al., 2009b). In investigating the possible influence of climate change on host and pathogen responses, Garner et al. (2011) used *B. bufo* to show that, while a warmer overwintering temperature was associated with an increased infection probability, it was also associated with a decreased infection burden, and overwintering temperature regime did not have a significant effect on eventual mortality. *B. bufo* has also been used to demonstrate that the consequences of exposure to *Bd* are not merely restricted to infection and direct mortality, but can manifest in lower mass at metamorphosis even in the absence of detectable infection, a possible result of the reallocation of energetic resources necessary in mounting a successful immune response against the pathogen (Garner et al., 2009). A consistent finding, however, in research on *Bd* and its effects on *B. bufo* has been the importance of body size in determining the consequences to the host of exposure. Lower post-metamorphic mass is associated with decreased survival probability as a result of *Bd* exposure, more so than dose of *Bd* administered and even infection status itself (Bielby et al., 2015; Farrer et al., 2011; Garner et al., 2011, 2009).

In this study, experimental *Bd* exposure was carried out on toadlets originating from four naturally fragmented wild *B. bufo* populations to test the hypotheses that heterozygosity and body size would be positively associated with survival time, and that survival would be influenced by population of origin. The study setup therefore aims to discern between phenotypic, genetic and geographic determinants to *Bd* susceptibility for *Bd*-naïve populations sharing an environment at a small spatial scale.

3.2 MATERIALS AND METHODS

3.2.1 ETHICS

All experiments were carried out under licence from the British Home Office according to the Animals (Scientific Procedures) Act 1986 and after ethical approval from the Zoological Society of London's and Salford University's Ethics Committees.

3.2.2 STUDY SITES AND SAMPLE COLLECTION

Spawn was collected in March 2016 from four of the breeding ponds examined in Chapter 2. Two of these were mainland sites (MAK, MAT), one site was located on Skye (SKB), and one site was located on an isolated island (CRO) in the Inner Sound between Skye and the Applecross Peninsula (see Figure 3.1). Different clutches were sampled by collecting sections of approximately 100 eggs from individual egg strings. Spawn was transported to animal facilities at the Institute of Zoology, Zoological Society of London, in March 2016. These are assumed to be *Bd*-naïve populations though no extensive survey of the *Bd* status of UK amphibian populations has taken place since 2008. During that survey, the closest and most northerly site with a positive record for *Bd* was over 280 km south of this area in Dumfriesshire, Scotland (Cunningham & Minting, 2008).

3.2.3 ANIMAL HUSBANDRY

Clutches (*n* = 19, 3-6 clutches per population) were hatched in captivity in outdoor animal facilities in the Institute of Zoology, Zoological Society of London, housed separately in 9 L polypropylene boxes (Really Useful Products LTD, UK) containing dechlorinated aged tap water and an air-driven sponge filter system. Partial (approximately 20%) water changes, as well as feeding using crushed Tetra Tabimin tablets, were carried out every two days. After metamorphosis, individuals were housed by clutch in 360 x 210 x 160 mm terrestrial tanks (Faunarium, ExoTerra ®, UK) lined with moist paper towelling and containing a cover object, and fed hatchling crickets *ad libitum.* 90 days after the start of metamorphosis, toadlets were weighed and housed individually in 1 L polypropylene boxes (Really Useful Products LTD, UK) lined with a moist paper towel and containing a cover object. Experimental procedures took place indoors in a temperature controlled room at 18°C with a 12:12 h light:dark cycle.

3.2.4 BD EXPOSURE TRIALS

202 metamorphosed individuals originating from 19 clutches were randomly allocated into one of two experimental treatments: (I) exposure to a high dose of active *Bd* culture or (II) a

control group exposed to culture media alone. Treatments consisted of 9 bath exposures (3 per week) over a 21-day period. Bath exposures were carried out by placing each metamorph in a Petri dish filled with 30 ml of aged tap water and the appropriate media for four hours per exposure. The average dosage applied per session in the exposed group was 148,500 (range 40,500 – 382,500) zoospores per exposure in 450 µl of culture media, a high dosage for this species (Blaustein et al., 2018). Zoospore concentrations were calculated by counting live spores using a haemocytometer. The *Bd* isolate used was UK CORN'12 3.1, part of the global panzootic lineage, *Bd*GPL, collected from an alpine newt in Cornwall, United Kingdom in 2012. Mortality was recorded daily, and dead animals were removed and stored in 70% ethanol. Previous *Bd* infection experiments on the same species and life stage have been run for 24 (Bielby et al., 2015) or 40 (Garner et al., 2009) days. For an improved measure of survival rates, this experiment was run until 50 days after the initial exposure, at which point all surviving animals were euthanised according to Schedule 1 of the Animals (Scientific Procedures) Act 1986.

3.2.5 DNA EXTRACTION AND PCR PROTOCOLS

Experimental metamorphs were genotyped at 11 previously-characterised *B. bufo* microsatellite loci (*Bbuf*µ11, *Bbuf*µ13, *Bbuf*µ15, *Bbuf*µ24, *Bbuf*µ39, *Bbuf*µ46, *Bbuf*µ49, *Bbuf*µ54, *Bbuf*µ62, *Bbuf*µ63, and *Bbuf*µ65 (Brede et al., 2001)) (Table 2.1). DNA was extracted from the hind foot using a Qiagen DNEasy extraction kit following the manufacturers protocol (Qiagen, UK). Concentration of extracted DNA was quantified using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA), and standardised to approximately 10 ng/µl. Microsatellite loci were amplified by PCR using the protocols described in Brede et al. (2001), with the exception of *Bbuf*µ11 which was found to yield more product at an annealing temperature of 56°C as compared with the recommended temperature of 44°C. All PCR reactions were carried out in a Veriti™ 96-well thermal cycler (Applied Biosystems™, UK). The total reaction volume was 10 µl, and contained 10 ng of genomic DNA, 5 pmol of each primer, 0.5 U Taq polymerase (BIOTAQ™, Bioline Reagents Ltd, UK), 0.15 mM of each dNTP, 2 mM MgCl2, and 1 µl of 10x NH4 reaction buffer. Primers were labelled with fluorochromes and amplified DNA fragments were separated by capillary electrophoresis using an ABI 3130 Genetic Analyser (Applied Biosystems). Fragments were sized using Peak Scanner Software v1.0 (Applied Biosystems). The hind foot of each dead experimental metamorph was also sampled for screening for the presence of *Bd*, using the

qPCR assay protocol described in Boyle et al. (2004). DNA was extracted using Prepman™ Ultra (Applied Biosystems), and samples were analysed in duplicate using a TaqMan ® qPCR assay (Applied Biosystems) with four size standards representing 100, 10, 1, and 0.1 *Bd* zoospore genomic equivalents (*Bd*GE), and a negative control.

3.2.6 GENETIC AND STATISTICAL ANALYSES

Observed (H_o) and expected (H_e) heterozygosities were calculated using the software GENEPOP 4.4 (Rousset, 2008). Allelic richness values for each population were calculated using FSTAT (Goudet, 1995). To evaluate the factors influencing survival in response to exposure to *Bd*, a set of survival analyses were performed. Survival analysis is a class of statistics that focuses on the distribution of survival times, or time to an event, for a set of individuals within a particular observation window. In this instance, a set of survival times of metamorphs recorded for 50 days after initial exposure to *Bd* is considered. A common feature of such data is censoring, which applies to missing data pertaining to individuals for whom no event (hereafter "death") is recorded, either because they were lost from the data set beyond a particular time point for reasons other than death, or because they were alive when the observation period was terminated. In the case of this study, for example, the survival times of individuals still alive at the termination point are unknown, but they are known to exceed 50 days. The Kaplan-Meier (hereafter "KM"), or product limit, estimate, is a nonparametric method for calculation of the survival function of a sample, i.e. probability of survival to a particular time, which incorporates these incomplete observations (Kaplan & Meier, 1958). A graphical plot of the KM estimate against time consists of a series of declining steps, with each vertical line indicating a time point at which one or more deaths has occurred. If the dataset contains two or more groups of interest, for example population or size class in this study, their contrasting survival functions can be compared by plotting the KM curves separately. The statistical significance of any difference between the survival times of two groups can be formally tested using the logrank test (Peto & Peto, 1972). This test is based on the chi-squared statistic, and calculates whether the observed number of deaths in each group differs significantly from that expected under the null hypothesis that the survival functions are identical between groups. A Cox Proportional Hazards (hereafter "CPH") regression model extends this test by modelling the effect of multiple predictor variables and allowing the incorporation of continuous variables (Cox, 1972). This model regresses the hazard function on the explanatory variables. The hazard function can be

regarded as the probability of an individual experiencing death during a particular time interval having survived to that time interval. In a proportional hazards model, each covariate has a multiplicative effect on the baseline hazard function, either increasing or decreasing risk of death. This proportional effect is assumed to be constant across time, e.g. a covariate that doubles the hazard function at one time point should do so at all time points. The assumption of time-independence can, and should, be tested by examining the relationship between Schoenfeld residuals and time (Schoenfeld, 1982). Schoenfeld residuals represent the difference between the observed covariate and that expected for each death given the risk set for that time point. This is calculated for each individual for each covariate. If the relationship between the set of Scheonfeld residuals and time is non-random, then the assumption of proportional hazards has been violated. The CPH coefficient expresses the proportional effect per unit of the explanatory variable on the hazard function per unit time. A positive coefficient indicates an increased risk of death, and a negative figure indicates that the covariate in question is protective against death.

KM curves were produced to compare the survival rates between populations and also between large and small animals (for this, the entire sample was median-split into two groups based on mass, following the approach used by Garner et al. (2011) in creating categories of high and low mass loss and infection burden). CPH analysis was carried out to examine the effect of population of origin, individual mean heterozygosity, Bd infection burden (log transformed zoospore genomic equivalents ($\log_{10}$(BdGE +1)) and body mass (cg). Each variable was tested to confirm it met the proportional hazards assumption and then evaluated individually with non-significant terms removed for the final model. These tests were carried out using the "Survival" package version 2.38 (Therneau, 2015) in *R* 3.5.0 (*R* Core Team, 2018)).

3.3 RESULTS

Table 3.1 summarises genetic variability parameters for each population. 202 individuals were genotyped across four populations (range 31-73 individuals) at 11 microsatellite loci, with a PCR success rate of 90%. High levels of allelic diversity were detected overall, with all loci polymorphic in all populations. CRO, the isolated island population, exhibited the lowest levels of heterozygosity and allelic richness.

Table 3.1 Genetic variability parameters for 4 *Bufo bufo* populations included in experiment. Characterised at 11 microsatellite loci.

Population type	Site	*n*	A/L	AR	H_o	H_e	PA
Island	CRO	31	4.73	4.48	0.56	0.61	4
Mainland	MAK	73	6.91	5.96	0.63	0.67	12
Mainland	MAT	54	7.27	6.14	0.59	0.68	11
Mainland (Skye)	SKB	44	6.27	5.73	0.58	0.67	9

n, number of individuals sampled; A/L, mean number of alleles per locus; AR, allelic richness; H_o, observed heterozygosity; H_e, expected hetrozygosity; ML, number of monomorphic loci; PA, number of private alleles.

Within 50 days of the initial exposure, 72 (71%) of 101 metamorphs exposed to *Bd* died, whereas all of the 101 control animals survived. All experimental mortalities showed detectable *Bd* infection; 35% (10/29) of exposed animals surviving to the end of the experiment were recorded as infected, and no *Bd* was detected in the unexposed control group. Increased infection burden ($\log_{10}$ (*Bd*GE +1)) significantly decreased survival probability (CPH coefficient 0.45, p <0.001, Table 3.3). There was a tendency for infection burdens of experimental mortalities to decrease with time, though this relationship was significant in only one population (CRO, Spearman rank correlation coefficient -0.67, p = 0.02, see Figure 3.4). Population of origin was a predictor of survival probabilities (logrank test = 12.6, df = 3, p = 0.006) with eventual survival varying between 18% (4/22) for SKB and 52% (14/27) for MAT (see Table 3.2). When compared to the population in which survival was highest (MAT), population membership significantly determined mortality when controlling for the effect of mass and infection (CPH coefficients ranging from 0.926 to 1.046, see Table 3.3). On a population level, isolation status and allelic richness did not predict survival. CRO, the isolated population showing the lowest allelic diversity, had higher survival than two of the three mainland sites (Figure 3.1). Individual mean heterozygosity did not predict survival rates (likelihood-ratio test = 0.12, df = 1, p = 0.729, and see Figure 3.4). Higher mass, however, significantly increased an individual's probability of survival following exposure to *Bd*, with an additional 1 cg leading to an approximately 4% decrease in mortality risk per day when controlling for population and infection burden (CPH coefficient in combined model: -0.038, p = <0.001, see Table 3.3 and Figure 3.4). The dataset was median split by mass into a large and a small group. Figure 3.3 shows Kaplan-Meier curves comparing survival between the two groups, which differed significantly in survival rates (logrank test = 26.8, df = 1, p <0.001). Median survival was 27 days for "small" individuals

and 46.5 days for “large” individuals. Individual mass itself did not vary significantly between populations (ANOVA: $p = 0.3$) and was not correlated with heterozygosity (Spearman rank correlation coefficient 0.05, $p = 0.6$).

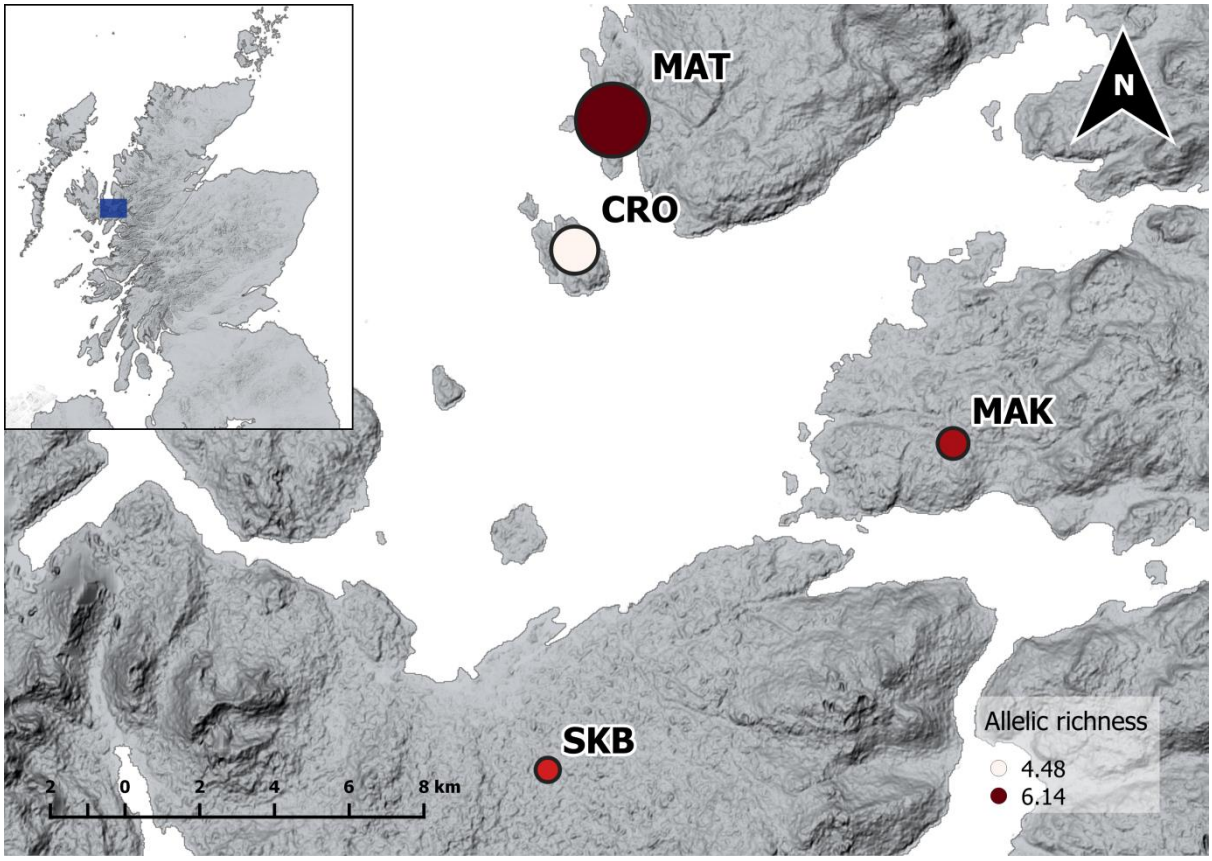

Figure 3.1 Map of source populations for *Bd* exposure experiments. Icons are sized according to proportional survival after *Bd* exposure. Icon colour intensity reflects allelic diversity. Inset shows the location of the study area in Scotland.

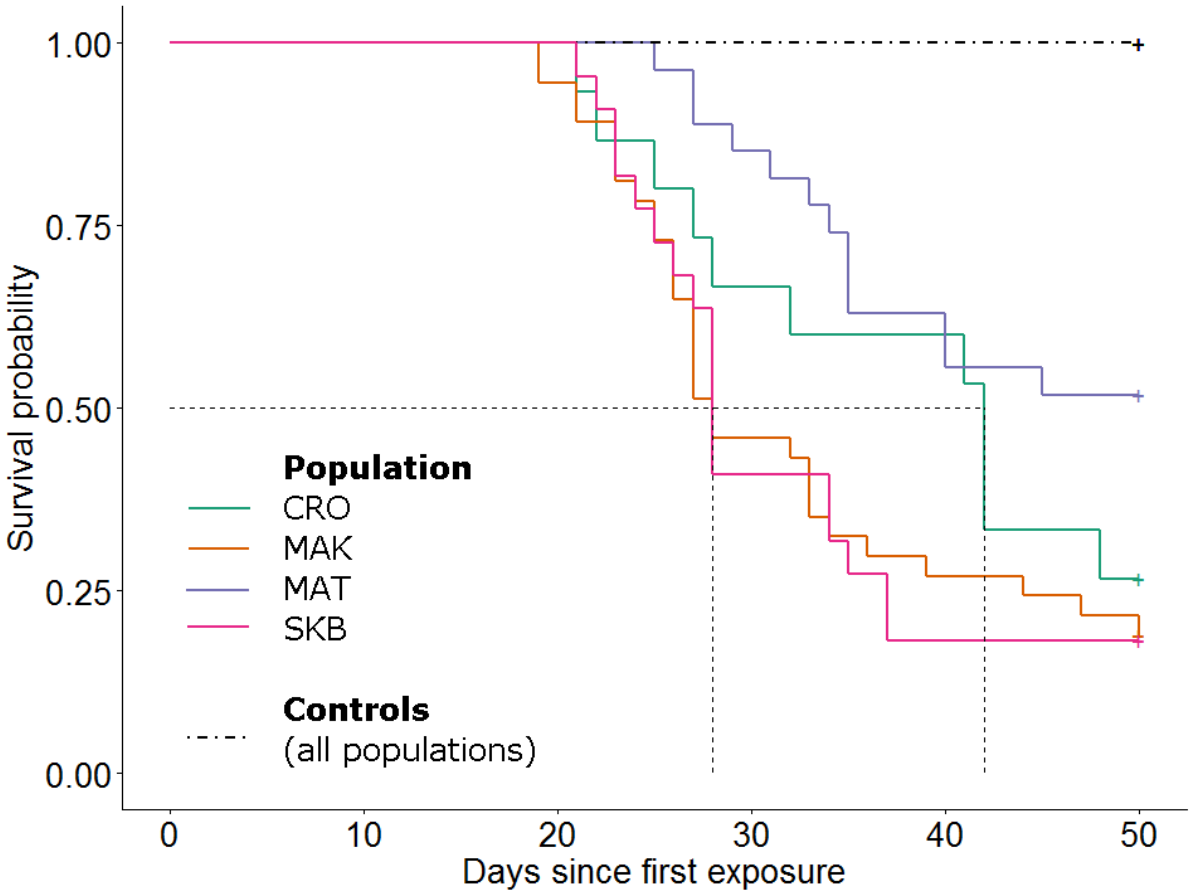

Figure 3.2 Kaplan-Meier curves comparing the survival of four common toad populations experimentally exposed to *Bd*. All control animals from all populations survived (dashed-dotted black line). Dotted black lines show median survival time of all populations excepting MAT, which exceeded 50% survival.

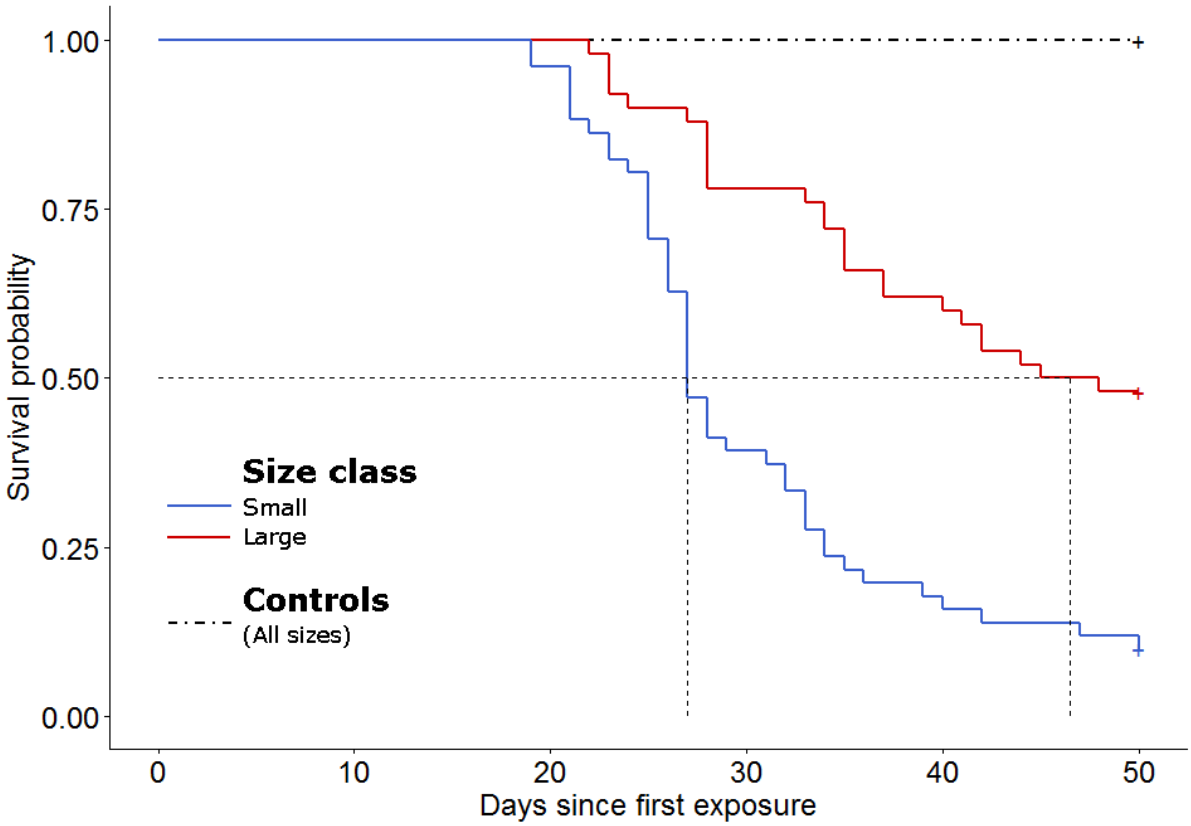

Figure 3.3 Kaplan-Meier curves comparing survival of different size classes of metamorphs. All control animals (dashed-dotted black line) survived. Size classes were formed by median-splitting by mass.

Table 3.2 Population survival rates, median survival duration, and infection prevalence subsequent to exposure to *Bd* in *Bufo bufo* metamorphs. *Median survival time not applicable to MAT population as survival to 50 days exceeded 50% in this group.

Population	Population type	Total exposed	Total surviving	Population survival rate	Median survival time (days)	*Bd* prevalence
MAT	Mainland	27	14	0.52	NA/>50*	0.70
MAK	Mainland	37	7	0.19	28	0.86
SKB	Mainland (Skye)	22	4	0.18	28	0.86
CRO	Isolated island	15	4	0.27	42	0.80

Table 3.3 Cox Proportional Hazard (CPH) model for survival of Common Toad metamorphs exposed to *Bd*. Model included mass (per cg), infection burden (log_{10} *Bd*GE), and population of origin (for which CPH coefficients are with reference to the population with the highest survival, MAT). The non-significant term of heterozygosity has been removed.

	Coefficient	SE	z	*P*
Mass (cg)	-0.038	0.009	-4.080	<0.001***
*Bd*GE	0.450	0.080	5.607	<0.001***
Populations (relative to MAT)				
CRO	0.926	0.418	2.216	0.03*
MAK	0.937	0.344	2.722	<0.01**
SKB	1.046	0.382	2.735	<0.01**

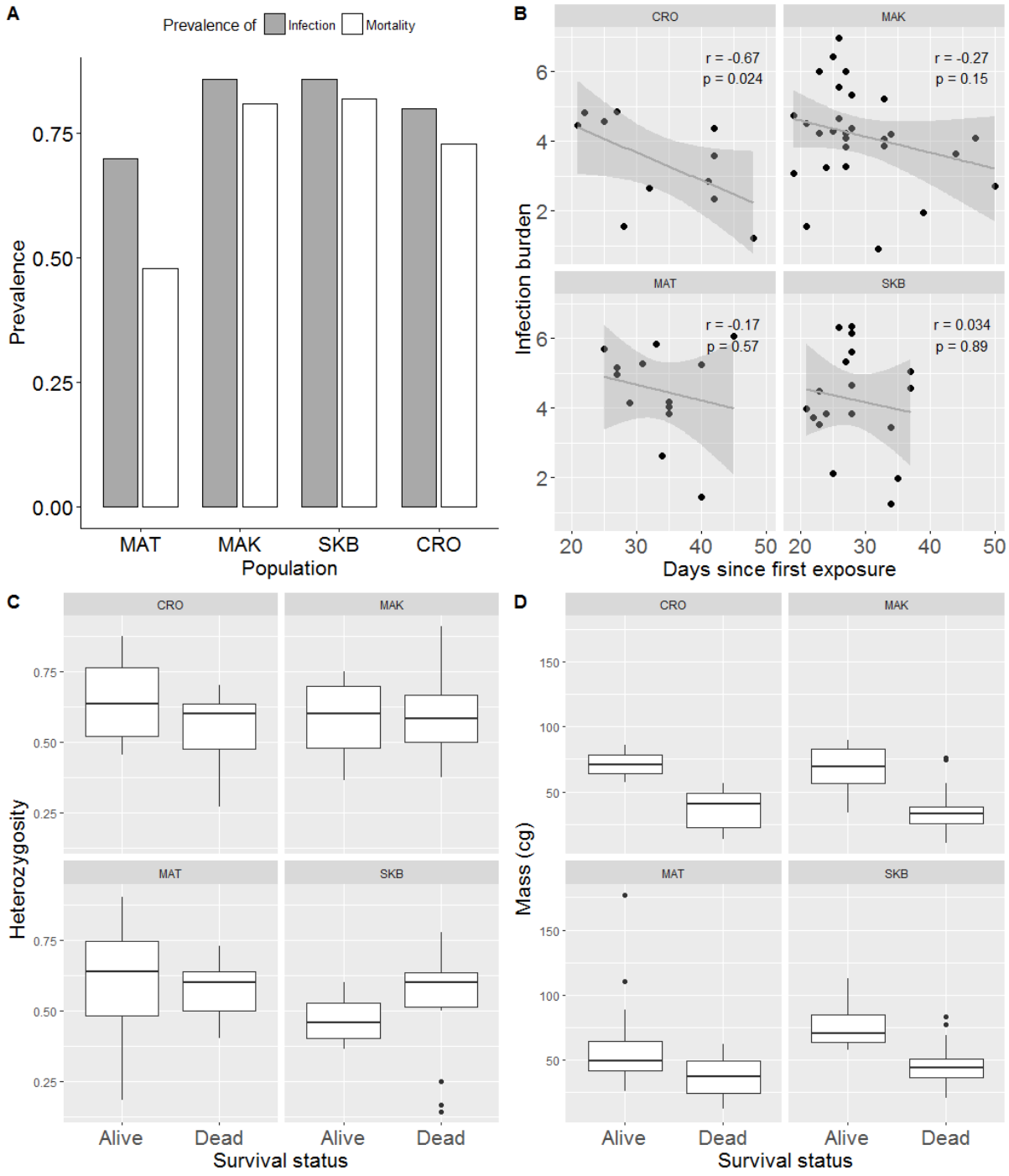

Figure 3.4 Responses of four *Bufo bufo* populations to exposure to *Bd* (**A**) Variation in infection prevalence and mortality rates between populations. (**B**) Spearman rank correlation of Infection burdens (log_{10} *Bd*GE) against time of death (in days since initial exposure to *Bd*) recorded for all individuals that died prior to termination of the experiment. (**C**) Boxplots comparing distributions of mean multilocus heterozygosity and (**D**) masses (cg) between individuals that survived until the end of the experiment and those that died, for each of four populations.

3.4 DISCUSSION

Emerging in the 20th century, the amphibian-killing pathogen *Bd* is the cause of the most devastating panzootic ever recorded. Its reach is global, and the species it is capable of

infecting number in the hundreds. The circumstances in which it is found are thus diverse, and its impact varies. Negative consequences, such as disease-driven mortality and extinction, are highly context dependent, and resolving the factors that mediate susceptibility remains challenging. Each affected wild population has its own history of exposure to the pathogen and exists in its own unique set of potentially shifting biotic and abiotic environmental parameters. Controlled experiments are therefore crucial in developing our understanding of this host-pathogen system and disentangling its principal drivers.

Since 1999, over 100 experimental studies have been carried out on the amphibian-*Bd* system (Blaustein et al., 2018). They have provided insight into the role played by factors such as temperature (Andre et al., 2008; Sonn et al., 2017), pathogen dose (Carey et al., 2006; Garner et al., 2011, 2009), pathogen strain (Doddington et al., 2013; Farrer et al., 2011; O'Hanlon et al., 2018), host species (Bielby et al., 2015; Gervasi et al., 2017; Searle et al., 2011), host developmental stage (Abu Bakar et al., 2016; Ortiz-Santaliestra et al., 2013; Rachowicz & Vredenburg, 2004), microbiome (Bates et al., 2018; Burkart et al., 2017; Jani & Briggs, 2018), host behaviour (Venesky et al., 2011), and, as is reviewed and investigated here, host size, population identity, and genetic architecture (Bataille et al., 2015; Bradley et al., 2015; Burrow et al., 2017; Palomar et al., 2016; Savage & Zamudio, 2011). This study harnessed the standardisation that a laboratory environment affords, and found that, once other biotic and abiotic factors are held constant, *Bd*-induced mortality in amphibians with no prior evolutionary or lifetime history of exposure to the pathogen was predicted by population of origin and individual size, but not by heterozygosity at microsatellite loci.

A significant relationship between body size and survival is consistent with previous findings, both in *B. bufo* (Bielby et al., 2015; Garner et al., 2011, 2009) as well as across several other species (Burrow et al., 2017; Carey et al., 2006; Kriger et al., 2006; Searle et al., 2011). In amphibians, size is generally associated with increased viability and probability of survival (e.g. Beck and Condgon, 1999; Altwegg et al., 2003; Cabrera-Guzmán et al., 2013). In the context of *Bd*, host body condition may confer a greater capacity to reallocate physiological resources in response to the costs associated with pathogen exposure, as has been proposed in other host-pathogen systems (Møller et al., 1998; Overturf et al., 2010; Wagland et al., 1984). In addition to the general increase in vigour that greater size can confer, allometric scaling relationships may render smaller animals especially vulnerable to a skin disease. *Bd* infection is confined to the epidermis in post-metamorphic animals, where it compromises

the osmoregulatory function of the skin, leading to electrolyte imbalance and associated pathologies (Voyles et al., 2009). Given that skin surface area and metabolic rate scale allometrically with mass in amphibians (Klein et al., 2016; White et al., 2006), smaller animals may be especially vulnerable to the disruption caused by cutaneous *Bd* infection. This phenomenon has been demonstrated in experimental *Bd* exposures of green tree frogs (*Litoria caerulea*), in which smaller infected animals exhibited higher rates of energetically costly skin sloughing, and suffered greater rates of ion loss than larger counterparts (Wu et al., 2018). While variation in body size in amphibians can be mediated by genetic factors (Lesbarrères et al., 2007; Rowe et al., 1999), this important phenotypic trait can also vary in response to environmental change. Research on long-term field data on *B.bufo* population demography, breeding success and morphometrics in the UK has identified a negative relationship between female toad body size and a warming climate, which was associated with decreased survival and fecundity (Reading, 2007). These findings, coupled with a projected expansion of the range of *Bd* in temperate zones of the Northern Hemisphere associated with a warming climate (Xie et al., 2016), suggest that the role played by host body size in amphibian chytridiomycosis may warrant greater attention.

Survival rates in response to exposure to *Bd* were also significantly associated with population of origin. This is consistent with previous experimental and field-based research (Addis et al., 2015; Luquet et al., 2012; Savage et al., 2015). It seems reasonable to propose that some characteristics of host genetic architecture drive such idiosyncratic population-level responses in standardised experimental conditions. It remains unclear what these characteristics are. Animals derived from an isolated island population (CRO) showed higher survival rates than those originating from two of the mainland populations (MAK, MAT), both of which displayed higher population-level allelic richness; moreover, individual-level heterozygosity was not predictive of survival probability. While susceptibility to *Bd*-driven mortality and infection has been linked to decreased variability in microsatellite markers (Luquet et al., 2012; Savage et al., 2015), this association has not been consistently identified (Wagner et al., 2017), and indeed the inverse relationship has been reported (Addis et al., 2015; Horner et al., 2017; May et al., 2011). Beyond neutral markers, characteristics of genes in the major histocompatibility complex (MHC), which encode a family of cell-surface receptors central to the adaptive immune response, have been found to be associated with response to *Bd*. While heterozygosity at MHC loci has been linked with resistance in at least one context (Savage & Zamudio, 2011), there is increasing evidence that *Bd*-resistant

populations and individuals share particular MHC alleles (May et al., 2011; Savage & Zamudio, 2011), or characteristics of MHC peptide-binding pockets (Bataille et al., 2015), and that there are signs of directional selection at a particular MHC allele associated with survival in a laboratory infection experiment (Savage & Zamudio, 2016). Such features, and other important aspects of genetic architecture, tend not to be detectable through microsatellite-based analysis, which can even show contrasting patterns. For example, in comparisons between *Bd*-infected and *Bd*-uninfected wild populations of natterjack toad (*Bufo calamita*), May, Zeisset and Beebee (2011) found that while the *Bd*-infected population had higher levels of microsatellite diversity, it had reduced levels of MHC diversity, compatible with directional selection for particular alleles. However, scope for interpretation remains limited in such studies, and discerning the role of host genetic architecture in shaping interactions with *Bd* is made more complicated if interactions with *Bd* have had the opportunity to shape host genetic architecture.

By conducting controlled exposure experiments on animals originating from fragmented populations naïve to *Bd*, but raised from egg stage in standardised conditions, the present study excluded the possible roles of environmental factors, epidemiological history, and evolutionary adaptation in explaining patterns of response to pathogen exposure. Population-level variation in mortality was clearly demonstrated, and warrants further examination, but the role of genome-wide heterozygosity as measured at 11 microsatellite loci was not predictive of survival. A more detailed investigation of host genome characteristics may therefore help reveal the genetic factors that predict susceptibility to this emerging infectious disease.

CHAPTER 4 PROBING THE POWER OF HIGH-THROUGHPUT SEQUENCING TO CHARACTERISE GENETIC STRUCTURE AND IDENTIFY LOCI INFLUENTIAL IN ADAPTATION AND DISEASE

4.1 INTRODUCTION

Since the advent of polymerase chain reaction technology in the 1980s, molecular markers such as microsatellites have become essential tools in addressing a wide range of ecological questions through the characterisation of polymorphisms between individuals and genetic groups (Selkoe & Toonen, 2006). Microsatellites offer the potential to affordably genotype non-model organisms at multiple homologous co-dominant loci and, given their high mutation rates, are especially attractive for studies examining recent population divergence. However, drawbacks such as unclear mutational mechanisms, and the need to develop distinct primer sets for each species, still limit their utility. More recently, Single Nucleotide Polymorphisms (SNPs) have emerged as a powerful alternative molecular marker, offering the potential to genotype individuals at thousands of loci.

A SNP is a specific single base pair position along a sequence at which the nucleotide present can vary between individuals in a population, with the least common variant existing in at least 1% of individuals (Brookes, 1999). SNPs generally exist in one of two alternate states and are therefore biallelic. Though tri- and tetra-allelic loci can and do exist, they are extremely rare (Brookes, 1999 and references therein). As a consequence, SNPs are less polymorphic than microsatellites when considered individually. However, they are far more abundant in the genome, accounting, for example, for 90% of sequence variation in humans (Collins et al., 1998).

The development and ever-decreasing cost of next generation sequencing (NGS) technologies, enabling the parallel sequencing of thousands of DNA molecules, has unlocked the utility of SNPs for ecological and evolutionary studies (Andrews et al., 2016). NGS involves sequencing-by-synthesis. In this approach, DNA is fragmented into small pieces, and modifications such as barcode indices for sample identification and adaptors to enable attachment to the flow cell of a sequencer are added. On the flow cell, each fragment is amplified into a clonal cluster of forward strands through bridge amplification. To initiate sequencing, primers and fluorescently labelled dNTPs, each of which has a reversible 3' blocker to ensure that only one nucleotide is incorporated per round of sequencing, are then added. After each nucleotide is incorporated into the complementary strand, it is identified and recorded through laser excitation and imaging of its unique florescent dye, before this is

enzymatically cleaved off for a new cycle to begin. The length of the read is determined by the number of cycles. Each strand is read simultaneously within a given cluster and this process is carried out across hundreds of millions of different clusters in parallel (reviewed in Metzker, 2010).

The rapidly increasing throughput and decreasing cost of these technologies has enabled whole genome sequencing of entire study populations of focal species, such as *Drosophila melanogaster* (Lara et al., 2012) and humans (Indap et al., 2010). However, costs are still sufficiently prohibitive that whole genome sequencing of populations of non-model organisms carried out by smaller teams of investigators remains unfeasible. Strategies to reduce the representation of the genome, and thus increase the number of individuals affordably assayed, are therefore necessary (Davey et al., 2011). Restriction site-associated DNA sequencing (RADseq) has arisen in the last decade as an attractive technique for identification and genotyping of thousands of genetic markers across large numbers of individuals in non-model organisms at reasonable costs (Andrews et al., 2016; Davey & Blaxter, 2010). RADseq combines NGS technology with the utilisation of restriction enzymes, similar to the approach used for amplified fragment length polymorphisms (AFLPs, Vos et al., 1995). Genomic DNA is digested using a restriction enzyme, which recognises and cuts the DNA at a specific sequence of nucleotides. These “cut sites” number in the thousands, are distributed throughout the genome and, if no mutations have occurred within each short sequence, are homologous between individuals of the same species. Sequencing adaptors and unique sample-specific molecular barcodes are ligated to the resulting DNA fragments. A consistent set of RAD loci can thus be produced by sequencing the fragments of DNA adjacent to each cut-site (Baird et al., 2008). While the earliest variants of RADseq involved a mechanical shearing step to reduce the fragment size to an appropriate length for sequencing, resulting in variable fragment lengths at each locus (Baird et al., 2008), recent developments of the method have used a second restriction enzyme to further reduce the complexity of the genome (Peterson et al., 2012; Truong et al., 2012). This double digest RADseq approach (dd-RAD) uses a combination of a rare-cutting enzyme and a frequent-cutting enzyme, creating an even distribution of fragments across the genome. Size selection is achieved either by gel cut or by preferential PCR amplification of small fragments (Andrews et al., 2016).

The power of RADseq techniques to affordably characterise thousands of genetic markers is increasingly being applied to ecological and evolutionary questions in non-model systems.

For example, Guo et al. (2016) examined the genetic basis for adaptation to high altitude in amphibians by pooling samples for each of 11 populations of Andrew's toad (*Bufo andrewsi*) distributed across an altitudinal gradient on the edge of the Tibetan Plateau. Using RADseq, they genotyped 15,557 SNPs and identified evidence of isolation by distance, as well as outlier loci putatively associated with local adaptation to altitude and temperature, though the functions of most of these were uncharacterised. Emerson et al. (2010) illustrated the potential for RADseq to reveal fine-scale phylogenetic divergence in a study-system for which no genomic resources had previously existed. They pooled DNA from six individuals from each of 21 populations of pitcher mosquito (*Wyeomyia smithii*) in eastern North America. Using 3,741 SNPs, they resolved both ancient divergence between a northern and a southern group, and recent postglacial divergence within the northern group. They highlight that these patterns were irresolvable using allozymes or mitochondrial sequences and further argue that the dense genome-wide sample of loci that RADseq provides removes the risk of sampling variation inherent in the significantly smaller number of loci typically assayed in microsatellite analysis.

The dd-RAD technique specifically has been deployed to investigate adaptation to local environmental conditions in wild populations. In investigating population structure of greenlip abalone (*Haliotis laevigata*) along an approximately 800km stretch of the southern Australian coast, Sandoval-Castillo et al. (2018) used dd-RAD to define a set of neutral and putatively adaptive loci. They showed low differentiation amongst neutral loci, but discrete structure was revealed in the distribution of adaptive loci, associated with sea surface temperature and oxygen concentration. Amongst these loci were genes associated with high temperature or oxygen tolerance. Similarly, dd-RAD was used by Termignoni-García et al. (2017) to highlight a pattern of isolation by environment in the distribution of putatively adaptive loci in the Yucatan jay (*Cyanocorax yucatanicus*), a bird endemic to the Yucatan peninsula. They showed low spatial genetic structure amongst neutral loci, but proposed that climatic conditions drove patterns of variation amongst candidate adaptive loci.

Studies using RADseq to examine host defence against pathogens in wild populations are beginning to emerge. Batley et al. (2018) used dd-RAD to identify candidate genes involved in susceptibility to Cetacean morbillivirus (CeMV), a highly infectious virus thought to be involved in unusual mortality events in cetaceans. They compared samples from 17 Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) who had died during an unusual mortality event in South Australia in 2013, and had tested positive for CeMV, with 21 "control"

samples taken from live, and presumably healthy, individuals within the same population. Out of 35,493 genotyped SNPs, they found 65 for which allele and genotype frequencies differed significantly between “cases” and “controls”, of which they identified eight candidate genes for disease risk, with functions relating to pain, stress, and immune responses.

The potential for SNP markers to reveal the genetic underpinnings of host defence against *Bd* remains largely unexplored. Kosch et al. (2018) used Diversity Array Technology sequencing (DArTseq) to genotype 3,245 SNPs for use in a Genome-Wide Association Study (GWAS) based on a *Bd* infection experiment using individuals sampled from four populations of the southern corroboree frog (*Pseudophryne corroboree*). DArTseq uses a combination of restriction enzymes to target low-copy DNA areas, thus preferentially representing areas more likely to contain genes (Melville et al., 2017, and references therein). The GWAS failed to find significant SNPs associated with *Bd* load or *Bd*-driven mortality, however the most significant variants were homologous to genes associated with immune response and host reproduction.

This chapter examines the increased capacity over traditional microsatellite markers of a SNP dataset, generated through dd-RAD, to characterise patterns of genetic variability in a set of individuals sampled from four common toad (*Bufo bufo*) populations. The same individuals are genotyped using both types of markers, with the same genetic analyses applied to the resulting datasets. Additionally, preliminary investigations are made into identifying loci that may be involved in defence against *Bd* exposure.

4.2 MATERIALS AND METHODS

4.2.1 STUDY SYSTEM AND SAMPLE ORIGINS

Samples originated from the individuals exposed to *Bd* in the experiment described in Chapter 3. Briefly, common toad (*B. bufo*) spawn was collected from a fragmented set of four populations on the range edge of the species distribution in north western Scotland. These were raised through metamorphosis in standardised conditions and exposed to *Bd* in a controlled experiment, with the consequent survival duration recorded. See Chapter 3 for details. In order to meet the constraints imposed by the dd-RAD procedure on number of individuals it was reasonably possible to process, the original set of 101 exposed individuals was reduced to 95 by randomly removing 6 individuals from the most well-represented

population (MAK). The resulting panel of samples constituted 15 individuals from CRO, 31 from MAK, 27 from MAT, and 22 from SKB.

4.2.2 DNA EXTRACTION PROTOCOL, DD-RAD LIBRARY PREPARATION, AND SEQUENCING

Chapter 3 describes the protocol for production of the microsatellite dataset. For dd-RAD SNP genotyping, the following procedure was followed: DNA was extracted from the hind foot of each toadlet using a Qiagen DNEasy Blood and Tissue extraction kit following the manufacturers protocol (Qiagen, UK). DNA concentration was assessed by fluorometry using a Qubit 3.0 (Thermo Fisher Scientific, MA, USA), and was normalised to 20 ng/uL. To verify standardised concentration, and ensure extracted DNA was of high molecular weight and free of contaminants and degradation, every sample was visualised on a 1.5% agarose gel. Preparation of dd-RAD libraries and next generation DNA sequencing were performed by Floragenex (OR, USA) following the protocol of Truong et al. (2012). Complexity of genomic DNA was reduced by double-digesting with a combination of rare and frequent cutting endonucleases (*Pst*I and *Mse*I, respectively), followed by ligation with Floragenex adaptors with individual indexes. 1x100bp single end sequencing was performed on the resulting PCR-generated library using an Illumina HiSeq 4000 (CA, USA).

4.2.3 DE NOVO SNP DISCOVERY AND GENOTYPING USING STACKS

Raw sequences were filtered and demultiplexed using the process_radtags pipeline in STACKS 2.0 (Catchen et al., 2013). Reads with an uncalled base were discarded, as were reads containing a 15 bp window in which the average quality dropped below a phred score of 10 (i.e. a 90% probability of being correct). Barcodes and RAD-tags containing 1 mismatch to an expected sequence were retained.

In the absence of a reference genome for *B. bufo*, a *de novo* assembly was produced and analysed using STACKS. STACKS consists of a number of pipelines that together take raw reads and, based on sequence similarity, identify putative alleles and loci within an individual sample, before building a catalog of alleles and loci for the population of samples. Broadly speaking, the principal parameters in STACKS control the levels of sequence dissimilarity tolerated within alleles and loci, and thus define the loci, and ultimately the reads, that are incorporated into the analysis. Parameter selection requires balancing the risk of failing to distinguish between convergent sequencing errors and genuine polymorphisms against that of merging paralogous loci together.

Firstly, across the thousands of raw reads within each individual sample, exactly matching

reads are assembled together into “stacks”. Each stack can be thought of as a putative allele. The minimum number of matching reads that must be present for a stack to be retained for further analysis is set by the *m* parameter. If, for example, *m* is set to 3, any stacks containing just two matching reads are set aside and considered secondary reads, which can be incorporated at a later stage. As *m* is increased, so too is the risk of rejecting genuine alleles and not incorporating them in future analyses. However, decreasing *m* increases the risk of erroneously considering reads with convergent sequencing errors as distinct alleles. Once a set of putative alleles is created, these are then merged into putative loci, which are identified based on sequence similarity between stacks. Within an individual sample, the level of dissimilarity permitted between alleles within a locus is controlled by the *M* parameter. Setting *M* therefore controls the balance between undermerging and overmerging loci. If set too low, a genuine polymorphic locus might be falsely considered as two monomorphic loci. If set too high, distinct repetitive or paralogous loci may be incorrectly merged together as single loci. Once a set of loci are identified, the secondary reads set aside in the initial step can be incorporated using a more relaxed nucleotide mismatch value (set by the *N* parameter). The next stage of the procedure takes the data thus far produced for each individual, and builds a catalog of loci containing all the loci and alleles in the population. A locus that is polymorphic across a population may be monomorphic and differentially fixed within individuals. It is therefore necessary to identify and merge such loci across samples to form the correct catalog locus. The number of nucleotide differences permitted within catalog loci is set by the *n* parameter. Similar considerations apply to setting this value as do to the *M* parameter (the number of mismatches permitted between stacks in merging them into loci within an individual): setting it too low risks falsely considering a single polymorphic locus as multiple independent loci, and setting it too high risks incorrectly merging together multiple distinct but similar loci. Failure to correctly characterise polymorphic loci differentially fixed in different groups of individuals is especially undesirable as these loci are particularly informative in population genetic analyses.

The optimal values for each parameter will vary dependent on the dataset involved, governed by multiple factors including the level of polymorphism and ploidy in the system being investigated, the type of RAD procedure used, and issues stemming from library quality such as degradation of DNA and contamination (Paris et al., 2017). Thus, given the idiosyncrasy of each dataset, there is no pre-defined set of parameter values to deploy, and

these must instead be ascertained by examining the effect of a range of values on the patterns and numbers of alleles and loci identified. For this study, the procedure developed by Paris, Stevens, & Catchen (2017), and further elaborated by Rochette & Catchen (2017), was followed to identify an optimal set of parameter values for analysis.

Firstly, to enable efficient testing of parameter values, a subset of samples representing the population was selected. This consisted of between one and two representatives of each clutch in the database, numbering 35 individuals in total. The STACKS *de novo* pipeline was then run a number of times while varying parameter values and measuring the number of polymorphic loci found across at least 80% of all samples (the *r80* loci, per Paris, Stevens, & Catchen, 2017) to identify a stable set of values. The minimum stack depth, *m*, was initially kept to the default value of 3, which performs well across a variety of datasets (Paris et al., 2017; Rochette & Catchen, 2017). *M* and *n,* which control the number of mismatches permitted between two alleles of an individual heterozygote locus, and two alleles in a locus across a population, respectively, were kept equal and run for every value from 1 to 9. By visualising the results of each of these runs, a set of parameter values was chosen to apply to the entire dataset. There are no explicit criteria that unambiguously define an optimal set of parameter values, but, largely following Rochette & Catchen (2017), values above which the number of widely shared polymorphic loci, the depth of coverage, and the distributions of SNPs per locus plateau were selected (see section 4.3.1 and Figure 4.2).

The chosen parameters were then applied to the whole dataset. Including every sample in the catalog of population loci adds noise (Rochette & Catchen, 2017), so the ten samples showing the highest coverage in each population (40 samples in total) were selected to form the catalog. SNP genotype calls were improved using the rxstacks component of STACKS. Using this unit, genotype calls were recomputed within an individual based on population-wide haplotype frequencies, and single catalog loci that matched multiple loci (i.e. confounded loci) in more than 10% of samples were removed. A new catalog was then produced with the improved genotype calls included and the problematic loci removed.

Prior to exporting the genotype calls from STACKS for downstream analysis, the dataset was filtered. In addition to only retaining loci present in at least 80% of samples (the *r80* loci), loci with a minor allele frequency lower than 0.05 and an observed heterozygosity greater than 0.70 were removed.

4.2.4 GENETIC ANALYSES

Exploratory genetic analyses were implemented using the ADEGENET package in *R* on both the microsatellite and SNP datasets (Jombart, 2008; *R* Core Team, 2018). Principal Component Analysis (PCA) was first performed to visualise structure in the patterns of genetic variability between individuals. After transforming the data using PCA, Discriminant Analysis of Principal Components (DAPC, Jombart et al., 2010) was used to identify clusters of genetically related individuals. While PCA characterises the overall variability between individuals, including both between-group and within-group variability, DAPC seeks to maximise between-group variation whilst minimising within-group variation. Individuals were assigned into genetic clusters using *k*-means, a clustering algorithm that identifies a given number (*k*) of groups. This was performed without providing prior information on the populations of origin, and was run sequentially using increasing values of *k*. These runs were compared using the Bayesian Information Criterion (BIC), with the preferred value of *k* defined by the elbow of the curve of BIC values when graphed against increasing values of *k.* To assess the number of SNP loci necessary to confidently assign individuals to the correct genetic cluster, the SNP dataset was sub-sampled with 10 random draws for successive numbers of loci with the proportion of individuals accurately assigned plotted against the number of SNP loci.

The *R* package PCADAPT 4.0.3 (Luu et al., 2017, 2018) was used to identify SNPs putatively marking local selection. This package calculates a Mahalobonis distance (a measure of the number of standard deviations between a given point and the mean of a distribution on a principal component axis) for each SNP and detects outliers that do not follow the distribution of the main bulk of points. A list of candidate outlier SNPs was generated with an expected false discovery rate set lower than 10%. A BLAST search (Zhang et al., 2000) was then run with the sequences associated with these SNPs against the NCBI nucleotide collection (nt) database with a $1e^{-05}$ e-value cut-off. To assess the influence of outlier loci in forming genetic structure as characterised through PCA, a second PCA was performed on the SNP dataset with outlier loci removed.

A preliminary analysis was carried out to identify SNP loci associated with survival after *Bd* exposure. Each population was median-split into “susceptible” and “resistant” groups based on number of days survived during the experiment detailed in Chapter 3. A DAPC was then performed within each population using each of these groups as priors. SNP loci most contributing to variation along the resulting discriminant function were identified.

4.3 RESULTS

4.3.1 DE NOVO SNP DISCOVERY AND GENOTYPING USING STACKS

The number of reads retained after demultiplexing, verification of RAD-tags, and quality filtering ranged between 1.30 and 5.11 million per individual, with mean number of reads at 3.50 million. This was after removal of one sample from the SKB population, which was excluded from the data set from this point forward due to the abnormally low number of reads returned (< 15,000). Average reads per individual ranged between 3.31 and 3.67 million across the four populations (see Figure 4.1).

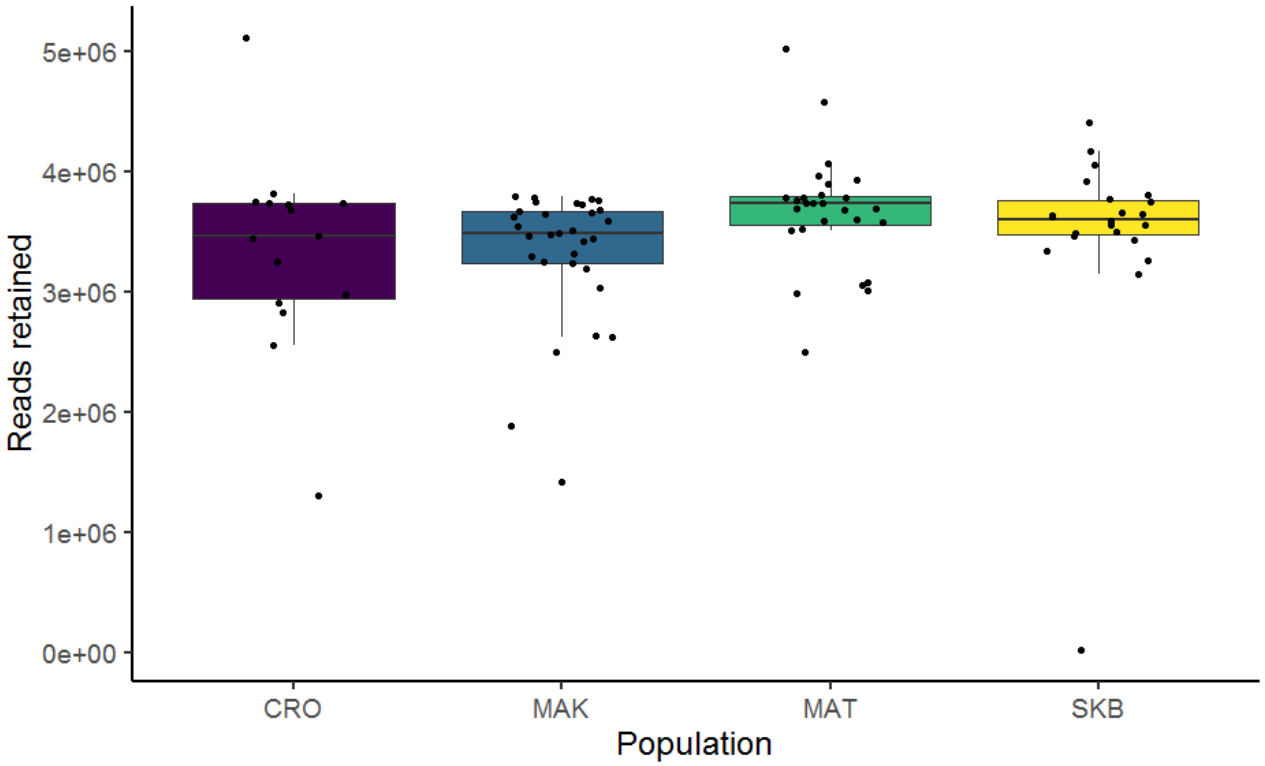

Figure 4.1 Number of reads retained for each population after initial demultiplexing and quality filtering. One sample from the SKB population was removed due to the low number of reads.

The number of SNPs and polymorphic loci identified by the STACKS pipeline on the test subset of samples steadily decreased with increasing values of the *M* and *n* parameters while the *m* parameter was fixed at 3. No single parameter value resulted in a major shift in the patterns of loci identified, but the depth of coverage per locus, the number of polymorphic loci returned, and the distribution of the number of SNPs per locus all stabilised at *M* and *n* = 4 (Figure 4.2). This value was chosen for further analysis on the entire dataset, resulting in a final selection of 10,884 polymorphic loci shared by 80% of all individuals after filtering.

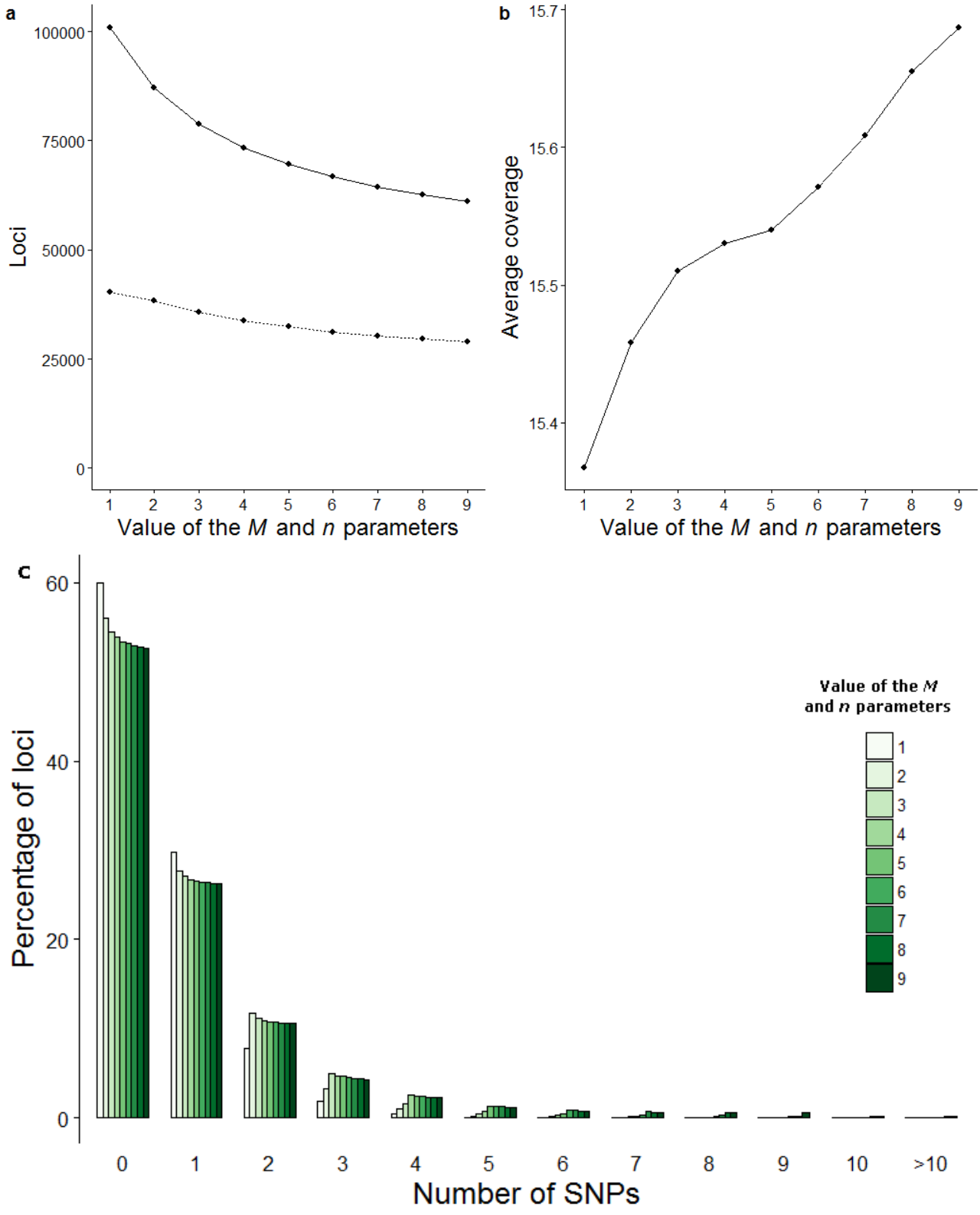

Figure 4.2 Selection of STACKS parameters for *de novo* analysis. The number of loci (solid line) and polymorphic loci (dashed line) shared by 80% or more of the test dataset gradually decreased with increasing value of the *M* and *n* parameters (**a**) with an accompanying minor increase in average coverage depth which plateaus between 3 and 5 (**b**). This is also the range at which the proportions of loci with 1-4 SNPs stabilises (**c**).

4.3.2 GENETIC ANALYSES

PCA on the SNP dataset revealed a probable occurrence of mislabelling, in which two samples which were originally processed consecutively were found to cluster well within each other's population. It was judged that these samples had been swapped, and they were removed from both datasets for any subsequent analysis.

The microsatellite dataset did not show discernible differentiation between the three mainland populations. However, CRO, the isolated island population, was clearly separated, especially along the second PC axis (Figure 4.3, a). Minimal structure was resolved along the third axis Figure 4.3, c). In contrast, the SNP PCA showed clear separation between, and tight clustering within, all four populations along the first three PC axes (Figure 4.3, b and d).

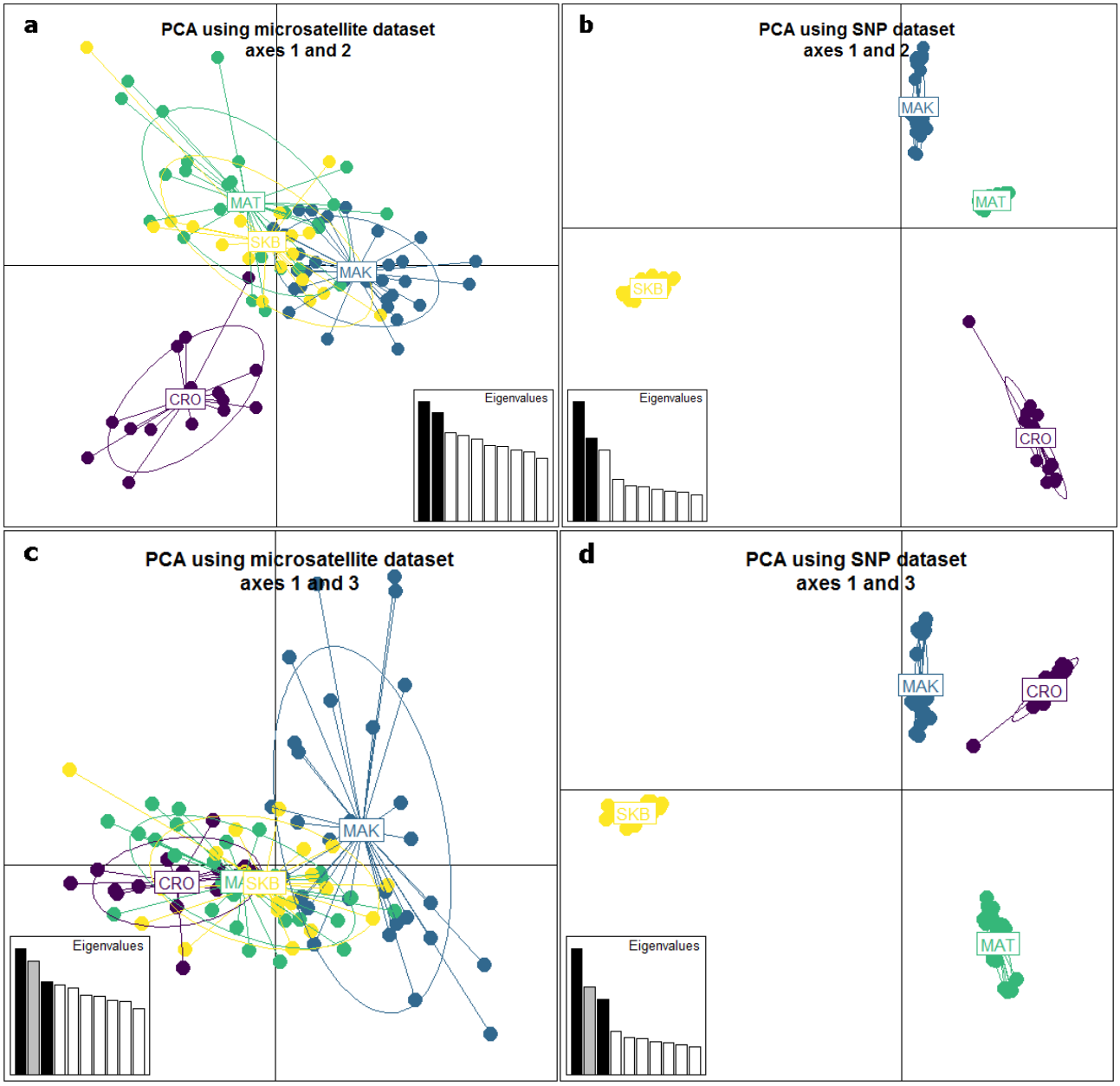

Figure 4.3 Comparison of PCA plots generated from microsatellite and SNP dataset analyses. Using the microsatellite dataset, the three mainland populations are not well differentiated along the first two PC axes (**a**) and no structure is apparent along the third axis (**c**). By contrast, PCA based on the SNP dataset reveals four well differentiated groups across the first three PC axes, corresponding to four populations from which the individuals were sampled (**b, d**).

The number of clusters inferred by the *k*-means algorithm, based on the value of *k* at which the elbow of the BIC curve occurred, was 4 for both the microsatellite and SNP datasets (Figure 4.4). This is equal to the number of populations from which the individuals were sampled. However, assignment of individuals into genetic clusters based on microsatellites did not universally correspond with the population of origin (Figure 4.5, a). The isolated island, CRO, was again well differentiated, with 13 out of 15 (87%) individuals assigned into

the same cluster. Assignment of individuals from the other three populations into clusters was more ambiguous: the inferred cluster containing the most individuals from the MAK population contained just 15 out of the 30 members of that population (50%). The equivalent figures for MAT and SKB were 14 out of 27 (52%) and 15 out of 20 (75%), respectively. Overall, interpreted this way, accuracy of assignment into inferred genetic clusters using the microsatellite dataset was 62%. Four clusters were also inferred based on the SNP dataset but, in this case, the assignment of individuals into these clusters corresponded 100% with the populations of origin (Figure 4.5, b). The filtered SNP dataset contained 10,884 loci, but random sub-sampling of these loci showed that assignment into the correct cluster was near perfect using just 30 loci or more (Figure 4.6).

A DAPC plot based on the microsatellite dataset illustrated its increased power to discriminate between groups, with the three populations MAK, MAT, and SKB, formerly indistinguishable based on PCA alone, mostly clustering together along the first two principal components (Figure 4.7, a). The corresponding plot based on the SNP dataset shows strong discrimination between the four original populations (Figure 4.7, b).

PCADAPT analysis identified 531 loci as potential outliers. The comparative lack of genomic resources for this species, and amphibians in general, limited interpretation of these. A BLAST search against the NCBI nucleotide collection (nt) database yielded results for 18 of the associated sequences (e-values ranging from $8e^{-06}$ to $1e^{-39}$). 5 of the 10 most significant matches were to amphibian species, but these were not adequately characterised to infer function. Removal of the outlier loci from analysis had a negligible effect on the PCA plot (Figure 4.8).

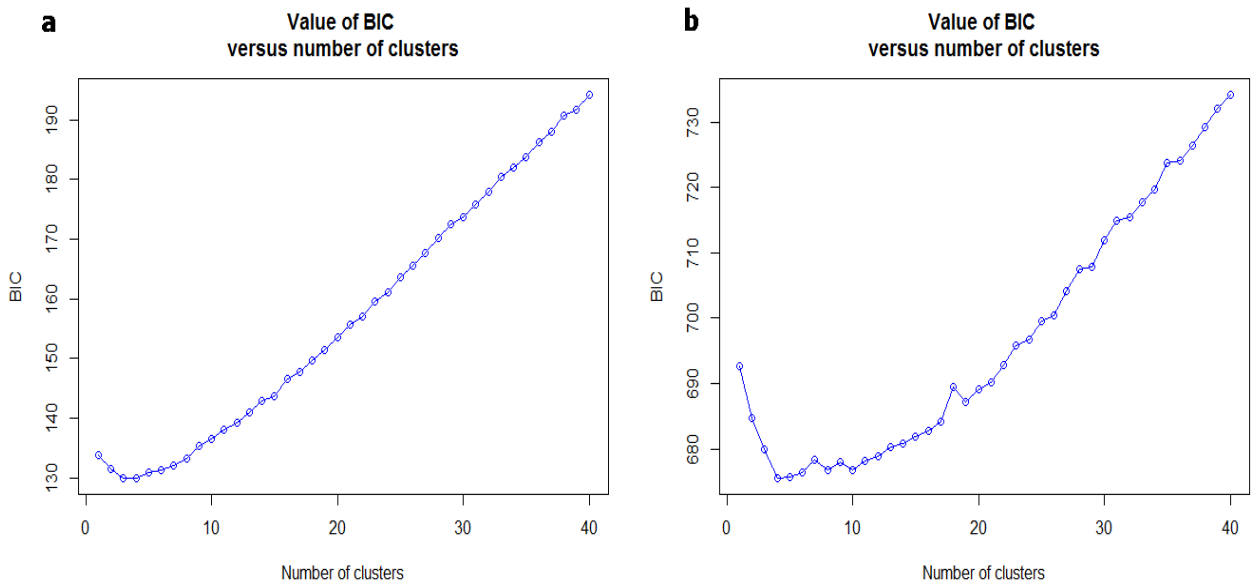

Figure 4.4 Inference of the number of clusters identified in the microsatellite and SNP datasets The chosen number of clusters, based on the elbow of the curve of BIC values, was 4 for both the microsatellite (**a**) and SNP (**b**) datasets.

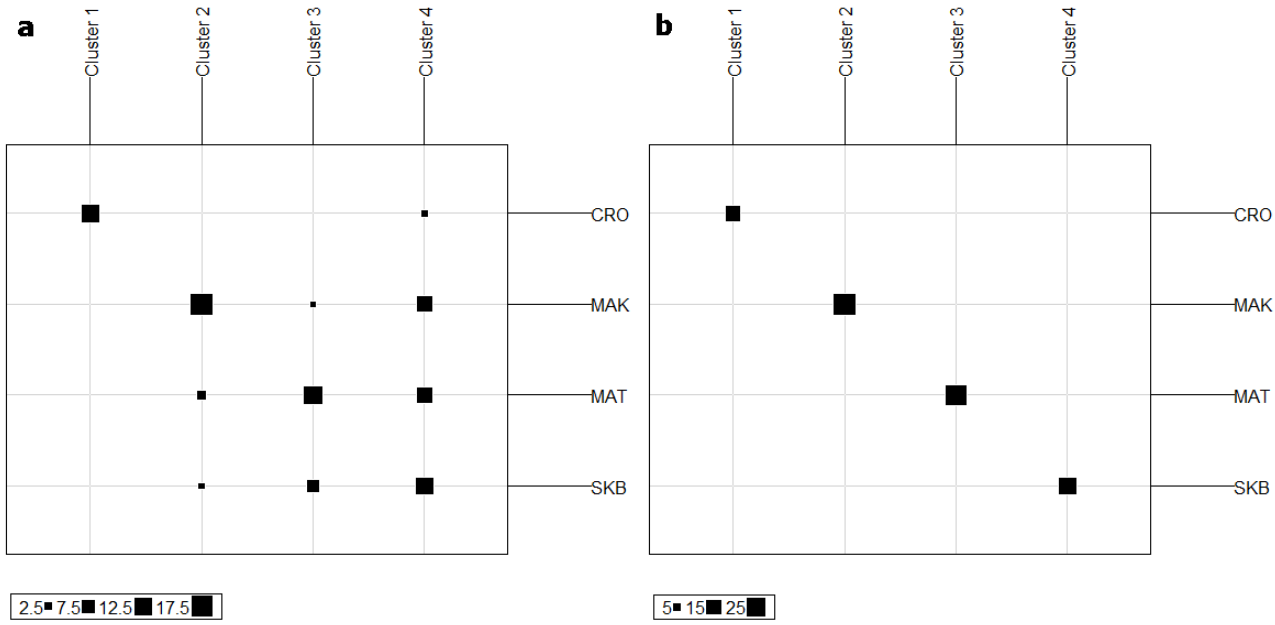

Figure 4.5 Assignment of individuals into genetic clusters inferred by *k* means algorithm. Using the microsatellite dataset (**a**), 62% of individuals were assigned into the cluster that best represented their population. Assignments into genetic clusters based on the SNP dataset corresponded perfectly with population of origin (**b**).

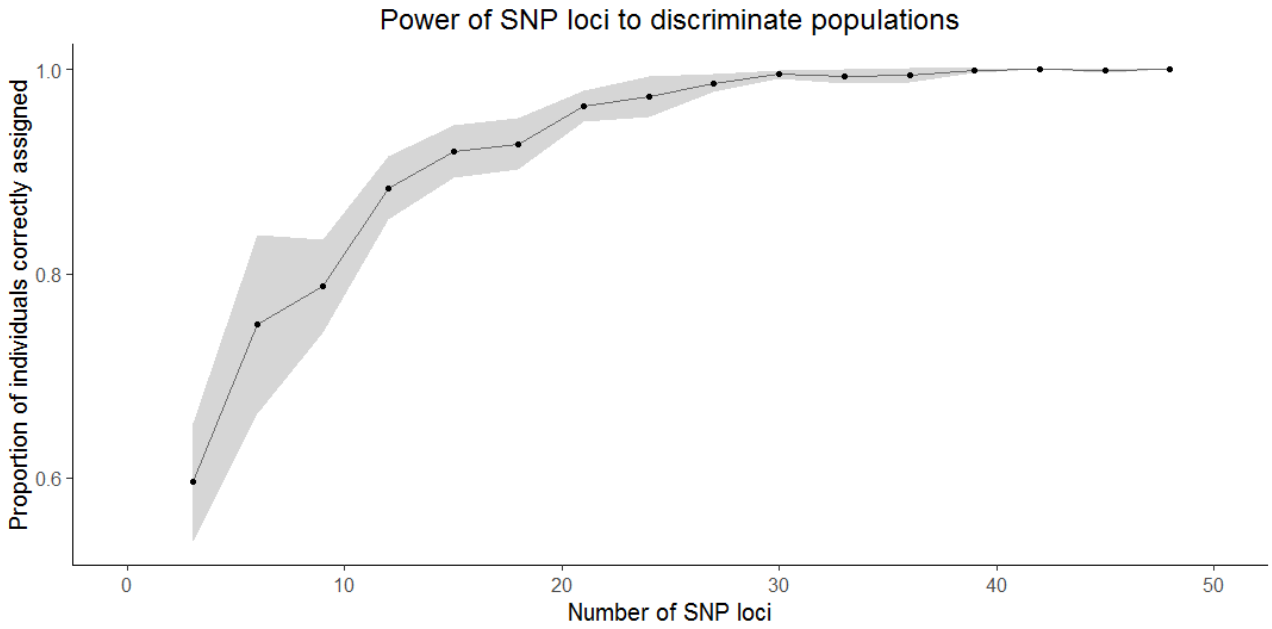

Figure 4.6 Power of SNP loci to correctly assign individuals to populations.

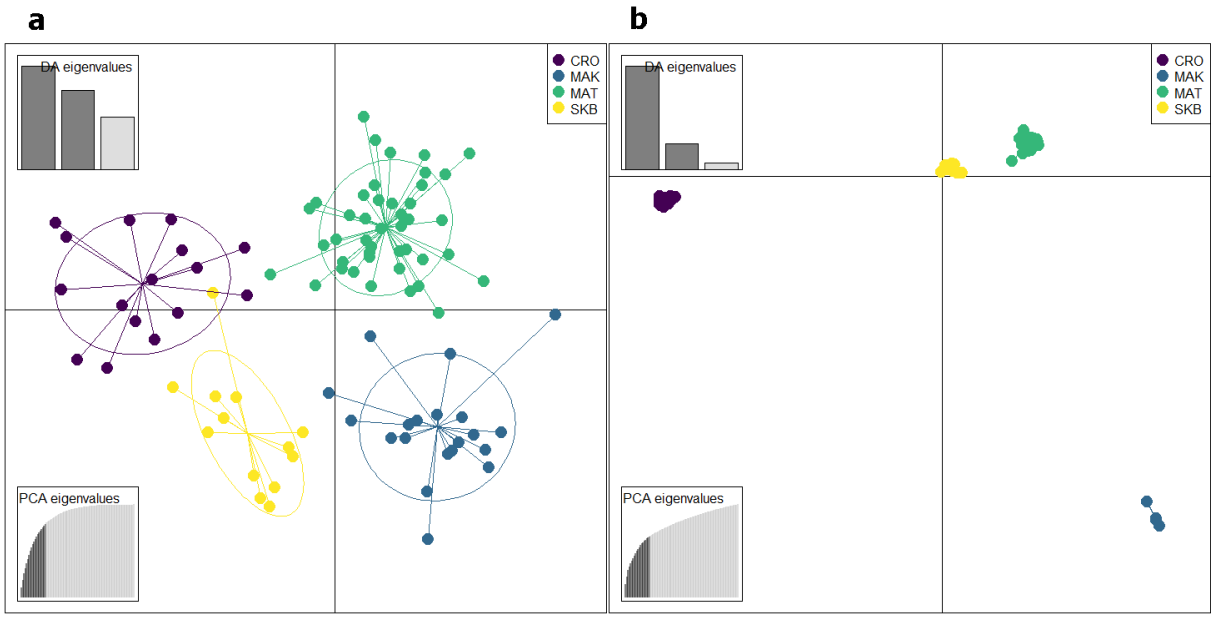

Figure 4.7 Comparison of DAPC plots generated from microsatellite and SNP dataset analyses DAPC based on the microsatellite dataset (**a**) shows increasing power to discriminate between groups, with however considerable overlap remaining along the first two PC axes. In contrast, DAPC on the SNP dataset (**b**) tightly clusters individuals within their respective populations, which are themselves well separated along both axes.

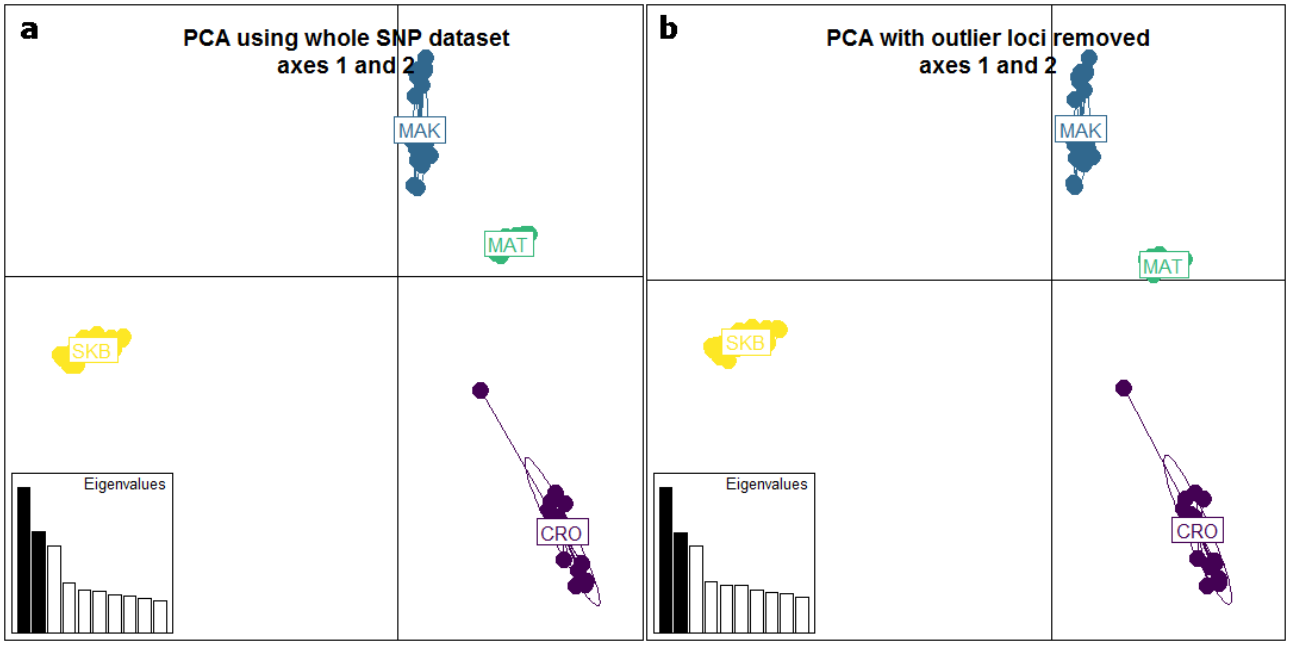

Figure 4.8 PCA plot generated using entire SNP dataset (a) compared with one produced after removal of outlier loci identified through PCAdapt analysis (b) Removal of 531 potential outliers resulted in a negligible change in the PCA.

Table 4.1 The 10 most significant matches arising from a BLAST search of outlier loci identified through PCAdapt analysis

Accession number	Description	Bit score	E value	Identities	Gaps
GU129140.1	*Salmo salar* IgH locus B genomic sequence	172	1.00E-39	93/93 (100%)	0/93 (0%)
AC146680.3	*Gasterosteus aculeatus* clone CH213-10I3, complete sequence	139	1.00E-29	81/84 (96%)	0/84 (0%)
AJ512448.1	*Rana lessonae* transposon lesBam, clone 2	128	2.00E-26	75/78 (96%)	0/78 (0%)
AC175575.2	*Xenopus tropicalis* clone ISB1-101C8, complete sequence	121	4.00E-24	65/65 (100%)	0/65 (0%)
XM_015958244.1	PREDICTED *Nothobranchius furzeri* alpha kinase 1 (alpk1), transcript variant X3, mRNA	119	1.00E-23	89/100 (89%)	5/100 (5%)
AC146873.3	*Xenopus tropicalis* clone CH216-71K21, complete sequence	115	2.00E-22	62/62 (100%)	0/62 (0%)
AJ512447.1	*Rana saharica* transposon sahBam	115	2.00E-22	77/84 (92%)	1/84 (1%)
LN590709.1	*Cyprinus carpio* genome assembly common carp genome, scaffold: LG5, chromosome: 5	110	8.00E-21	84/96 (88%)	2/96 (2%)
CP020796.1	*Oryzias latipes* strain HNI chromosome 18	99	2.00E-17	81/95 (85%)	0/95 (0%)
AY037817.1	*Bufo bufo* microsatellite Bb46 sequence	73.1	1.00E-09	45/48 (94%)	0/48 (0%)

DAPC analysis did not show clear discrimination between “susceptible” and “resistant” groups of individuals within each population (Figure 4.9). None of the 20 alleles most contributing towards variation along the discriminant function within each population were shared across populations.

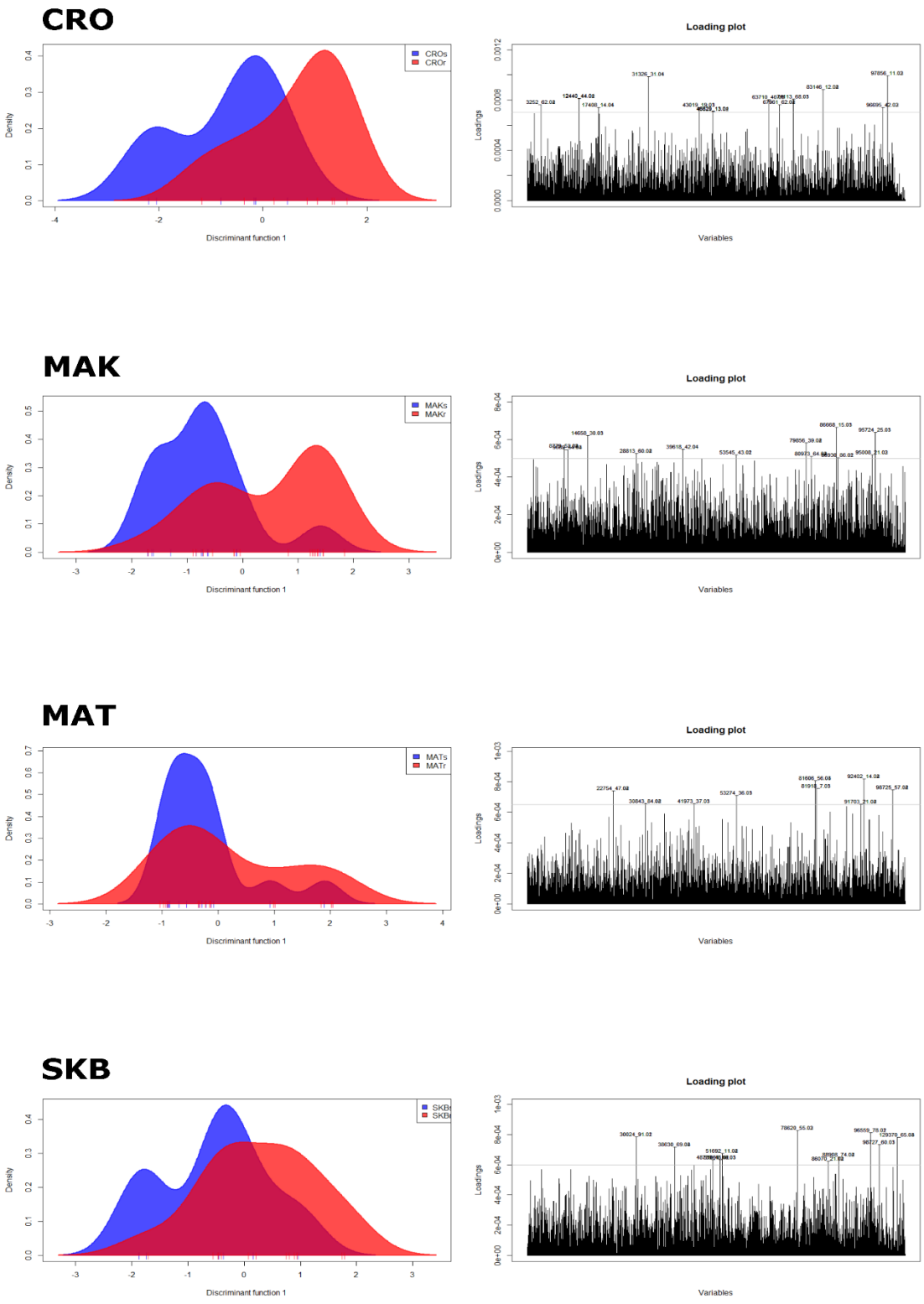

Figure 4.9 DAPC analyses of susceptible versus resistent toadlets within each population
DAPC failed to show strong discrimination between individuals who had short versus long survival times subsequent to *Bd* exposure (left). Loading plots (right) show the alleles most contributing to variation along the discriminant function within populations, none of which were shared across populations.

4.4 DISCUSSION

An asset of microsatellite markers over older technology is the potential to genotype individuals at multiple loci simultaneously (Selkoe & Toonen, 2006). This chapter illustrates the ability of SNP markers to inflate that asset almost a thousandfold. The microsatellite dataset comprised 11 loci, a number itself exceeding previous microsatellite studies on this species (Brede & Beebee, 2004; Luquet et al., 2015b; Martinez-Solano & Gonzalez, 2008; Roth & Jehle, 2016; Wilkinson et al., 2007), but this was compared with a SNP dataset numbering 10,884 loci after filtering (though it should be remembered that, while microsatellites are highly polymorphic, SNPs are biallelic). While the increase in power offered by such a dataset is impressive, great care must be taken in the type of bioinformatic processing undertaken, as choices taken here can have major impacts on the outputs of any downstream population genetic analysis (Shafer et al., 2017). In this study, the STACKS *de novo* pipeline was used, which has been shown to yield consistent results if a reference genome is absent (Shafer et al., 2017). Current best practice was followed in selecting the parameter values in this analysis, resulting in values consistent with those that perform well in other datasets (Paris et al., 2017; Rochette & Catchen, 2017).

The capacity of the increase in density of the resulting dataset to discern patterns in genetic variability was obvious. While PCA and DAPC analyses on the microsatellite dataset showed separation of the isolated island population, reinforcing the observations made in Chapter 2 on the importance of seawater in influencing population divergence in this system, there was overlap between the remaining three more connected populations, which were well resolved under the equivalent analyses using the SNP dataset. While this is likely due to the lower power offered by microsatellites, it is also worth bearing in mind that differences in patterns of variation between SNPs and microsatellites do not arise solely through the different numbers of loci assayed. The mutation rates of microsatellites are significantly higher than those of SNPs (Morin, Luikart, & Wayne, 2004, and references therein) and the markers therefore inherently reflect dynamics on different timescales. However, microsatellites are thus expected to be more sensitive to more recent population divergence, which would imply historical allopatry followed by more recent sympatry in these populations if the varying patterns reported here are taken as reflecting biological reality.

A striking aspect of this analysis was the ability of SNP genotypes generated through dd-RAD, combined with modern analytical techniques, to accurately detect patterns of genetic

variability in set of samples not optimised for population genetic inferences, taken as it was from a limited number of families. Unlike Bayesian clustering algorithms such as STRUCTURE, DAPC does not rely on pre-defined population genetics models which depend on assumptions which may be violated in this dataset. No prior information on population identities was incorporated into this analysis, yet the number of clusters, and assignment of individuals into those clusters, perfectly corresponded the origins of the samples. Figure 4.5 and Figure 4.7 offer compelling illustrations of the power of these techniques.

A feature of a set of SNP loci produced through RADseq is that it will contain a sample of coding, noncoding, and regulatory regions of the genome. It is therefore possible to identify loci involved in local adaptation or in traits such as disease response. Though in this analysis 531 loci were identified as outliers from neutral expectations with respect to population divergence, the absence of a reference genome for the study species precludes characterisation of the function of these loci. Also, in contrast to the study of Guo et al. (2016), who reported directional selection along an altitudinal gradient, this chapter did not set out to measure local adaptation, and the study sites were chosen based on ecological similarity.

DAPC failed to identify alleles consistently associated with disease response, i.e. varying survival, across populations. Similarly, Kosch et al. (2018) found no SNPs showing significant associations with *Bd*-driven mortality or infection load in their experiments. These findings are perhaps not surprising. Disease response can be a complex trait, dependent on a modest contribution of many genes together with complex gene regulatory networks (Boyle et al., 2017). Nevertheless, the dataset considered here may yet yield more insights, and be amenable to a GWAS approach similar to that employed by the Kosch et al. (2018) study. There are important considerations to bear in mind before such future research is conducted, however. It must be emphasised that RADseq only samples a subset of the genome, a matter of particular relevance for studies of amphibians, whose genomes are comparatively large. For example, the genome of *B.bufo,* the species studied here, is approximately 11 Gb in size (Vinogradov, 1998). This does not render invalid any findings of strong associations between particular loci and phenotypic traits, it is simply a reminder that a large fraction of the genome is left out of consideration in GWAS analyses based on RADseq (Lowry et al., 2017, but see McKinney et al. 2017 and Catchen et al., 2017).

For over a decade, GWASs have yielded important discoveries in human population genetics, disease biology, and therapeutics (reviewed in Visscher et al., 2017). However, association studies in wildlife populations present particular challenges (reviewed in Santure and Garant, 2018). A principal constraint is the number of wild individuals that can practicably be genotyped and phenotyped, as well as the density of markers and proportion of the genome missing from reduced representation approaches. Studies on wild populations may also involve samples that contain related individuals which, if left unaccounted for, can lead to spurious associations between genomic traits and phenotype.

While the application of GWAS to investigate the genetic traits underpinning response to disease in wild populations remains limited, there is some promise in this approach. Wright et al. (2017) sequenced the genomes of Tasmanian devils (*Sarcophilus harrisii*) that had recovered from the otherwise fatal devil facial tumour disease (DFTD), a transmissible cancer that threatens the species with extinction. They compared these to the genomes of individuals who had died, and found that survival was linked with two key genomic regions. The confirmation of a genetic basis for survival in the face of this disease may suggest the potential for the phenotypic traits associated with recovery to naturally increase in frequency in the wild, and, as the authors speculate, may even lead to novel treatments in the future.

The pursuit of an understanding of the genetic underpinnings of response to *Bd* would be greatly aided by an increased availability of amphibian genomic resources. There is information latent in the present dataset that awaits a reference genome to unlock it. Calboli et al. (2011) have argued that, given the recency and scale of the *Bd* epizootic, amphibian genomics could provide an unprecedented real-time multi-species insight into the evolution of host pathogen interaction. This, coupled with the potential utility that such insight could bring to the conservation of the world's most threatened vertebrate group, highlights the missed opportunity that the current underrepresentation (Calboli et al., 2011; Koepfli et al., 2015) of amphibians in the list of completed vertebrate genomes constitutes.

CHAPTER 5 DISCUSSION AND CONCLUDING REMARKS

5.1 KEY FINDINGS

This aim of this thesis was to examine how natural amphibian populations vary in their genetic characteristics and responses to disease and to identify predictors, both genetic and phenotypic, of *Bd*-driven mortality. In investigating these questions, the following key findings were made:

1. The populations of common toads around Skye, north west Scotland, were characterised by strong genetic fragmentation and varying levels of allelic diversity. Seawater shaped the spatial genetic structure of this system by isolating island populations, which showed lower levels of genetic variability (Chapter 2).
2. Survival in response to exposure to *Bd* varied significantly depending on the population of origin of the individuals concerned, but this was not associated with population-level allelic diversity (Chapter 3).
3. Individual heterozygosity was not associated with *Bd*-driven mortality. Body size, however, was significantly positively correlated with survival time (Chapter 3).
4. Analysis of a SNP dataset revealed genetic structuring not discernible based on microsatellite genotyping of the same individuals (Chapter 4).
5. Outlier SNPs putatively related to local adaptation were identified, but it was not possible to characterise their function. No loci with a clear association to disease response were identified (Chapter 4).

5.2 GENERAL DISCUSSION

Bd has, over the course of decades, attained a global distribution to affect over 500 species of amphibians. The global trade in amphibians has facilitated its spread but, once established in an environment, it can disperse naturally as quickly as tens of kilometres per year (Lips et al., 2008). In light of the role of chytridiomycosis as an emerging infectious disease, and its capacity to spread into otherwise pristine regions, it is of critical importance that we develop ways to identify at-risk populations in areas that have yet to be exposed to the pathogen. Through field and experimental studies, multiple putative drivers of susceptibility to disease and declines have been identified. Species identity (Gervasi et al., 2017), host immune factors (Bataille et al., 2015), host microbiome (Woodhams et al., 2014), density-dependent host-pathogen dynamics (Briggs et al., 2010) and environmental variables such as

temperature (Cohen et al., 2017), have been shown to have an influence over disease outcomes and population persistence. The role of host genetic factors, particularly genetic diversity, is a fundamental area that remains poorly understood, however.

Natural animal populations with lower levels of genetic variability are expected to be less able to respond to environmental changes and novel threats, have lower levels of fitness, and show reduced disease resistance (reviewed in Reed, 2010). Allentoft and O'Brien (2010) have proposed that amphibians are particularly vulnerable to the loss of genetic variability and its consequent deleterious effects. They argue that amphibian life-history traits, such as low dispersal rates and a tendency towards small effective population sizes, have acted synergistically with anthropogenic impacts, such as habitat fragmentation and the spread of novel pathogens, to amplify the impact of genetic effects. They further demonstrate that correlations between genetic variability and fitness traits are consistently found in amphibian studies, presenting a review of 19 publications, of which 15 found a positive correlation. The extent to which this relationship applies in the amphibian-*Bd* system remains underexplored.

Findings arising from study systems in which *Bd* is already established are poorly suited to identify predictors of disease response in hitherto unexposed populations, as they are drawn from contexts in which the pathogen and host have undergone an unrecorded period of sympatry. There is therefore a remarkable lack of research characterising the response to *Bd* exposure of amphibian populations naïve to the novel pathogen. Bataille et al. (2015) included a naïve population in their infection experiments, finding it to be intermediate in its response when compared with two historically-exposed populations, but this does not examine variation between *Bd*-naïve populations. Luquet et al. (2012) examined the responses of amphibians originating from five different populations to *Bd* exposure, suggesting that genetically eroded populations suffered greater negative impacts. However, the animals involved in the experiments were not genotyped, and the authors don't indicate whether or not there may have been any history of *Bd* exposure in the populations they investigated.

This thesis approached the question using a study system of putatively *Bd*-naïve naturally fragmented common toad populations. Chapter 2 characterised the patterns of genetic variability across 11 breeding sites, showing strong divergence between populations, especially between those separated by sea. More isolated island populations formed unique genetic clusters and were marked by lower levels of genetic diversity. This corresponds with

features that may make amphibians uniquely predisposed to a loss of genetic diversity: high philopatry to small discrete breeding sites coupled with low vagility, and in this case an inhibited capacity to disperse across seawater.

Chapter 3, however, showed that, while naïve populations showed inherently idiosyncratic responses to novel pathogen exposure, these responses were not readily predictable by population-level measures of allelic diversity or individual heterozygosity. These findings caution against a simplistic expectation that genetically eroded populations or individuals will be especially vulnerable to novel pathogens. Some animals can persist and, indeed, flourish despite low levels of genetic diversity. The Mauritius kestrel and the northern elephant seal, for example, have experienced remarkable population recoveries from severe bottlenecks, with minimal apparent impacts on fitness arising from their extremely low levels of genetic variability (Bonnell & Selander, 1974; Groombridge et al., 2000). It is also possible for populations and species to have naturally low levels of genetic variability even in the absence of recent bottlenecks. The wandering albatross, for example, may have persisted in this state for millennia while enjoying high breeding success (Milot et al., 2007). While small, genetically depauperate populations are expected to suffer inbreeding depression as a result of the expression of deleterious recessive alleles in a homozygous state, this can also lead to selection purging such alleles from the population (Pekkala et al., 2012). Populations with a history of inbreeding may therefore be less susceptible to contemporary inbreeding depression than comparatively outbred populations, a phenomenon that has been empirically demonstrated in *Drosophila melanogaster* (Swindell & Bouzat, 2006a, 2006b). The identity of the populations studied here may need to be considered in this light. I sampled from a set of sites on the range edge of a species, with populations in isolation potentially for generations. This is quite a different situation from, for example, the study performed by Hitchings & Beebee (1998). They identified deleterious consequences of genetic erosion driven by habitat fragmentation in *Bufo bufo*, but the fragmentation in this case was caused by recent barriers to migration created by urban development.

Bearing in mind the absence of any obvious increase in susceptibility in this study, it could be argued that, in some contexts, genetically eroded populations may in fact be at a lower risk of *Bd*-driven declines. The very factor that can lead to a population-wide loss of genetic variability, demographic isolation, may also serve to inhibit pathogen dispersal in a natural context. For example, Millins et al. (2018), found that, in a landscape of island and mainland

sites, the prevalence of the mammal specialist zoonotic tick-borne pathogen *Borrelia afzelii* was significantly lower on isolated islands, which they attributed to the lower densities, stronger population fluctuations, and lower immigration experienced by host populations. Both Addis et al. (2015) and Horner et al. (2017) have reported a perhaps unexpected positive relationship between *Bd* prevalence and genetic diversity in field-sampled amphibian populations. While they were unable to explore the mechanisms for this pattern in their studies, further research into the interplay between the genetic and epidemiological consequences of demographic isolation may yet prove to be an interesting line of investigation.

Ultimately, what can be learned from the genome of an organism depends on the molecular marker used. In 1994, Taylor et al. published a ground-breaking study using microsatellite markers to study a remnant population of northern-hairy nosed wombat (*Lasiorhinus krefftii*), discerning patterns of genetic variation not previously possible using mitochondrial DNA or other methods of DNA fingerprinting. Since then, microsatellites have revolutionised population genetics, providing insights into the structure and demographic history of populations and species, the relationships and origins of individuals, dispersal patterns of offspring, and the impact of landscape features on migration and population structure (Sarre & Georges, 2009; Selkoe & Toonen, 2006). Today, as novel technologies such as RADseq offer the potential to genotype individuals at thousands of loci in both coding and non-coding regions, a revolution of similar magnitude is underway (Andrews et al., 2016). Chapter 4 demonstrates the power of these technologies to discern genetic structuring in wild populations, and offers a preliminary glimpse of the potential to identify SNPs associated with local adaptation and disease response. Over the coming years, studies using these techniques to better understand the amphibian-*Bd* system are sure to multiply, and the potential to use the current dataset for deeper insight will grow in line with increased availability of amphibian genomic resources. The clarity of any findings that arise will ultimately depend on the underlying biological reality, however. The advent of next-generation SNP datasets combined with GWAS analytical techniques has revealed the complexity of the genetic underpinnings of many traits, for which even the most significant loci explain just a small proportion of the predicted genetic variance (Manolio et al., 2009), with the “missing heritability” accounted for by many SNPs across the genome with effect sizes too small to pass significance tests (Yang et al., 2010). Furthermore, the main drivers behind complex traits can often be noncoding, highlighting the role of gene regulation in

determining phenotype (Boyle et al., 2017; Li et al., 2016). It may not be realistic, therefore, to expect that GWAS studies in the amphibian-*Bd* system will reveal a handful of genes with clear mechanistic links to disease response accounting for a significant amount of the variation observed. This does not rule out a future role for genetic modification of susceptible amphibian species, as discussed, for example, by Garner et al. (2016), but it underscores the complexity of the task.

The work presented here emphasises that, in order to continue to develop our understanding of the drivers of the most devastating vertebrate disease ever recorded, there is a need for a greater investment on research on *Bd*-naïve amphibian populations, more investigations into the interaction between epidemiology and genetic factors, a more explicit focus on the influence of body size on disease susceptibility, and further investment in amphibian genomic resources. Abstract knowledge is of limited use if it is not applied, however. The research-implementation gap between science and real-world action is a significant issue in conservation biology, in which much of what is published is not of any meaningful use to conservation managers on the ground (Cook et al., 2013; Knight et al., 2008; Sunderland et al., 2009). In a recent review, Garner et al. (2016) point out that, despite decades of research into the amphibian-*Bd* system, remarkably little progress has been made in actually developing measures to mitigate the effects of the disease in wild populations, even while many researchers invoke amphibian conservation as a potential output of their work. The authors call for a more collaborative effort involving interdisciplinary teams integrating with conservation managers and deploying tools such as decision analysis to deliver informed management actions. Decision analysis refers to a group of formalised approaches to integrating available knowledge in making rational choices in the presence of uncertainty (Keeney, 1982). This technique was recently applied by Canessa et al. (2018) to examine management options in the event of an outbreak of *Batrachochytrium salamandrivorans* in the fire salamander (*Salamandra salamandra*) in Europe. While their analysis mostly served to highlight the challenges and low probability of success of the management options considered, the methodology applied serves as a template for harnessing the considerable scientific knowledge available to inform applied conservation actions in mitigating the devastating impacts of both of these widely destructive fungal pathogens.

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