Conservation Management of the Mountain Chicken Frog

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Declaration

All of the work presented in this thesis is my own, with the following acknowledgements.

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Abstract

Global biodiversity is being lost at an unprecedented rate, such that we have entered the sixth mass extinction in the history of the earth with emerging infectious diseases (EID) recognised as an important contributor to this loss. Amphibian chytridiomycosis is an EID that has driven very rapid declines in, or even extinctions of, hundreds of amphibian species. Infectious diseases such as chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), often persist in biological and non-biological reservoirs making them difficult to eradicate. In turn, this makes reintroductions of target species challenging due to the risk of infection. This thesis investigates the critically endangered mountain chicken (*Leptodactylus fallax*) as a case study of the population impacts of a chytridiomycosis epidemic and to test the effectiveness of strategies to mitigate the effects of the disease. Specifically, this research (1) charts the decline of the mountain chicken on the only two islands on which it exists, and determines the impact on genetic diversity; (2) tests whether anti-fungal treatment can improve the survival of mountain chickens with Bd infection in the wild; (3) examines the role of Bd reservoir species in causing Bd infections of reintroduced mountain chickens; and (4) determines habitat features that are predictors of infection at release sites. Chytridiomycosis drove the mountain chicken to near extinction on Dominica in 2002 and Montserrat in 2009, in one of the fastest recorded vertebrate species declines, leading to a significant loss of genetic diversity. On Montserrat, treating mountain chickens with an anti-fungal drug (itraconazole) during the chytridiomycosis epidemic improved survival rates and reduced Bd infection rates in the short term, but did not provide long-term protection. Although mountain chickens have been driven to near-extinction by Bd infection on Montserrat, the pathogen persists in two sympatric reservoir species which are not impacted by Bd infection, the most prolific of which (*Eleutherodactylus johnstonei*) displays strong seasonality in Bd infection prevalence and load. Timing mountain chicken reintroduction to occur during the period when tree frog Bd infection was at its lowest was tested to determine the impact on reintroduction success. Multi-state mark-recapture modelling applied post-release showed that optimising the timing of release reduced Bd infection rates and increased survival. Radio-tracking was utilised with geographic profiling to determine that release site water bodies were likely sources of Bd infection in reintroduced mountain chickens. This could inform targeted mitigation of the pathogen and improve future reintroduction success. Where species have been extirpated in the wild, and an irreversible threat such as an EID persists, novel reintroduction strategies are required. These include optimising the timing and conditions of release in order to minimise the impact of the threat along with targeted mitigation measures such as individual level treatments.

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1 Introduction

1.1 Global biodiversity crisis

Global biodiversity is currently facing a sixth mass extinction (Ceballos *et al.* 2015) defined by a loss of over 75% of species (Jablonski and Chaloner 1994). As we enter the Anthropocene marked by the increasing impact of humans on the ecology and geology of the globe (Corlett 2015), current extinction rates are estimated to be 100 - 1000 times higher than the background rate with 0.01-0.1% of species going extinct annually (Ceballos *et al.* 2015; De Vos *et al.* 2015). The loss of even individual species has been shown to impact the resilience of ecosystems (Gaston and Fuller 2008; Ceballos and Ehrlich 2002) which could in turn lead to the loss of further species in a 'chain of extinction'.

'Chains of extinction' are one of four threats described by Diamond (1989; 1984) as 'the Evil Quartet' alongside overhunting, habitat destruction and fragmentation and invasive alien species (IAS), and as being responsible for most of the extinctions in recent history. The pressure exerted on wildlife by each of these threats is increasing with a growing human population. Economic prosperity is driving globalisation, creating a more interconnected world and removing geographic barriers to anthropogenic threats that are readily transported to locations containing naïve species, and systems (Marano, Arguin and Pappaioanou 2007; Meyerson and Mooney 2007; Cunningham, Danzak and Rodriguez 2003).

Whilst habitat loss and fragmentation is often cited as the greatest threat to biodiversity, a recent analysis suggests that IAS are the threat most commonly associated with vertebrate extinctions post AD 1500 (Bellard, Cassey and Blackburn 2016). There are numerous mechanisms through which IAS might drive biodiversity loss, for example: predation (e.g. Ogutu-Ohwayo 1990), competition (e.g. Strayer 1999), habitat destruction (e.g. Barrios-Garcia and Ballari 2012), and as vectors (e.g. Woodworth *et al.* 2005), reservoirs (e.g. Sainsbury *et al.* 2000), and causal agents of disease (e.g. James *et al.* 2009). Whilst expensive, removal of invasive alien predators from isolated locations such as islands, where they are the greatest threat, is one of the most successful conservation measures utilised today and has a measurable conservation benefit (Jones *et al.* 2016). Invasive pathogens represent a more difficult problem which cannot be easily mitigated.

1.2 Emerging infectious diseases of wildlife

An emerging infectious disease (EID) is an infectious disease that has recently increased in incidence, impact, pathogenicity, host range or geographic range (Daszak, Cunningham and Hyatt 2000; Lederberg, Shope and Oaks, Jr. 1992). Historically, EIDs have attracted limited scientific attention unless they impacted humans or agriculture (Harvell *et al.* 1999; McCallum and Dobson 1995). However, due to the relatively recent recognition of their impact on the conservation status of wild animals (Cunningham, Danzak and Rodriguez 2003; Daszak, Cunningham and Hyatt 2000; Scott 1988) - for example avian malaria on Hawaiian birds (van Riper *et al.* 1986), rinderpest in African undulates (Plowright 1982) and canine distemper on the black footed ferret (*Mustela nigripes*; Thorne and Williams 1988) - the study of EIDs in wildlife has increased (Cunningham 2005; Daszak, Cunningham and Hyatt 2000).

Infection with a pathogen might not always result in an animal suffering from disease. Some hosts are able to persist infected with a pathogen with reduced negative fitness consequences (tolerance), whilst others might limit pathogen infection and growth (resistance) (Roy and Kirchner 2000). The ability to tolerate infection may also vary in an individual, with changing physiological states, or with changing environmental conditions (Cunningham, Danzak and Rodriguez 2003). When infection with a pathogen results in disease, it can reduce host fitness in a number of ways including death, increased susceptibility to further disease or predation, or by lowered reproductive rate (Cunningham 1996). These might have both individual and population level impacts.

Until relatively recently, there was a reluctance to acknowledge the impact of infectious disease on wildlife, in part due to classical infection theory describing a reduction in disease pressure between susceptible hosts as they undergo infectious disease driven declines resulting from reducing contact and transmission rates (McCallum and Dobson 1995). More recently however, there has been an acceptance that the conditions required for infectious disease to cause extinction are more common than previously thought. For example, the presence of multiple hosts (especially tolerant reservoir species) reduces the likelihood of pathogen extinction as one susceptible host declines. This allows for a disease threat to persistently act upon declining populations which are sensitive to even modest mortality (De Castro and Bolker 2005).

Efforts to understand and combat the impact of EIDs are now at the forefront of biodiversity conservation as diseases are acknowledged to be occurring at an increased rate globally (Jones *et al.* 2008). This is likely to result in the continuing loss of biodiversity, which in turn could result in the increased susceptibility of the remaining species to disease (Young *et al.* 2014; Keesing *et al.* 2010). The resulting disease-extinction vortex could lead to a massive loss of species and perpetuate the mass extinction that we are currently witnessing.

1.3 Fungal disease

Alongside the trend of increasing number of infectious diseases emerging, there has been an increase in proportion of those EIDs of which a fungus is the causative agent (Fisher *et al.* 2012). These include white-nose syndrome in bats (Lorch *et al.* 2011; Blehert *et al.* 2009), ash die-back disease in European Ash trees (Kowalski 2006), snake fungal disease in North America (Allender *et al.* 2015; Lorch *et al.* 2015), crayfish plague on European crayfish (Holdich *et al.* 2009) and chytridiomycosis in amphibians globally (Martel *et al.* 2013; Skerratt *et al.* 2007; Berger *et al.* 1998). Despite previous assumptions that fungal diseases had little impact on the health of wild animal populations, the loss of biodiversity caused by chytridiomycosis is the greatest disease driven loss ever recorded (Fisher *et al.* 2012).

1.4 Reintroductions

Population restoration is a form of conservation translocation which has become an important, often last resort action used to improve the conservation status of endangered species with small wild populations. The IUCN defines two forms of population restoration: reinforcement and reintroduction (IUCN/SSC 2013). Reinforcement is defined as the translocation of individuals into an existing population of conspecifics. Reintroduction is the release of individuals in an area within the historical range from which it has been extirpated in an attempt to establish a viable population.

Reintroductions have been taking place for many years (Seddon, Armstrong and Maloney 2007) with many of the early reintroductions regarded as failures (e.g. Dodd and Siegel 1991; Griffith *et al.* 1989). In response to the high failure rates and the growing use of reintroduction as a conservation tool, the first reintroduction guidelines were published by the IUCN in 1998 by the IUCN/SSC Reintroduction Specialist Group (RSG) (IUCN 1998). The RSG sought to reduce the failure rate of reintroductions by highlighting the importance of thorough prior planning. When planning

a reintroduction, practitioners were encouraged to consider habitat quality at the release site, the origin of the animals for release, and the reintroduction design so that explicit hypotheses could be tested and results reported.

In 2013, the most recent version of the IUCN reintroduction guidelines were published with an improved focus on assessing the risks of reintroduction for the focal species and the associated ecological communities both at source and destination (IUCN/SSC 2013). For example, the intrinsically small size of reintroduced populations puts them at high risk of extinction through processes such as environmental and demographic stochasticity, inbreeding depression and genetic drift (Caughley 1994). In the 2013 guidelines and more recent literature active management of the reintroduced population has been championed in order to maximise success. This is representative of a recent blurring of the distinction between ex-situ and in-situ conservation, with actions akin to captive management applied to reintroduced populations to ameliorate threats and maximise success. Examples include supplementary feeding of reintroduced hihi (*Notiomystis cincta*) in New Zealand (Chauvenet *et al.* 2012), and proposed low-level assisted gene flow between reintroduced populations of Mauritius kestrel (*Falco punctatus*) in Mauritius (Ewing *et al.* 2008). Utilising an adaptive management approach alongside post-release monitoring, the best species specific management options can be identified and implemented (IUCN/SSC 2013; Armstrong, Castro and Griffiths 2007).

Around the time of the first IUCN reintroduction guidelines, Caughley (1994) argued that under the 'declining population paradigm', the threats which had caused the original decline must be identified and removed before reintroduction was attempted. This was associated with the rise of the concept of 'zoos as arks' whereby species could be held in captive collections until threats could be removed from their wild habitat (Balmford, Mace and Leader-Williams 1996; IUDZG/CBSG (IUCN/SSC) 1993). The 2013 guidelines move on from this concept in two important ways. Firstly, they acknowledge that wild habitat is not static, and in the period between extirpation and reintroduction, the habitat at the intended release site may change such that it is no longer suitable for the focal species. These differences must be monitored and accounted for in order for reintroduction success to be maximised (Norris 2004).

Secondly, the 2013 guidelines acknowledge that many of the processes which threaten wildlife today such as climate change, extreme climatic events and EIDs cannot be simply removed prior to

reintroduction. There are many species threatened by infectious diseases and for which reintroductions are likely to be required, but where disease eradication is problematic. For example, black footed ferrets (*Mustela nigripes*) are threatened by canine distemper virus in reservoirs such as domestic dogs and foxes (U.S. Fish and Wildlife Service 2013), wild dog (*Lycaon pictus*) translocations have been impacted by canine distemper virus (van de Bildt *et al.* 2002) and rabies (Hofmeyr *et al.* 2004) from domestic dogs and jackals (*Canis mesomelas*), and Madagascar pochard (*Aythya innotata*) reintroductions could be impacted by infectious disease from domestic fowl (Woolaver *et al.* 2015). Also, Bd infection persists in many amphibian (Reeder, Pessier and Vredenburg 2012), and potentially non-amphibian (McMahon *et al.* 2013), host reservoirs and represents a continuing threat to reintroductions of any of over 200 species which have already suffered disease-driven declines and even extinctions (Skerratt *et al.* 2007). These 'irreversible' threats require imaginative solutions with which to facilitate the persistence of reintroduced populations in the face of the threats which caused their original decline (Harding, Griffiths and Pavajeau 2015).

1.5 Amphibian declines

Prior to the 1990s, amphibian species were thought to be declining at the same rate as other vertebrate groups (May, Lawton and Stork 1995) and amphibian conservation was targeted to generic threats such as habitat degradation and alteration (Beebee 1996). In the 1990s and 2000s severe amphibian declines and even extinctions were reported to be occurring globally at an unprecedented rate with particularly great losses reported in Australia (Gillespie and Hollis 1996; Richards, McDonald and Alford 1993; Czechura and Ingram 1990; McDonald 1990), Central America (Cheng *et al.* 2011; Lips *et al.* 2006a; Lips *et al.* 2004; Lips 1999; Lips 1998; Pounds *et al.* 1997) and South America (Ruiz and Rueda-Almonacid 2008; Lampo *et al.* 2006; La Marca *et al.* 2005; Ron *et al.* 2003). This led to increased attention on amphibian conservation and the launch of the Global Amphibian Assessment, which confirmed that amphibians were declining at a greater rate than any other vertebrate taxon (Stuart *et al.* 2004).

At least 41% of all extant amphibian species are classified as being at risk of extinction (IUCN 2015). Multiple threats cause amphibian declines both independently and synergistically (Sodhi *et al.* 2008; Gascon *et al.* 2007). Some complex threats have well understood mechanisms while others remain to be identified (Collins and Storfer 2003). These threats include habitat loss and fragmentation (Gallant *et al.* 2007; Blaustein and Wake 1995), UVB and chemical pollution (Brühl

et al. 2013; Hayes *et al.* 2006; Blaustein *et al.* 2003), overexploitation for food and trade (Warkentin *et al.* 2009; Schlaepfer, Hoover and Dodd 2005), invasive alien species (Vredenburg 2004; Kats and Ferrer 2003), climate change (Carey and Alexander 2003; Pounds, Fogden and Campbell 1999) and infectious diseases such as chytridiomycosis and ranavirosis (Daszak, Cunningham and Hyatt 2003). Additionally, 48% of rapidly declining species were found to face one or more as yet unidentified threats (Stuart *et al.* 2004). The magnitude of these threats has led to the Amphibia being described as biggest victims of the sixth mass extinction (Wake and Vredenburg 2008).

Some species of amphibian often play important roles in ecosystems meaning their loss would be near impossible to mitigate (Ranvestel *et al.* 2004; Daszak, Cunningham and Hyatt 2000). The loss of amphibians would not only likely have impacts on their immediate environment, but also on the human populations which are increasingly interacting with them. For example, the tadpoles of some amphibian species are known to predate on mosquito larvae and their disappearance could therefore cause an increase in the mosquito population and the human and animal diseases of which they are vectors (Mokany and Shine 2003).

Many of the declines observed during the 1990s and 2000s occurred in relatively pristine environments and without the presence of an obvious driver, leading to them being described as 'enigmatic'. It was later discovered that the EID, chytridiomycosis, was playing a central role in many of these declines (Lips *et al.* 2006a; Berger *et al.* 1998).

1.6 Chytridiomycosis

Chytridiomycosis is caused by one of two waterborne chytridiomycete fungi: *Batrachochytrium dendrobatidis* (Bd) (Berger *et al.* 1998) or *Batrachochytrium salamandivorans* (Bsal) (Martel *et al.* 2013). Bd has an extremely broad host range infecting over 500 species of amphibian (*Bd-Maps.net* 2016; Olson *et al.* 2013) across the taxonomic range and occurs on every continent on which there are amphibians (Fisher, Garner and Walker 2009). *Batrachochytrium salamandivorans* has a more limited host range infecting only urodeles (newts and salamanders) and is currently only known to occur in the wild in Belgium (Martel *et al.* 2014), Germany (Sabino-Pinto *et al.* 2015) and the Netherlands (van der Sluijs *et al.* 2013), but has also been detected in the pet trade in the U.K. (Cunningham *et al.* 2015).

Bd virulence varies by strain (Fisher *et al.* 2009; Berger, Marantelli, *et al.* 2005), with at least six strains currently identified including the hypervirulent global panzootic lineage (Bd-GPL) which, probably during the 20th century, has been spread globally and is the primary cause of chytridiomycosis-driven declines (Bai *et al.* 2012; Schloegel *et al.* 2012; Farrer *et al.* 2011). The origins of Bd remain poorly understood, with two main hypotheses emerging (Rachowicz *et al.* 2005). Firstly, it is possible that Bd was locally endemic in Africa and transported round the globe from Africa with the African clawed frog (*Xenopus laevis*) through trade during the 1930s (Weldon *et al.* 2004). Similar hypothesis on global transport from Asia (Fong *et al.* 2015; Bai *et al.* 2012) or Brazil (Rodriguez *et al.* 2014; Rosenblum *et al.* 2013) have also been proposed as Bd has been identified in earlier museum specimens at these locations. Alternatively, Bd was endemic in many amphibian populations, but became increasingly pathogenic due to changing environmental conditions that might increase host susceptibility or result in increased virulence of Bd (Pounds *et al.* 2006). The prevailing opinion is that Bd is novel in some locations and endemic in others (Rosenblum *et al.* 2013).

Bd infects the keratinised skin of an amphibian, although the infection is most often located in the ventral abdomen (especially in the drink patch, if present), hind limbs, feet and toes (Longcore, Pessier and Nichols 1999; Pessier *et al.* 1999; Berger *et al.* 1998). Chemotaxis has been reported in the mobile water-borne Bd zoospores which have been recorded using their flagellum to move towards sources of keratin (Moss *et al.* 2008). The zoospores of Bd then encyst on the epidermal surface of an amphibian host and each infects an epidermal cell via a germ tube (Greenspan, Longcore and Calhoun 2012). The Bd zoosporangia then grow inside the amphibian epidermal cells and are moved towards the surface as the amphibian epidermal cells mature and migrate towards the *stratum corneum* (Berger, Hyatt, *et al.* 2005). During this process, within each zoosporangium, zoospores reproduce clonally and, when mature, are released via a discharge papillae onto the surface of the epidermis (Berger, Hyatt, *et al.* 2005). During its relatively hardy zoospore lifestage, Bd has been shown to survive outside a host in sterile water or damp substrate for up to three months during which time it remains viable and is able to infect new hosts (Kilpatrick, Briggs and Daszak 2010; Mitchell *et al.* 2008; Johnson and Speare 2005; 2003). There is, however, little understanding of the potential survival of Bd in a natural environment where it would need to compete with other micro-organisms.

Infection with Bd causes a thickening of the epidermis, including the outer keratin layer, which disrupts sodium and potassium ion transport leading to osmotic imbalance and eventually to death by cardiac arrest (Campbell *et al.* 2012; Marcum *et al.* 2010; Voyles *et al.* 2007). Tadpoles are less susceptible to chytridiomycosis as Bd infection is limited to the keratinised mouth parts in those species in which these are present. Following metamorphosis, however, the infection can spread to the newly keratinised skin (Marantelli *et al.* 2004; Rachowicz and Vredenburg 2004). Clinical signs of Bd infection in susceptible amphibians include weight loss, lethargy, abnormal posture, lack of righting reflex and skin ulceration, although death might be the only presentation seen (Berger, Hyatt, *et al.* 2005; Berger *et al.* 1998).

On histological examination, it is rare to see any inflammatory response to Bd infection and this may be due to suppression of the adaptive immune response by the pathogen (Poorten *et al.* 2016; Fites *et al.* 2014; 2013). The expression of genes associated with an adaptive immune response has been found to be downregulated after exposure to Bd in *Xenopus tropicalis* when compared to animals not exposed to Bd (Rosenblum *et al.* 2009). Contrastingly, alleles of class II major histocompatibility genes (MHC), which encode cell surface glycoproteins that bind pathogen molecules and initiate the acquired immune response (Jones *et al.* 2006), were found to be an important predictor of survival of *Lithobates yavapaniensis* when infected with Bd (Savage and Zamudio 2011). Evidence of positive selection of MHC alleles associated with survival of Bd infected hosts has also been observed in wild populations of *L. yavapaniensis* (Savage and Zamudio 2016) and *Bufo calamita* (May, Zeisset and Beebee 2011). Exposure of *Xenopus laevis* to heat-killed Bd appeared to stimulate an increase in Bd-specific antibodies (Ramsey *et al.* 2010), but exposure of *Rana muscosa* to formalin-killed Bd failed to increase survival or resistance (Stice and Briggs 2010). Similar, contrasting findings have been reported for prior exposure to live Bd, with both increasing and decreasing survival when re-exposed to the pathogen being reported. Exposure to Bd with clearance of infection through anti-fungal treatment did not increase survivorship of *Litoria booroolongensis* on subsequent re-exposure (Cashins *et al.* 2013). Multiple exposures of *Osteopilus septentrionalis* to live Bd, followed by clearances by heat-treatment, did result in increased survival following re-exposure to Bd (McMahon *et al.* 2014). Most research on amphibian resistance and immunity to Bd has investigated the innate immune response, such as anti-microbial skin peptides (Holden *et al.* 2015; Rollins-Smith 2009; Woodhams *et al.* 2007) and to indirect immunity such as commensal skin bacteria that produce antifungal compounds (Becker *et al.* 2015; Pask, Woodhams and Rollins-Smith 2012; Becker and Harris 2010; Harris *et al.* 2009).

Most amphibians known to be resistant to lethal chytridiomycosis appear to rely on one or both of these mechanisms to combat infection with Bd or the development of disease (Rollins-Smith *et al.* 2011). Unfortunately, innate immune responses are generally short lived and are not pathogen specific, resulting in limited efficacy (Stice and Briggs 2010).

Along with the apparent variation in ability to mount an immune response to infection with Bd, some species exhibit a behavioural response to Bd infection. For example, Panamanian golden toads (*Atelopus zeteki*) exhibited modified thermoregulatory behaviour during a chytridiomycosis epidemic, increasing their body temperatures resulting in lower likelihood of Bd infection (Richards-Zawacki 2010). As a result of these variations in response to infection with Bd, there is strong variation in the impact of Bd on wild amphibian species (Searle *et al.* 2011). Some species appear able to survive with Bd infection (e.g. Kielgast *et al.* 2010; Pearl *et al.* 2009; Longcore *et al.* 2007), whereas over 200 species globally have declined or gone extinct as a result of the emergence of chytridiomycosis resulting from Bd infection (Skerratt *et al.* 2007). There is evidence that a small number of populations have undergone some level of recovery following initially severely declining due to the emergence of chytridiomycosis (e.g. Newell, Goldingay and Brooks 2013; Briggs *et al.* 2005; Retallick, McCallum and Speare 2004). Populations of most species have continued to decline (e.g. Hunter *et al.* 2010), mostly due to continued mortality caused by Bd infection (Phillott *et al.* 2013; Murray *et al.* 2009) and are at continued risk of extinction. Given this huge impact, chytridiomycosis has been described as “the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction” (Gascon *et al.* 2007).

The internationally-recognised and IUCN established Amphibian Conservation Action Plan (ACAP) advocates captive assurance programmes to prevent extinction from chytridiomycosis (Zippel *et al.* 2011). Unfortunately, there is estimated to be the capacity to hold viable populations of only 50 highly threatened amphibian species in zoos around the world and it has been estimated that a total of 943 amphibian species are in need of captive assurance colonies (Zippel *et al.* 2011). Some amphibians are also poorly suited to captivity, with complex breeding systems or husbandry requirements (Tapley *et al.* 2015). Also, there is no realistic exit strategy from a captive breeding programme as Bd has been removed from the environment only once (Bosch *et al.* 2015) and this was achieved using a largely non-transferable method. As a result there is a requirement for the

development of novel, effective in-situ mitigation methods to negate the threat of chytridiomycosis (Harding, Griffiths and Pavajeau 2015).

In captivity, many species infected with Bd respond well to treatment with a range of anti-fungal drugs (Brannelly, Richards-Zawacki and Pessier 2012; Martel *et al.* 2011; Nichols and Lamirande 2001) or to treatment with increased temperature (Chatfield and Richards-Zawacki 2011; Woodhams, Alford and Marantelli 2003). The variation in impact of Bd on different amphibian species (Searle *et al.* 2011) and the commonality of multispecies assemblages means that, in most cases in the wild, Bd is able to persist in sympatric reservoir species in the wild meaning reinfection after in-situ treatment is likely. This has led to a reluctance to trial in-situ conservation measures (Scheele, Hunter, *et al.* 2014), while logistical difficulties of recapture and correct dosing of animals, has additionally inhibited the use of antifungal drugs in the wild (A. Cunningham, pers. comm.). There are, therefore, only a small number of amphibian conservation projects which are implementing in-situ conservation management in an attempt to mitigate the impact of chytridiomycosis. These comprise the southern corroboree frog (*Pseudophryne corroboree*) project in Australia (corroboreefrog.com.au), the Panama Amphibian Rescue and Conservation Project (amphibianrescue.org) using the Panamanian golden toad (*Atelopus zeteki*) as a flagship, the mountain yellow-legged frog (*Rana muscosa*) recovery programme in California (sandiegozooglobal.org) and the mountain chicken frog (*Leptodactylus fallax*) recovery programme in the Caribbean Lesser Antilles (mountainchicken.org).

1.7 The mountain chicken frog

1.7.1 Biology

The mountain chicken frog is the largest frog native to the Caribbean, which can grow up to 1 kg in weight, 21 cm snout-vent length (SVL) and which can live for over 12 years (Martin *et al.* 2007; Daltry and Gray 1999). The mountain chicken has powerful hind legs which are capable of propelling it in jumps of nearly 3 m in length. The males are generally smaller than the females and more brightly coloured. Males can be identified by the black spurs that occur on their forelegs during the breeding season which are thought to be used during amplexus. Males are also extremely territorial and 'wrestle' one another to defend their territories (Daltry 2002).

Mountain chickens have an unusual breeding system involving strong maternal care and no reliance on water bodies for breeding. During the breeding season, the males produce an

underground burrow in which the female produces a foam nest into which she deposits her eggs (Gibson and Buley 2004). Once the tadpoles have hatched and utilised their yolk sacs, the female feeds them with infertile eggs (Gibson and Buley 2004). Prior to the juveniles leaving the nest, the female aggressively defends the nest from predators. Juveniles appear to remain with the female near the nest for several weeks post metamorphosis, after which they disperse and receive no further parental care.

1.7.2 Distribution

The mountain chicken is endemic to the Lesser Antilles, and was once found on at least six islands: St. Kitts, Montserrat, Guadeloupe, Dominica, Martinique and St. Lucia. A combination of threats including habitat loss, introduction of alien predators (most importantly, mongoose), and hunting, led to the species being extirpated from much of its former range. It is now found on only two islands: Dominica and Montserrat (Hedges and Heinicke 2007; Lescure 1979). It is culturally important on both islands, being a part of the national crest and national bank logos on Dominica as well as being, until recently, the national dish on this island. Mountain chickens are also the top predator on Montserrat, and one of the few top predators on Dominica. They have been recorded as consuming prey including large spiders, snakes, small frogs, small mammals and crop pests including slugs (Rosa *et al.* 2012; Brooks 1982).

1.7.3 Threats

The status of the mountain chicken as a traditional dish on Montserrat and as the national dish on Dominica, indicates that human consumption is a possible threat to the long-term survival of the species (McIntyre 2003). In addition, invasive species including rats, cats, dogs and livestock and multiple eruptions of the Soufriere hills volcano since 1995 on Montserrat have put pressure on the mountain chicken populations (Fa *et al.* 2010). The volcanic activity on Montserrat has caused both loss of parts of the historical mountain chicken range, and the persistent production of acid rain and ash throughout the 1990s-2010s, both of which might have negatively impacted the mountain chicken and its prey (Daltry and Gray 1999).

The mountain chicken is now critically endangered, partly as a result of the ongoing threats described above, but most particularly because of a rapid and a catastrophic decline as a result of the introduction of chytridiomycosis on both islands (Fa *et al.* 2010; Martin *et al.* 2007; Magin 2003). In response, the Mountain Chicken Recovery Programme (mountainchicken.org) was

established with the goal of 'enabling the restoration of the mountain chicken frog within its native range on Montserrat and Dominica'. The partnership comprises the Zoological Society of London, Durrell Wildlife Conservation Trust and Chester Zoo along with the governments of Dominica and Montserrat. The members of the partnership lead conservation efforts on the ground, manage a captive breeding and reintroduction programme and carry out research to gain a greater understanding of the epidemiology and impact of chytridiomycosis to inform conservation measures and ensure the persistence of mountain chickens in the wild.

The research described in this thesis forms part of the mountain chicken recovery programme and incorporates the use of data that has been collected since 2002 on Dominica and since 1998 on Montserrat. The mountain chicken represents a good model species for the study of the chytridiomycosis management strategies as it is a large-bodied frog which is relatively easy to catch and which is the subject of long term monitoring on both Dominica and Montserrat. Also, the cultural importance of this species means that there is a strong local desire to enact conservation measures to ensure its long-term survival. The aim of this thesis is to analyse existing and newly collected data in order to generate conservation management recommendations for the mountain chicken frog in the continued presence of Bd throughout its historical range. The intention is for the results of this work to be applicable to amphibian conservation elsewhere through the provision of recommendations and improvement for current and future conservation efforts for amphibians affected by chytridiomycosis globally.

This research set out to achieve the following objectives:

I. To understand and quantify both the demographic and genetic impact of the emergence of chytridiomycosis on the mountain chicken populations of Dominica and Montserrat.
II. To test in-situ anti-fungal treatment, which has been used successfully ex-situ, as a mitigation strategy for epidemic chytridiomycosis in mountain chickens.
III. To determine the Bd status of sympatric amphibians on Dominica and Montserrat and understand the seasonal dynamics of Bd infection in these species.
IV. To assess whether reintroductions of captive bred mountain chickens represents a viable conservation strategy to enable long term persistence of mountain chickens in Montserrat. Reintroductions of mountain chickens that took place in different seasons will be analysed in order to test for variation in seasonal success.

V. To investigate the movement patterns of released mountain chickens and to ascertain whether there are particular movement behaviours or habitat features that represent an increased risk of infection with Bd.

2 Dynamics and genetics of a disease-driven species decline to near extinction: lessons for conservation

This chapter is currently in review in Scientific Reports as: **Hudson, M.A.**, Young, R.P., D'Urban Jackson, J., Orozco-terWengel, P., Martin, L., James, A., Sulton, M., Garcia, G., Griffiths, R.A., Thomas, R., Magin, C., Bruford, M.W., and Cunningham A.A. Dynamics and genetics of a disease-driven species decline to near extinction: lessons for conservation.

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Abstract

Amphibian chytridiomycosis has caused precipitous declines in hundreds of species worldwide. By tracking mountain chicken (*Leptodactylus fallax*) populations before, during and after the emergence of chytridiomycosis, we quantified the real-time species level impacts of this disease. We report a range-wide species decline amongst the fastest ever recorded, with a loss of over 85% of the population in fewer than 18 months on Dominica and near extinction on Montserrat. Genetic diversity declined in the wild, but emergency measures to establish a captive assurance population captured a representative sample of genetic diversity from Montserrat. If the Convention on Biological Diversity's targets are to be met, it is important to evaluate the reasons why they appear consistently unattainable. The emergence of chytridiomycosis in the mountain chicken was predictable, but the decline could not be prevented. There is an urgent need to build mitigation capacity where amphibians are at risk from chytridiomycosis.

2.1 Introduction

Recent studies indicate that the Earth has entered a sixth period of mass extinction (Ceballos *et al.* 2015). Unlike previous mass extinction events, the current situation stems from human activities and mitigation measures have been agreed upon internationally in the form of the Convention on Biological Diversity (CBD). This treaty's undertakings include the prevention of extinction of known threatened species and the safeguarding of genetic diversity for a range of organisms, including those of socio-economic or cultural value. The Aichi 2020 Targets for the CBD specify a range of measures, including those designed to "improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity" (www.cbd.int). These targets were driven by a generally perceived failure of the international community to achieve the CBD's goal of halting biodiversity loss by 2010, yet it is becoming apparent that many of the 2020 targets will also be missed (Tittensor *et al.* 2014). It is important to evaluate the reasons why these targets appear consistently unattainable if lessons are to be learned. Within this context, case studies of success and failure in conservation planning and action can provide important examples of the challenges of meeting global biodiversity targets and on how success rates may be improved.

With over 40% of species currently threatened with extinction (Sodhi *et al.* 2008; Stuart *et al.* 2004), amphibians are a disproportionately affected group. This rate of loss is increasing (Collins 2010), with emerging infectious diseases, and specifically amphibian chytridiomycosis, being one of the primary drivers of this unprecedented level of threat (Collins 2010; Skerratt *et al.* 2007). Amphibian chytridiomycosis, due to infection with the non-hyphal chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), is thought to have caused the decline or extinction of over 200 species of amphibian world-wide in recent decades (Fisher, Garner and Walker 2009; Skerratt *et al.* 2007).

Chytridiomycosis-induced amphibian declines usually occur following the introduction of Bd to a naïve population. The precise timing of disease introduction, however, is rarely identified and often the first recognition of Bd emergence in an amphibian population is the loss or rapid decline of that species at a study location (Lips *et al.* 2006a; Lips 1999). As a result, few studies have captured the trajectory and rate of chytridiomycosis-driven amphibian declines from the time of disease onset. Where data are available, population collapses of more than 90% in as little as 1-3 years from first recorded death have been reported repeatedly (Vredenburg *et al.* 2010;

Woodhams *et al.* 2008; Lips *et al.* 2006a; Rachowicz *et al.* 2006; Briggs *et al.* 2005; Lips 1999; Laurance, McDonald and Speare 1996).

A lack of monitoring and the speed of chytridiomycosis-driven amphibian declines have resulted in few data being available for assessing the genetic impact of the disease. Despite the numerous population declines reported, only one study has investigated the associated genetic impact, providing evidence of a disease-induced population bottleneck in the common midwife toad (*Alytes obstetricans*) (Albert *et al.* 2014). This is important as amphibians, which often have small effective population sizes, fragmented populations and low dispersal rates, are particularly vulnerable to loss of genetic diversity (Allentoft and O'Brien 2010). The importance of conserving genetic diversity has recently been recognised by its inclusion in the CBD's 2020 Targets (Strategic Goal C, Target 13).

The mountain chicken (*Leptodactylus fallax*) is the largest frog endemic to the Lesser Antilles which can live 12 years (Martin *et al.* 2007). This species is now confined to only two islands, Montserrat and Dominica, after being driven to extinction on other east Caribbean islands by introduced predators and over-hunting for food prior to the global emergence of Bd (Breuil 2009; Hedges and Heinicke 2007; Daltry 2002; Schwartz and Henderson 1991). With relevance to the CBD's 2020 Target 13, the species is culturally valuable; it features on Dominica's official coat-of-arms and is incorporated into local folklore and proverbs. The mountain chicken was also of socio-economic importance as, prior to the current declines, the frog was the national dish of Dominica and was a source of income for hunters, restaurants and the tourism industry. A monitoring programme for the mountain chicken was initiated in 1998 on Montserrat and in 2002 on Dominica by Fauna & Flora International and the respective Forestry Departments to investigate the impacts of hunting that, along with invasive rats and pigs, was considered the main threat to the species (Daltry 2002).

In December 2002, reports of dead and sick mountain chickens were first received by forestry officers from members of the public in Dominica. Initially, only isolated reports were made but within weeks widespread mortality was apparent. A diagnosis of chytridiomycosis due to Bd infection was made on carcases shipped to the Zoological Society of London for pathological examination (Magin 2003). Following the epidemic on Dominica, the mountain chicken was listed as critically endangered by the IUCN in 2004 (Fa *et al.* 2010). Targeted surveillance of amphibians

in 2005 showed Bd to be absent from Montserrat (Garcia *et al.* 2007), therefore a risk analysis was conducted to identify potential pathways for introduction of the pathogen to the island. The highest risk identified was the accidental importation of infected tree frogs (*Eleutherodactylus* spp.) within shipments of produce, most of which was imported directly from Dominica (Horton 2005). Recommendations were made and communicated to minimise this risk, including the removal of any amphibians found in produce prior to shipping, the capture and euthanasia (in contrast to the common practice of release into the wild) of any amphibians found in produce on arrival in Montserrat, and awareness-raising for exporters, importers and the public (Horton 2005). However, in February 2009, Montserrat forestry officers reported multiple dead mountain chickens during a routine visit to one of the population monitoring sites. We rapidly diagnosed the cause as chytridiomycosis due to infection with Bd by histopathology which has been more recently verified by real-time PCR on archived samples (authors' unpublished data). Further investigations showed evidence of an eastward wave of mass mortality across the species' range on Montserrat. In response, 50 mountain chickens were captured from the last intact population in front of the epidemic wave to set up a biosecure conservation assurance population in Europe.

On both Dominica and Montserrat, long-term monitoring programmes produced mountain chicken population demographic data and genetic samples before, during and after the onset of epidemic chytridiomycosis. Here, we quantify the population and genetic impacts of the disease, discuss the management responses to this crisis and evaluate their effectiveness in terms of conserving biodiversity. This study of the predictable near-extinction of a culturally iconic species due to a process addressed by Aichi Target 9 (invasive species and pathways) is a useful model for understanding how failures in national and international governance and support mechanisms are impeding our ability to meet the CBD 2020 targets.

2.2 Methods

2.2.1 Study sites

Montserrat and Dominica are small volcanic islands in the Lesser Antilles chain in the Caribbean. Montserrat, approximately 102 km^2, has a central active volcano creating its highest peak at 915m. The Centre Hills to the north, with a maximum altitude of 740m, are characterised by deep 'ghauts' (river valleys) in a radial pattern from a central peak (Young 2008). The climate on Montserrat is characterised by two distinct seasons, a drier, cooler season running from January to June and a wetter, warmer season from July to December. Dominica is larger, approximately 754

km^2, and more mountainous than Montserrat, with steep interior slopes, most of which are covered in native forest. It has a tropical climate with year-round rainfall. Dominica lies approximately 150 km South East of Montserrat with the island of Guadeloupe situated between them.

2.2.2 Population monitoring

A full description of transect monitoring techniques is provided by Daltry (2002). Briefly, the relative mountain chicken population abundance was measured across multiple 10-metre-wide 200-250 m long transects on each island using visual encounter surveys (VES). Two or more observers walked slowly along the transect midline shining torches into the vegetation on each side and every mountain chicken seen was recorded in a single dataset. Each survey was conducted shortly after nightfall when the frogs are active (Daltry 2002). Each VES lasted approximately 1 hr per 200 m. At the beginning and end of each survey, relative humidity (%) and ambient temperature (^oC) were recorded.

On Dominica, nine transects were established in 2002 in areas with mountain chicken populations, typically in lowland coastal areas. These were surveyed on an approximately monthly basis from August 2002 to March 2004. Surveys were conducted on eight occasions from January 2006 to July 2007. From January to September 2014, the established transects and an additional 13 (selected on local knowledge of historical distributions and patches of potentially suitable habitat) were surveyed on three occasions to search for any surviving mountain chickens. On Montserrat, mountain chickens inhabit upland areas in which 17 transects of 200 m were established in 1998; 13 were monitored regularly from 1999 to 2005: transects were surveyed four times in 1999, biannually (wet and dry seasons) from 2000 to 2005 and once per year in 2007, 2011 and 2012. From 2003, some transects were extended to a maximum of 400m to capture a greater proportion of the available habitat at each site, with effort per transect-metre maintained.

Following the onset of declines on Montserrat, an extended 800 m length of the transect with the last known intact population (Fairy Walk) was surveyed using VES on a thrice-weekly basis from August 2009 to January 2010 as part of an amphibian chytridiomycosis treatment trial (Hudson *et al.* 2016). Only individuals in the treatment trial control group were included in the generalised linear model (GLM) of the Fairy Walk decline to omit any treatment effects. Ad-hoc monitoring of

these transects was carried out between 2012 and 2014 to identify any remnant mountain chicken populations or individuals.

2.2.3 Disease diagnosis

Systematic pathological examinations were conducted on frogs found dead during the initial stage of epidemic mortality on each island by examining formalin-fixed samples of hind toe and skin from the ventral pelvic area (drink patch) from each carcase. Following the emergence of chytridiomycosis on each island, the epidemic was tracked via skin-swabbing of frogs found sick or dead using rayon-tipped swabs (Hudson *et al.* 2016). Swabs were refrigerated until Bd analyses were undertaken in the laboratory. Fieldwork biosecurity measures were undertaken as recommended by the Amphibian and Reptile Groups, UK ARG Advice Note 4 (http://www.arguk.org/advice-and-guidance/view-category) to minimise Bd contamination of samples or the spread of pathogens between study sites by researchers. DNA was extracted from fresh/frozen hind toe and ventral pelvic skin samples and examined for the presence of Bd DNA using a Bd-specific qPCR (Boyle *et al.* 2004) but adding bovine serum albumin to the PCR mix to minimise any reaction inhibition (Garland *et al.* 2010). Negative controls and four standards (100, 10, 1 and 0.1 zoospore equivalents) were included on each plate and all samples, standards and controls, were tested in duplicate. Samples were defined as positive if both duplicates showed a successful PCR product. However, if only one duplicate worked a third qPCR was run. If the third repeat showed no PCR product, the sample was considered negative. Genomic equivalent (GE) values for each sample were calculated by multiplying the mean quantity of the duplicate dilutions by 120 (4 µl from 60 µl total elute used to make 1 in 10 dilution (x15) and 5 µl of 40 µl dilution was used in qPCR (x8) [15 x 8 = 120]).

2.2.4 Analysis of population data

To ensure encounter rates were comparable across transects of different lengths and between islands, a nightly average encounter rate per 200 m of transect was calculated, excluding two Dominican transects where no visual encounters were made. Generalised linear mixed models (GLMM) in R (R core team 2015) were used to assess temporal trends in the encounter rate on Dominica between 2002-2004 with transect name as a random effect, assuming the counts were Poisson distributed, and using a logit link function. Transect surveys on Dominica in 2006 and 2007 were not included in the analysis as no individuals were seen during this period, however the model from the 2002-2004 data was used to predict the expected encounter rates in those years.

On Montserrat, visual encounter rate trends from 1999 to 2005 also were analysed using a Poisson GLMM with a logit link and with transect name as a random effect. To examine the trends at the intensively-monitored Fairy Walk, the pre-decline VES data for this site were modelled independently with a Poisson GLM with a logit link function, and the decline between August 2009 and January 2010 was modelled using a Poisson GLM with a logit link function.

In each model, encounter rate per 200 m of transect was used as the response variable. In order to test for a temporal trend, 'time' was included as an independent variable, calculated as the number of months from the first survey in the model. In the GLM of the Fairy Walk decline, time was measured in weeks due to the shorter duration of this model. The environmental variables collected for each survey, the number of observers, the season (Wet/Dry), and the average rainfall in the month of, and in the month prior to, the survey were included as covariates in the models to account for mountain chicken detectability. Models were developed using stepwise elimination of the least significant variable and a likelihood ratio test (LRT) used to assess whether the model fit was reduced by exclusion of the variable compared to the model containing that variable at an alpha value of 0.05. This was continued until only variables that significantly improved model fit were retained.

2.2.5 Range change estimates

As widespread systematic monitoring of the mountain chicken distribution was not available outside the transects, two standardised range metrics used in the red listing process (IUCN 2001) were calculated: i) extent of occurrence (EOO) and ii) area of occupancy (AOO) for both the pre-epidemic distribution and the present day distribution. The EOO is defined as "The area contained within the shortest continuous imaginary boundary which can be drawn to encompass all the known, inferred or projected sites of present occurrence of a taxon" (IUCN 2001). This was calculated by drawing a minimum convex polygon around the midpoints of all transects on which mountain chickens had been observed. This likely represented an overestimate as it almost certainly included areas of unsuitable habitat. The AOO is defined as "The area within its 'extent of occurrence', which is occupied by a taxon, excluding cases of vagrancy" (IUCN 2001). This was calculated by overlaying a 500 m grid on both islands and assuming full occupancy of a grid square if mountain chicken presence had been confirmed on a transect which had its midpoint within a grid square. A 500 m grid size was chosen as it represents the best current approximation of the

home range size of the mountain chicken based on local knowledge (authors' unpublished data).Each metric was independently calculated for each island.

We used Figure 1 in McIntyre (2003), which estimates the mountain chicken distribution prior to 2003 using polygons and points, to estimate its pre-epidemic distribution on Dominica. Any grid cell which was more than half filled with a polygon from McIntyre's map or occupied by a transect point was considered occupied. Present day estimates of the distribution on Dominica were generated using the data from the 2014 extended transects and personal communications with forestry staff. On Montserrat, pre-epidemic distributions were generated using transect data from the routine monitoring carried out from 1998-2007. Present day estimates on Montserrat were generated using the non-systematic monitoring carried out between 2012 and 2014 and personal communications with forestry staff. In both cases, grid squares of current distribution were randomly assigned to within a 3 square linear distance of their location to protect the location of the populations.

2.2.6 Population genetics

To assess if there were differences in genetic variation between islands or between populations *pre-* and *post-* declines (including between wild and captive populations), genetic analyses were conducted using microsatellite markers. Blood, buccal swabs or tissue samples from live and dead wild mountain chickens were collected from Montserrat and Dominica (Fig. 2.1) between 2002 and 2014. For founders and first generation captive-bred individuals from Montserrat, buccal mucosa was sampled by swabbing with rayon-tipped swabs (Pidancier, Miquel and Miaud 2003) and stored in 99% ethanol. All extractions were performed using the Roche High Pure PCR Template Preparation Kit (Ref: 11796828001; Version 16). DNA from buccal swabs was extracted following the same tissue protocol with modifications as follows: swabs were removed and allowed to air dry for 5 minutes for ethanol to evaporate, after which they were placed in individual micro-centrifuge tubes with 40 µl Proteinase K and 200 µl tissue lysis buffer and incubated at 56^{o}C overnight. After this extended incubation, the tissue protocol steps were followed. Eluted DNA was stored at -20^{o}C.

Eight newly-developed polymorphic microsatellite markers were genotyped for each animal (Appendices A and B) and the resultant genotypes were checked for null alleles and allelic drop out using MicroChecker v.2.2.3 (van Oosterhout *et al.* 2004). Hardy Weinberg equilibrium (HWE)

and linkage disequilibrium, were calculated with Genepop v.4 (Rousset 2008; Raymond and Rousset 1995), and observed and expected heterozygosity, unbiased allelic richness, and genetic differentiation among populations (F_{ST}) were estimated with MSA v4.05 (Dieringer and Schlötterer 2003). Per locus and per population inbreeding coefficient (F_{IS}) was estimated by FSTAT v.2.9.4. (Goudet 2003).

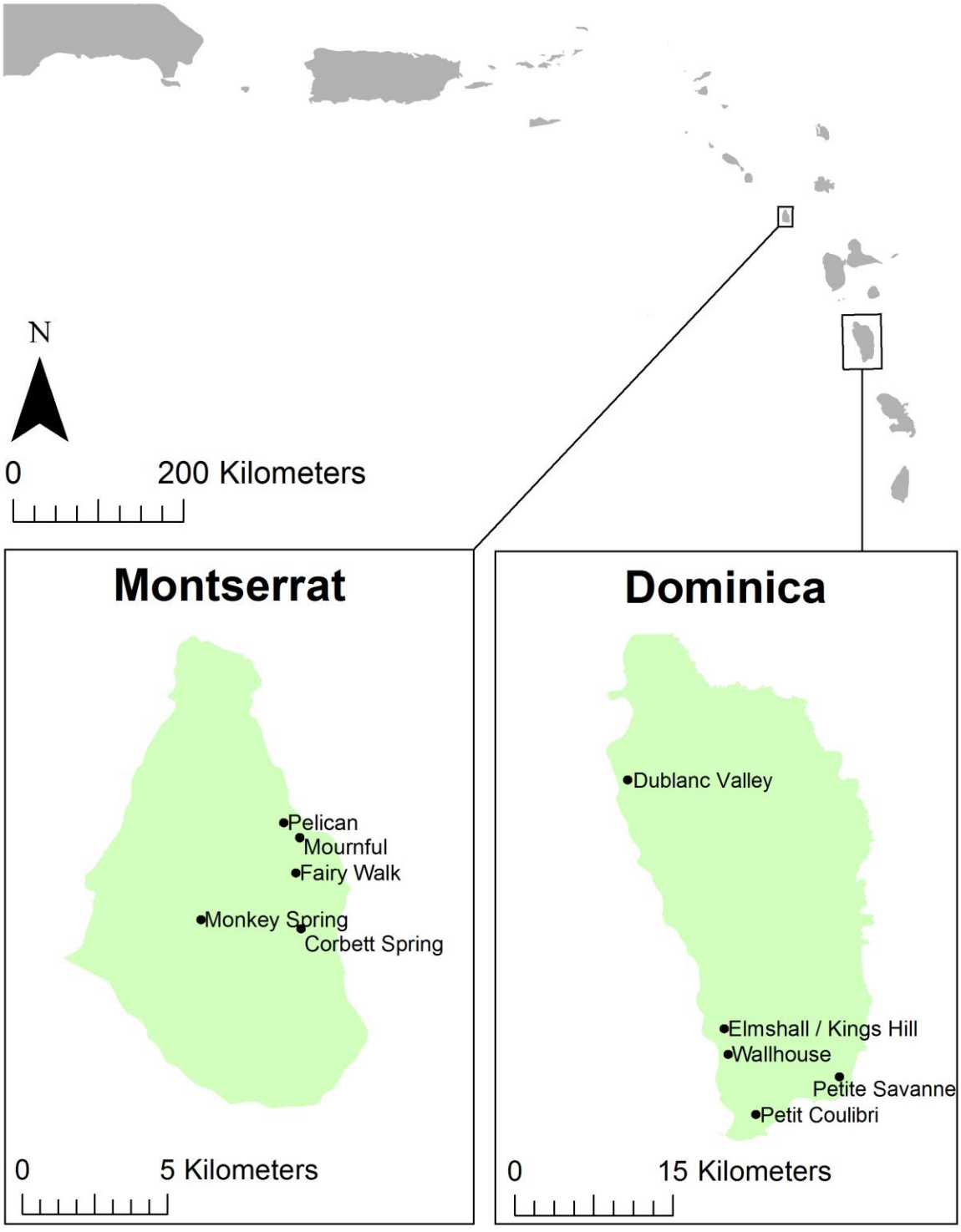

Figure 2.1 Location of pre-decline samples collected and used in genetic analysis.

For the *pre-* and *post-* decline genetic comparison of the Dominican samples we use a decline end-date of 2006 by which time no animals could be detected in the wild. The *post-decline* (recovering) population was defined as animals caught in 2014 when mountain chickens began to be detected on three of the historical transects in the absence of chytridiomycosis driven mortality. In the 2014 surveys, a large number of ~2 year old animals were detected alongside a small number of older animals, suggesting that breeding had occurred at increased levels in 2012, although no samples were collected in 2012 or 2013. Although 6 juveniles were found at one location in 2011, they were infected with Bd and disappeared shortly after (presumed to have died of chytridiomycosis) and so these animals were not considered part of a recovering population (authors' unpublished data). As these animals were caught so long after the initial decline but before the apparent population recovery began, the animals were excluded from the *pre-*/*post- decline* comparison but included in the general inter island genetic diversity statistics. The *pre-*/*post- decline* groups of samples were compared using standard population genetic diversity statistics described above to assess if genetic diversity in the *post-decline* population was significantly smaller as a consequence of the Bd-induced population crash. Additionally, signatures of demographic contraction in the 17 wild Dominican individuals caught in 2014 were tested using the Wilcoxon test in the software Bottleneck v. 1.2.02 (Piry, Luikart and Cornuet 1999). For this analysis, 1000 simulations were carried out under the stepwise mutation model (S.M.M. Ohta and Kimura 1973) and the Two-Phased mutation model (T.P.M. Di Rienzo *et al.* 1994) using default parameters.

2.2.7 Representatives of the founder population

A resampling analysis was carried out to establish whether the Fairy Walk (Montserrat) individuals collected in 2009 used to establish the captive founder population (*Founders* herein), were representative of the genetic diversity in the larger wild population (*Wild* herein). For this purpose, we compared the average number of alleles per locus, observed and expected heterozygosity of the Fairy Walk individuals against 100,000 random samples of eleven individuals from the allele frequency distribution of the 74 wild individuals from Montserrat. We selected these statistics as they are affected by dramatic changes in effective population size (Hoban *et al.* 2014). In order to test for a difference, we examined whether the statistics for the observed data were within the 95 percentiles of the random sample distribution.

2.3 Results

2.3.1 Demography and disease

Detailed pathological examinations were conducted on four adult frogs found dead between 19th February and 28th March 2003 from three sites across Dominica. Histological examination of hind-leg skin and feet showed large numbers of intracellular sub-spherical, septate structures characteristic of Bd (Berger *et al.* 1998). All of the extracted DNA samples tested positive for Bd DNA using qPCR (4567, 4780 and 12643 genomic equivalents). No other findings that could be related to the cause of death were observed. Twenty-eight additional mountain chickens were found dead and 21 found alive with signs of severe chytridiomycosis (See Appendix C for more details).

Dead mountain chickens were first reported on Montserrat in February 2009 on the eastern side of the island. Twelve transects were surveyed across the island during the following two weeks, and epidemic mortality was observed at three sites in the east. No mountain chickens were found at five long-term population monitoring sites in the north or west of the Centre Hills, and severely depleted populations at sites in the south and east. Only two sites, Fairy Walk and Corbett Spring, were found to contain apparently healthy mountain chicken populations. Both of these sites are in the extreme south-east of the Centre Hills. Using qPCR, we confirmed the presence of Bd DNA in skin swabs at five of the seven sites in which mountain chickens were found, including all five with observed mortality. Only skin swabs from Corbett Spring and Fairy Walk did not test positive for Bd DNA. On returning to Montserrat in August 2009, no mountain chickens were found at any site other than Fairy Walk and one other site nearby, where epidemic mortality was observed. Of 120 mountain chickens skin-swabbed during two weeks of monitoring at Fairy Walk in August 2009, 105 (87.5%) tested positive for Bd DNA. The temporo-spatial pattern of mortality and decline suggested a north-west to south-east epidemic wave with an active front in the remaining populations in the south-east.

No explanatory variables other than time were retained in the final GLMM of the Dominica decline (Chi-sq = 20.31, df = 1, p = 0.01). Mean visual encounter rates per 250 m declined by more than 50% from 2 (SE = 0.31) to 0.85 (SE = 0.18) within 12 months from August 2002 (Fig. 2.2). By March 2004, encounter rates on the transects had fallen to 0.29 (SE = 0.11), a decline of c. 85% in 18 months. When the surveys restarted in 2006 no frogs were seen on any transect, despite

increased efforts, until 2014 when a single population of 14 individuals was found on one of the original transects.

On Montserrat, season (LRT Chi-sq = 20.997, df = 1, p <0.0001) and time (LRT Chi-sq = 3.8476, df = 1, p = 0.04) were retained as fixed effects in the GLMM for the 1999-2007 pre-decline data. A greater number of individuals were encountered on transects during the dry than the wet season, and the visual encounter rate increased between 1999 and 2007 (Fig. 2.3). However, the VES in June 2011 and 2012 did not detect any mountain chickens.

Time was the only significant parameter retained in the GLM of the Fairy Walk decline (z = -5.41, df = 21, p < 0.0001), with the encounter rate per 250 m of transect declining from a maximum of 10 (week 5) individuals to 2 (week 18) a decrease of c. 80% in just 13 weeks (Fig. 2.4). Continued monitoring of the Montserrat transects, 2012-2014, identified only four individuals at three sites.

2.3.2 Range change estimates

Intensive surveys on Dominica in 2014 encountered a total of 44 mountain chickens across six sites. On Dominica, the estimated EOO of the mountain chicken was reduced by 95% (from 663.63 km² to 38.08 km²) and the estimated AOO was reduced by 94.4% (from 35.25 km² to 1.75 km²) between 2002 and 2014 (Fig. 2.5). The mountain chicken population caught in 2014 was Bd-negative and it is possible that a degree of nascent post-epidemic population recovery was present.

The island-wide decline on Montserrat, 2009 to 2012, reduced the species' estimated EOO by 87.9% (from 12.68 km² to 1.53 km²) and the estimated AOO by 80% (from 3.75 km² to 0.75 km²; Fig. 2.5). There is no apparent population recovery on this island to date.

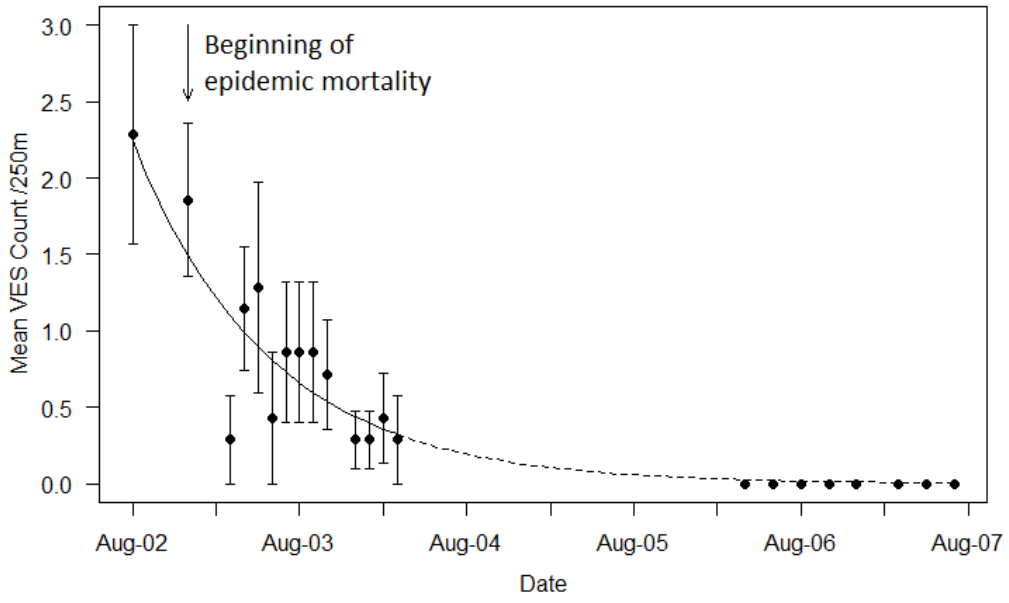

Figure 2.2 Generalised linear mixed effects model of encounter rate of mountain chickens on Dominica. Dashed line indicates model extension to cover period in which no data were collected and the data not included in the model. Error bars represent standard error around the mean of count across the transects.

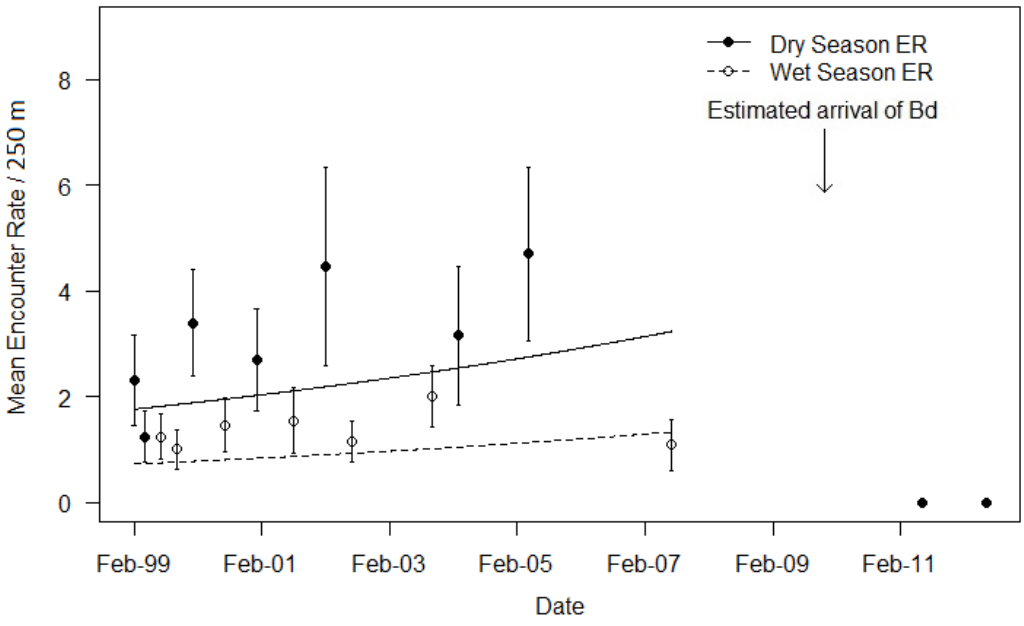

Figure 2.3 General linear mixed effects model of mountain chicken encounter rate on historically monitored transects on Montserrat. Error bars represent the SE around the mean encounter rate across the transects.

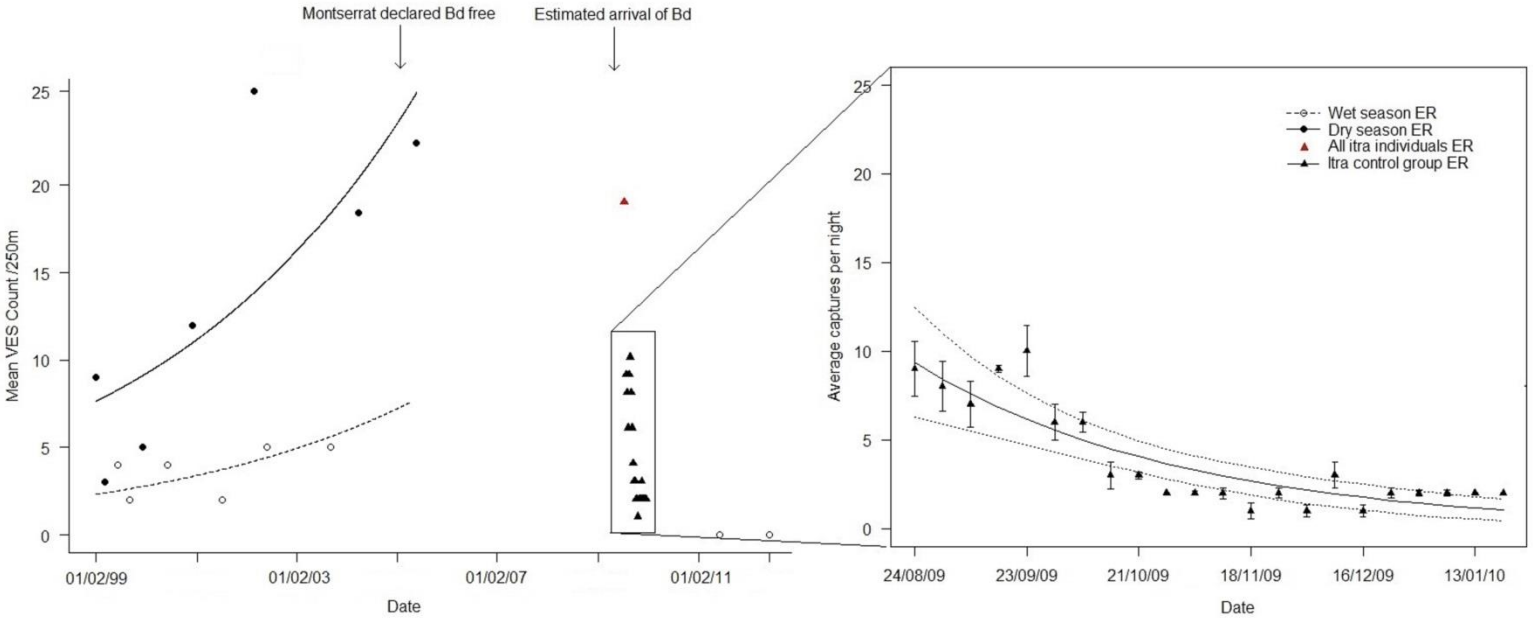

Figure 2.4 Decline in mountain chicken encounter rate at Fairy Walk on Montserrat. LHS shows general linear model of historical monitoring and RHS shows close up of general linear model of rapid decline during epidemic chytridiomycosis observed in 2009. Red triangle shows total encounter rate in first week of intensive monitoring during the study described in Hudson *et al.* (2016). Black triangles indicate only the control groups (~50 % of the population) to exclude the impact of the treatment used in Hudson *et al.* (2016).

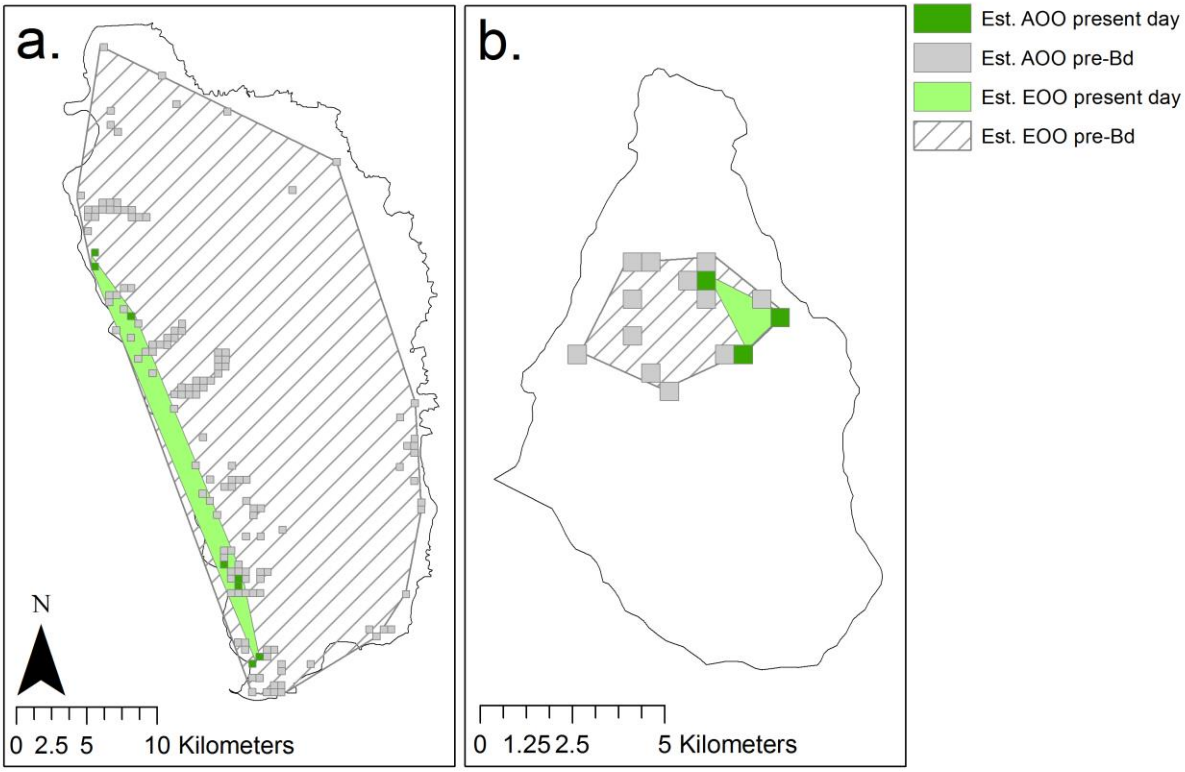

Figure 2.5 Mountain chicken range collapse on a) Dominica and b) Montserrat. Area of occupancy (AOO) is shown as grid squares and extent of occurrence (EOO) as a minimum convex polygon.

2.3.3 Population genetics

Analysis of eight microsatellites showed a total of 52 alleles (Dominica: 48, Montserrat: 38) with an average of 6.5 per locus and 34 shared between both islands (Table 2.1). The expected heterozygosity ranged from 0.39 to 0.75 in Dominica and from 0.48 to 0.75 in Montserrat. Microchecker analysis identified the markers Lepfal_0867, Lepfal_3035, Lepfal_1628 and Lepfal_3956 as having potential null alleles in Dominica. The same analysis within Montserrat identified markers Lepfal_1628 and Lepfal_3956 as potentially having null alleles. As Lepfal_1628 and Lepfal_3956 show high probability of null alleles in both islands, we carried out analyses to assess the effect of the missing alleles on the summary statistics describing the genetic variation in these populations. The results for both analyses were similar with no statistical associations changing significance and/or direction, with the exception of the number of alleles per locus (uncorrected for sample size). When comparing the number of alleles in *pre*- and *post*- decline populations within Dominica using the remaining six loci, the difference was not significant, but with eight loci (including the null alleles) there was a significant decline in diversity ($p = 0.03$). We present the results including all markers for the remainder of this section. No significant linkage disequilibrium was found between any pair of loci in each island's population.

To avoid the confounding effects of rapid population decline on genetic diversity, the interisland genetic comparison was conducted using only samples collected prior to the arrival of Bd. The comparison between Dominica and Montserrat *pre-decline* showed no significant differences in expected heterozygosity (Welch t-test $p = 0.727$; Table 2.1). However, the allelic richness (corrected for differences in sample size) within the Dominican *pre-decline* population showed a consistent but non-significant trend towards a higher allelic richness than the *pre-decline* population on Montserrat (Table 2.1; Welch t-test $p = 0.06$). The population on Montserrat was not in HWE due to three loci showing significant excess of homozygosity and one locus showing a significant excess of heterozygosity (HWE exact test, $p = 0.016$, Appendix D). Consistent with these results, the inbreeding coefficient of Montserrat was significantly positive ($p < 0.001$), and was not significantly different than that of the Dominican datasets (Welch t-test, *pre-decline* Dominica comparison $p = 0.814$; *post-decline* Dominica comparison $p = 0.421$; Table 2.1). Differentiation between the *pre-decline* Dominican vs. *pre-decline* Montserrat and *post-decline* Dominican vs. *pre-decline* Montserrat datasets showed significant F_{ST} values of 0.2 and 0.25 respectively ($p < 0.001$).

Table 2.1. Statistical descriptors of genetic variation. Descriptor values are averaged across all 8 loci. For each population the corresponding sample size is shown (n), the observed average number of alleles per locus (ANA), allelic richness (AR), the observed heterozygosity (H_O), the expected heterozygosity (H_E), and the population's inbreeding coefficient (F_{IS}). * indicates F_{IS} values significantly different from 0 (P<0.001). Populations deviating from Hardy Weinberg Equilibrium are indicated with bold F_{IS} values. Among the Dominican samples, six collected between 2011 and 2012 were excluded from the *pre-* and *post-* population decline analyses as they did not clearly belong to either group. Among the Montserrat individuals, 35 samples correspond to animals that had been captive-bred and thus were excluded from the comparison between the *Founders* and *Wild* samples.

Population	n	ANA	AR	H_O	H_E	F_{IS}
Dominica	52	6.0	6.0	0.45	0.59	0.233*
Montserrat	120	4.8	4.3	0.49	0.59	0.167*
Dominica: pre-decline (2002 - 2004)	29	5.9	5.2	0.49	0.61	0.197*
Dominica: post-decline (from 2014)	17	3.5	3.5	0.35	0.49	0.283*
Montserrat: *Founders* (collected 2009)	11	3.5	3.5	0.51	0.58	0.116
Montserrat: *Wild* (2009)	74	4.4	3.3	0.48	0.59	0.180*

Within Dominica, the expected heterozygosity and allelic richness (corrected for sample size) for samples collected from 2002 to 2006 (*pre-decline*, n = 29) were both greater than those of the wild individuals sampled in 2014 (*post-decline*, n = 17). Expected heterozygosity declined from 0.61 to 0.49 and allelic richness reduced from 5.2 to 3.5, although only the latter was significant (Table 2.1; Welch t-test p = 0.17 and p = 0.05 respectively). Hardy Weinberg exact tests indicated that neither dataset was in equilibrium, with heterozygote deficiency at four loci in the *pre-decline* dataset (p =< 0.05) and three loci in the *post-decline* dataset (p =< 0.05; Appendix D). We also observed significant positive inbreeding coefficients in the *pre-* and *post-* decline populations (Table 2.1), although they were not significantly different between the *pre-* and *post-* decline datasets (Welch t-test p = 0.493). While these results identify a trend towards reduced genetic diversity after the population crash, demographic analyses using Bottleneck did not find evidence for a genetic bottleneck in the *post-decline* population on Dominica (Heterozygote excess Wilcoxon's Sum Rank test TPM p = 0.58 and SMM p = 0.88).

2.3.4 Representativeness of the founder population

Genetic diversity and differentiation was assessed to ascertain whether the individuals removed from Montserrat in 2009 to establish the captive breeding program (*Founders*, n = 11) were a genetically representative sample of the pre-epidemic wild Montserrat individuals (*Wild*, n = 74). The *Founders* and *Wild* populations were both overall within HWE. The differences in average number of alleles per locus, allelic richness and heterozygosity between these populations (Table 2.1), were not statistically significant (all Welch t-tests: $p > 0.05$). The *Wild* population had a positive inbreeding coefficient ($F_{IS} = 0.18$) that was significantly larger than zero ($p < 0.001$), but which did not statistically differ from that in the *Founder* population (Welch t test $p = 0.705$, Table 2.1). As expected from these results, the differentiation between the *Wild* and *Founder* individuals resulted in an F_{ST} value of 0.

The genetic variability of the *Founder* population used for captive breeding was compared to the *Wild* population accounting for sample size using 100,000 random samples of eleven animals among the *Wild* samples. The average number of alleles per locus, the observed and the expected heterozygosity (Table 2.1) fell well within the 2.5 (Lower Boundary – LB) and 97.5 (Upper Boundary – UB) percentiles of the distribution of those statistics in the random samples of the *Wild* population. The range between the LB and UB for these statistics was 2.9 - 3.6 (average number of alleles per locus), 0.39 - 0.58 (observed heterozygosity), and 0.52 - 0.64 (expected heterozygosity). Thus, the resampling analysis showed that the genetic variation captured by the *Founder* animals was within the expected distribution of genetic variation in the *Wild* population (Fig. 2.6).

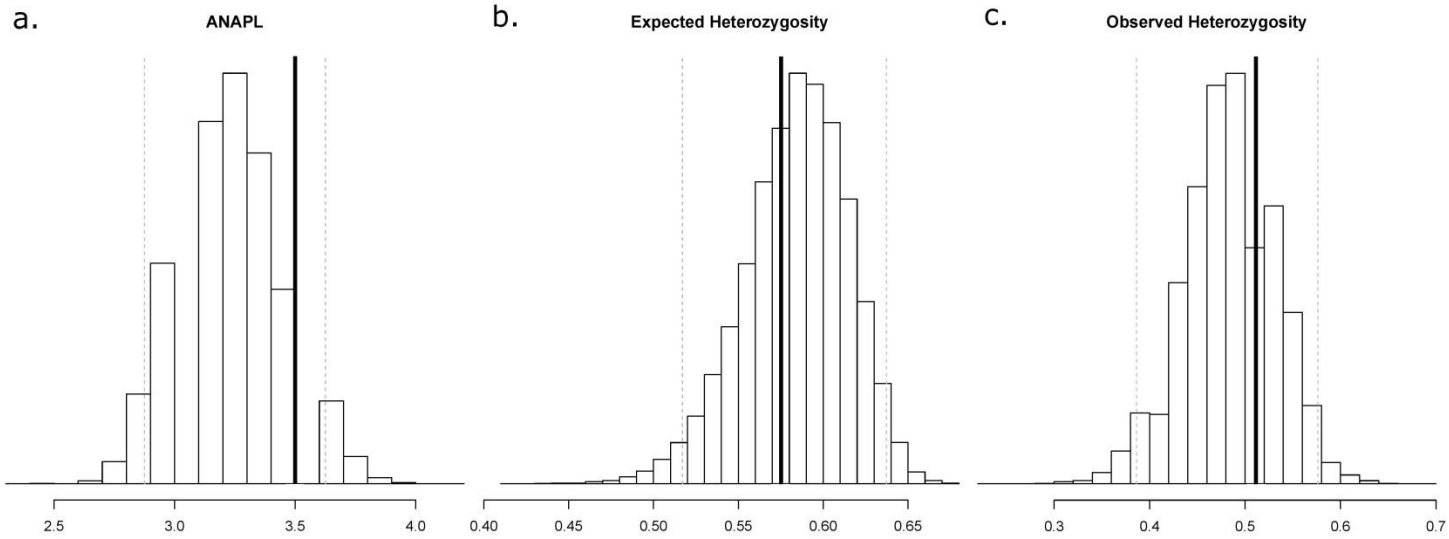

Figure 2.6 Histograms of the expected distributions of three genetic variation statistics. Each histogram describes the expected distribution of a summary statistic in 100,000 bootstrap resamples of eleven individuals in the wild population. The bold line is the observed value of the statistics in the eleven founders, and the grey dotted lines show the 2.5th and 97.5th percentiles of the distribution. a) Average number of alleles per locus - ANAPL; b) Expected Heterozygosity; c) Observed Heterozygosity.

2.4 Discussion

This study shows that the first recorded outbreak of chytridiomycosis in the Lesser Antilles caused the rapid decline and near extinction of the mountain chicken on Montserrat and Dominica. The decline of the mountain chicken across its range is amongst the fastest recorded for any species, with island-wide population collapses due to chytridiomycosis occurring within 18 months on Dominica and under one year on Montserrat. On Dominica, the visual encounter rate halved every seven months between August 2002 and March 2004. It is not clear when the mountain chicken population reached undetectable levels, but by 2006 no individuals could be found despite repeated efforts in prime habitat. A similar but more rapid trajectory of decline was observed on Montserrat. The last intact population on this island, at Fairy Walk, was monitored intensely from August 2009 shortly after chytridiomycosis reached this site. Initial encounter rates were similar to those seen pre-epidemic (between 1999 and 2007), but the encounter rate halved every seven weeks until monitoring had to be suspended after five months due to volcanic activity.

Detectability of mountain chickens on transects was likely not 100%. We attempted to fit an open population N-mixture model (Dail and Madsen 2011) to account for detection probability, but these models failed a goodness of fit test and were not used in analysis despite producing similar estimates for the rate of decline as those produced by the GLMM. Despite this inability to assess detection probability, we maintain that VES is an effective tool for measuring terrestrial amphibian abundance especially when there are a large number of sites in a variety of habitats (Ribeiro-Júnior, Gardner and Ávila-Pires 2008; Rödel and Ernst 2004; Doan 2003). Although presence/absence surveys may have allowed an even greater number of sites to be surveyed, they do not account for unequal abundance between sites (Pollock 2006), which was evident for this species from pre-decline transects. The population coverage was also maximised as transects were originally set up to monitor its status across both islands and so were positioned in known population sites that covered the geographical extremes of the species range.

Amphibian chytridiomycosis has caused similarly rapid declines in other populations (Vredenburg *et al.* 2010; Lips *et al.* 2006a; Rachowicz *et al.* 2006; Briggs *et al.* 2005; Lips 1999; Laurance, McDonald and Speare 1996), but rarely have entire species declines across their entire range been recorded within such a short time frame (85% in 18 months on Dominica, 85% in 13 weeks at Fairy Walk on Montserrat). No other threat has been recorded as having driven such a rapid range-wide decline of a species. The brown tree snake on Guam caused site level extirpations of several bird species within 18 months, however range-wide declines

occurred more slowly (Wiles *et al.* 2003). The saiga antelope (*Saiga tatarica*) declined by 95% over 15 years due to hunting (Milner-Gulland *et al.* 2001) and the Oriental white-rumped vulture (*Gyps bengalensis*) declined in India by 99.9% over 15 years as a result of secondary poisoning (Prakash *et al.* 2007).

The range collapse of the mountain chicken was also precipitous, with a decline of over 80% in both AOO and EOO on Montserrat and of ~95% on Dominica. The species' range on Dominica has collapsed to a small part of the west coast and has undergone severe fragmentation. On Montserrat, only four individuals at three sites have been discovered since 2012 despite repeated intensive surveys; since 2014 only two of these individuals have been recaptured, both at the same site. While the persistence of individuals after a chytridiomycosis epidemic has been recorded in other studies (Scheele, Guarino, *et al.* 2014; Doddington *et al.* 2013; Tobler and Schmidt 2010; Retallick, McCallum and Speare 2004), the extent of the mountain chicken range collapses are similar to those reported by Vredenburg *et al.* (2010), where the majority of mountain yellow-legged frog (*Rana muscosa*) populations were extirpated over a four year period by epidemic chytridiomycosis. The reduction in mountain chicken population connectivity, however, undoubtedly affects the level of gene flow between remaining individuals, increasing the risk of extinction (Allentoft and O'Brien 2010; Dixo *et al.* 2009).

The optimal temperature for the growth of Bd is 17 - 25 °C (Piotrowski, Annis and Longcore 2004) and this is why montane amphibians in the tropics are thought to be at greatest risk (Bielby *et al.* 2015). In Dominica mountain chickens occur in the lowlands where temperatures regularly exceed 28 °C (authors' unpublished data) at which Bd stops growing (Piotrowski, Annis and Longcore 2004). It is not clear therefore why this species has been so badly affected by chytridiomycosis on this island. One possible mechanism might be if the behaviour of this species maintains a lower temperature microclimate in burrows or other refugia. Also, when sick with chytridiomycosis, mountain chickens seek water bodies (Hudson *et al.* 2016), which in Dominica are primarily rivers fed by cool mountain streams.

Previous analyses of mitochondrial DNA sequence data (mtDNA; cytochrome B; 804bp) for the mountain chicken (Figure 3 within Hedges and Heinicke 2007), found a single haplotype across the islands (n=2). Our own analysis of a 463bp portion of the cytochrome oxidase 1 mtDNA gene corroborates this finding (DNA sequences available from Cardiff University upon request), strongly indicating that Dominican and Montserrat mountain chickens represent the same species and the same evolutionary significant unit (ESU).

While the inbreeding coefficient was significantly larger than zero on both islands, results suggest that, before the population crash, the Dominican population was larger than that of Montserrat, harbouring greater genetic variation. The island of Dominica is approximately eight times larger than Montserrat and both island biogeography theory and population genetics studies indicate that populations persisting on smaller and more-isolated islands tend to have lower genetic diversity than those living on larger and more-connected islands (e.g. Gonzalez *et al.* 2014; Wang *et al.* 2014; Ciofi and Bruford 1999). It has been previously hypothesised that Amerindian dispersal from South America into the Lesser Antilles may explain the presence of mountain chickens on these islands (Breuil 2009). If such hypotheses are correct, it could also be expected that Dominica would harbour more genetic variation as it is closer to South America than Montserrat. We did not test for this hypothesis explicitly and no mountain chicken samples from South America are known. Nevertheless, the genetic diversity of the Montserrat mountain chicken population is likely to have been further affected by volcanic events that periodically eliminated mountain chickens from pyroclastic sites on the island (Young 2008; Daltry and Gray 1999). While prior to the chytridiomycosis-driven decline some differentiation between the mountain chicken populations in these islands was observed (F_{ST} ~ 0.2), it is likely that further population size reductions will exacerbate the differences between the relic populations, as well as further reducing the overall genetic variation of this species.

Within Dominica, a temporal trend in genetic diversity could be seen. Whilst comparisons of *pre*- and *post*- decline heterozygosity and inbreeding coefficients were not significant, the difference between *pre*- and *post*- decline allelic richness was close to significant (a metric found to be much more sensitive for detecting demographic change over short time scales (Hoban *et al.* 2014; Spencer, Neigel and Leberg 2000). Recently, genetic bottlenecks were detected in Bd-affected populations of the common midwife toad (*Alytes obstetricans*) in central Spain (Albert *et al.* 2014). They were not, however, able to compare pre- and post-decline genetic diversity at the same location. In the present study we were unable to detect evidence of a genetic bottleneck in the *post-decline* population on Dominica using the program Bottleneck, despite seeing both a trend of loss of heterozygosity and some evidence for differences in genetic diversity metrics for the *pre*- and *post*- decline populations. However, very recent bottlenecks often go undetected in genetic data using limited sample sizes, even when it is known that those bottlenecks did occur (Peery *et al.* 2012). In our case, the *post-decline* dataset for the Dominican mountain chicken population was confined to 17 samples taken in 2014 and this sample size might not have been high enough to detect a genetic bottleneck signature.

On Montserrat, a comparison between the founders of the captive population (*Founders*) and the wild individuals sampled pre-decline (*Wild*) showed very little difference in expected heterozygosity or allelic richness, and genetic differentiation (F_{ST}) between the two populations was zero. Also, the resampling analysis showed that the genetic diversity captured by the founder stock adequately represented the wild population, while the F_{IS} indicated no significant evidence of inbreeding. Captive breeding programs for wildlife conservation require careful genetic management to avoid further reductions of genetic diversity (Russello and Amato 2004). In this context, molecular analysis can be helpful in establishing the comparative diversity of wild and captive populations and in managing that genetic diversity in future captive generations (Ramirez *et al.* 2006). In the case of the mountain chicken, care should be taken to ensure that the captive population continues to represent the founding gene-pool in subsequent generations (e.g. by controlling mating to avoid increasing inbreeding, and avoiding population decreases of the capture population), especially as eventual reintroduction is the primary aim (Mountain Chicken Recovery Programme 2014; Iyengar *et al.* 2007).

Shortly after the onset of the chytridiomycosis epidemic on each island, mountain chickens were provided full legal protection and their hunting was banned. The Dominican government also banned the import of amphibians or amphibian products following the discovery of chytridiomycosis on that island. During the seven-year gap between the emergence of chytridiomycosis in the Lesser Antilles (i.e. on Dominica) and its incursion to Montserrat, amphibian populations on the latter island were shown to be free from Bd infection and a risk analysis identified the most likely routes of introduction. Unfortunately, no identified mitigation measures were implemented. Capacity for sanitary measures that remove amphibian stowaways and contaminated soil from produce prior to export was developed in Dominica in 2007 to enable exports to the Lesser Antillean islands of Guadeloupe and Martinique. Had this requirement been imposed for produce shipped to Montserrat, the risk of Bd incursion would likely have been much reduced.

There is evidence of enzootic Bd infection in Caribbean tree frogs such as *Eleutherodactylus coqui* from Puerto Rico (Longo, Burrowes and Joglar 2010) and *E. Johnstonei* on Montserrat (authors' unpublished data) indicating some tolerance to Bd infection. The intentional or accidental transport of amphibians such as these between islands is a likely route of transmission of Bd throughout the Caribbean (http://www.mountainchicken.org/wp-content/uploads/2010/11/Chytridiomycosis-Management-Plan.pdf). The World Organisation

for Animal Health (OIE) listed Bd in May 2008, making the pathogen internationally notifiable and thus subject to OIE standards to assure the sanitary safety of international trade in live amphibians and their products. Despite this, few if any of the OIE's 180 member states have imposed relevant sanitary requirements on traded amphibians. This might be because, although it is a huge industry involving tens of millions of animals annually (Schloegel *et al.* 2010), the international amphibian trade is generally unregulated and unrecorded (Peel, Hartley and Cunningham 2012). Neither Dominica nor Montserrat are members of the OIE, although other island states with high levels of amphibian endemicity which would be threatened by Bd incursion are (e.g. Sri Lanka). If lessons to help the world meet its Aichi 2020 targets are to be learned from the demise of the mountain chicken, these member states should be implementing the necessary sanitary regulations and instigating Bd surveillance and control measures.

3 In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis epidemic

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Abstract

The emerging infectious disease, amphibian chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (Bd), threatens hundreds of amphibian species globally. In the absence of field-based mitigation methods, the Amphibian Conservation Action Plan advocates captive assurance programmes to prevent extinction from this infectious disease. Unfortunately, with the cooperation of the entire global zoo community, the International Union for the Conservation of Nature Amphibian Ark estimates only 50 species could be saved. Clearly, if catastrophic losses are to be averted, alternative mitigation techniques need to be developed. There has been an absence of trialling laboratory proven interventions for chytridiomycosis in field settings, which must change in order to allow informed management decisions for highly threatened amphibian populations. We tested the in-situ treatment of individual mountain chicken frogs (*Leptodactylus fallax*) using the antifungal drug, itraconazole. Multi-state mark recapture analysis showed increased probability of survival and loss of Bd infection for treated frogs compared to untreated animals. There was evidence of a prophylactic effect of treatment as, during the treatment period, infection probability was lower for treated animals than untreated animals. Whilst long term, post-treatment increase in survival was not observed, a deterministic population model estimated antifungal treatment would extend time to extinction of the population from 49 to 124 weeks, an approximated 60% increase. In-situ treatment of individuals could, therefore, be a useful short-term measure to augment other conservation actions for amphibian species threatened by chytridiomycosis or to facilitate population survival during periods of high disease risk.

3.1 Introduction

Emerging infectious diseases are a growing threat to both humans and biodiversity globally (Morens and Fauci 2013; Daszak, Cunningham and Hyatt 2000). Three main strategies exist for the management of wildlife disease: prevention of introduction, mitigation of impact, and eradication (Wobeser 2002). Globalisation, with its increased rate and volume of trade and travel, means preventing the introduction of novel diseases is increasingly difficult (Marano, Arguin and Pappaioanou 2007). Whilst neutralisation of threats has long been considered a pre-requisite for successful wildlife conservation (Caughley 1994), the emergence of threats which cannot be negated poses a difficult challenge to conservation managers. One example is amphibian chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), which is implicated in the rapid decline or extinction of over 200 amphibian species globally (Skerratt *et al.* 2007), and has been described as "the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and it's propensity to drive them to extinction" (Gascon *et al.* 2007). This rapid global loss of amphibians is likely to have major implications for the environment (Whiles *et al.* 2006).

In the absence of in-situ mitigation for amphibian chytridiomycosis (Woodhams *et al.* 2011; Joseph *et al.* 2013), the Amphibian Conservation Action Plan advocates the creation of Bd-free captive populations for eventual release as a key conservation strategy (Gascon *et al.* 2007). Currently, conservation practitioners rely on such captive assurance programmes to prevent species extinctions (Mendelson *et al.* 2006), but this is only a short to medium term solution and Amphibian Ark estimates that only around 50 species can be saved in this way (Zippel *et al.* 2011). Even so, zoos are currently failing to prioritise species that are likely to require captive breeding programmes to prevent their extinction (Dawson *et al.* 2016). There is, therefore, an urgent need to change the research focus from the treatment of captive animals to in-situ mitigation (Harding, Griffiths and Pavajeau 2015; Scheele, Hunter, *et al.* 2014).

A range of potential in-situ interventions to mitigate the impacts of chytridiomycosis have been suggested, but so far these remain largely untested in the field (Scheele, Hunter, *et al.* 2014; Berger and Skerratt 2012). These include habitat manipulation to inhibit Bd (Scheele, Hunter, *et al.* 2014), reintroduction after selection for tolerance in captivity (Venesky *et al.* 2012), and in-situ use of antifungal treatments (Berger and Skerratt 2012). Some antifungal drugs, including itraconazole, are effective in the treatment of Bd infection in captivity, but only following multiple daily applications (e.g. Brannelly, Skerratt and Berger 2015; Georoff *et al.* 2013; Jones *et al.* 2012; Tamukai *et al.* 2011; Forzán, Gunn and Scott 2008). In addition to being effective, the application of itraconazole is relatively easy, being via immersion in an

aqueous solution – albeit that repeated administration is required for successful treatment (Nichols and Lamirande 2001). Whilst there have been some reported side-effects in certain species (Brannelly 2014; Brannelly, Richards-Zawacki and Pessier 2012) and life stages (Woodhams *et al.* 2012; Garner *et al.* 2009), itraconazole is considered to be the treatment of choice for amphibian chytridiomycosis (Holden *et al.* 2014). Reducing the dose from 0.01% for 11 days to 0.0025% for 5 days has been shown to reduce side effects while maintaining efficacy (Brannelly 2014). Bosch *et al.* (2015) described the eradication of Bd from the wild Mallorcan midwife toad (*Alytes muletensis*) tadpoles by treating them with itraconazole in captivity and returning them to the wild following chemical disinfection of their breeding ponds and surrounding rocks. As other amphibians and vegetation were absent from the disinfected sites, and as these were rock pools containing little organic matter (which rapidly inactivates most disinfectants), this technique is unlikely to be transferable to many other species or locations.

In-situ treatment regimens provide challenges in field settings due to, for example, large target population sizes, low capture rates the potential of reinfection and the need for a continuous supply of labour. As a result, previous studies have treated individuals with itraconazole in captivity prior to re-release rather than treating them in-situ (Hardy *et al.* 2015). Environmental persistence of Bd zoospores (Johnson and Speare 2005; 2003) and the possible presence of infected sympatric amphibians (Daszak *et al.* 1999) mean animals treated in-situ would likely be exposed to Bd both throughout and after the treatment period, increasing the likelihood of their extirpation (Mitchell *et al.* 2008; Retallick, McCallum and Speare 2004). Antifungal treatment in a field setting, however, might enable treated animals to persist by lowering their Bd infection load until the initial epidemic has passed (Briggs, Knapp and Vredenburg 2010; Vredenburg *et al.* 2010). There is some evidence that animals surviving the epidemic phase persist by tolerating subsequent lower levels and frequencies of infection (Briggs, Knapp and Vredenburg 2010; Retallick, McCallum and Speare 2004). Also, repeated infection and clearance of Bd might allow the development of resistance in some species (McMahon *et al.* 2014).

The Caribbean is a global hotspot of amphibian endemism, with 99% of the 197 species being endemic (Fong, Viña Dávila and López-Iborra 2015), and it has the highest proportion (84%) of threatened amphibians within a region (Stuart *et al.* 2008). One species, the mountain chicken frog (*Leptodactylus fallax*), has suffered a precipitous decline due to chytridiomycosis (Mountain Chicken Recovery Programme 2014; Fa *et al.* 2010; Magin 2003). The mountain chicken is classified as critically endangered on the IUCN Red List of Threatened Species (Fa *et*

al. 2010) and is restricted to only Dominica and Montserrat in the Lesser Antilles. A 2005 survey found no evidence of Bd in amphibians on Montserrat (Garcia *et al.* 2007), but in January 2009 mountain chicken mortality due to chytridiomycosis was first discovered on Montserrat and this was rapidly followed by epidemic mortality across the island (Mountain Chicken Recovery Programme 2014). The characteristically rapid rates of chytridiomycosis-driven declines (Lips *et al.* 2006b), such as those observed in the mountain chicken, limit the time available to react effectively. Interventions that can reduce rates of decline can be valuable for providing extra time to implement further conservation actions.

In this study we report the use of itraconazole treatment in a field setting in an attempt to mitigate the impact of epidemic chytridiomycosis. We assess whether in-situ antifungal treatment is a feasible and effective method for improving the survival of a critically endangered species undergoing a precipitous decline due to epidemic chytridiomycosis. The mountain chicken is an ideal species to use as a model for such in-situ treatment as it is a large territorial animal with predictable behaviours, making it relatively easy to detect and individually identify. Also, the species has been studied for over ten years on Montserrat, so there is a great deal of knowledge about its distribution, abundance and behaviour and field sites were already established (Garcia *et al.* 2007; Martin *et al.* 2007). On Montserrat the presence of a sympatric amphibian fauna of species (*Eleutherodactylus johnstonei* and *Rhinella marina*) able to carry Bd renders an in-situ treatment study realistic for extrapolation to other species and regions where sympatric amphibians act as Bd reservoirs. Effective treatment of chytridiomycosis in captive mountain chickens using itraconazole has shown the drug to be safe for this species (authors' unpublished observations). Finally, the mountain chicken has a voracious appetite and requires large enclosures in captivity, therefore it is difficult and expensive to hold a large enough captive population for a viable, long-term conservation breeding programme.

3.2 Materials and methods

3.2.1 Study site

Montserrat is a U.K. overseas territory in the Eastern Caribbean (16.45°N, 62.15°W). The centre of the island comprises an active volcano which has been erupting regularly since 1995. As a consequence the mountain chicken is restricted to a circa 17 km^2 mountainous area; the Centre Hills region which is typified by montane rainforest and deep valleys (or ghauts – Fig. 3.1) (Young 2008).

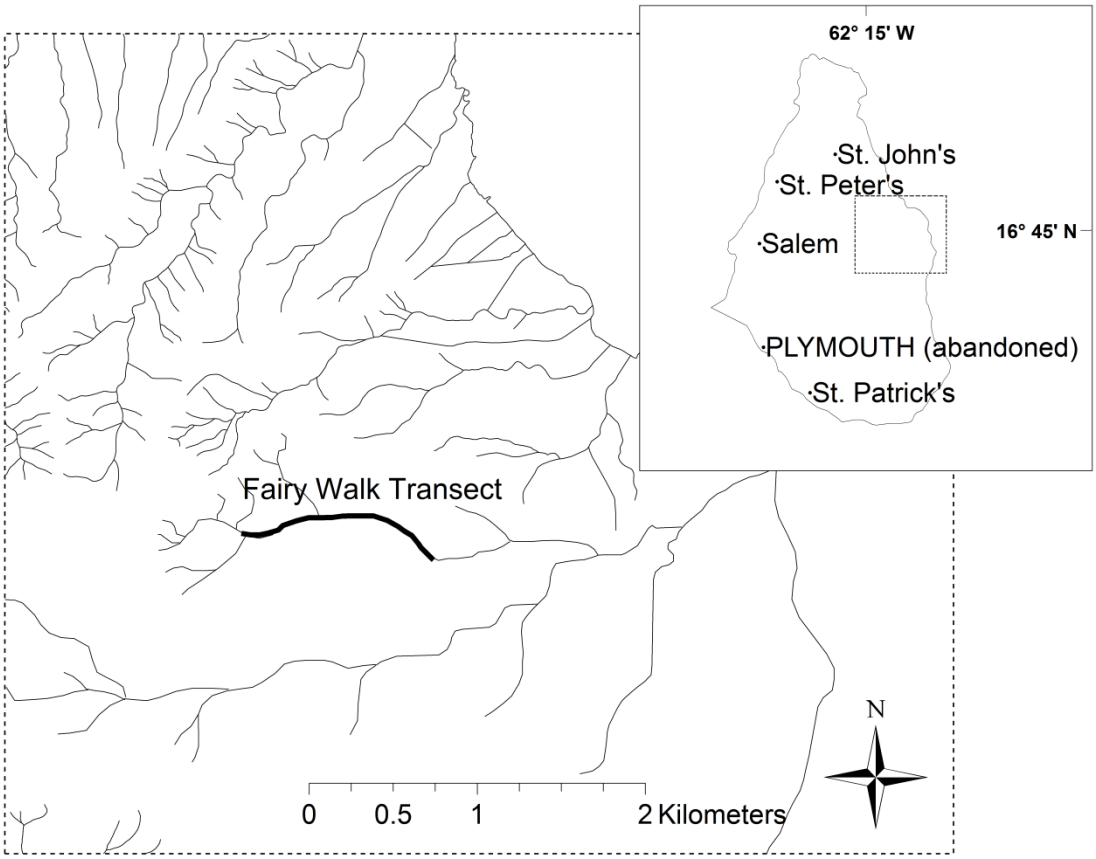

Figure 3.1 Map of Montserrat and Fairy Walk study site. The ghauts (steep sided valleys) of Montserrat with the study transect in Fairy Walk ghaut highlighted, downstream of the Fairy Walk spring on the East of Montserrat.

The field site (Fairy Walk) is a forested relatively-shallow-sloped ghaut of approximately 1 km^2 on the eastern flank of the Centre Hills at an approximate elevation of 250 m asl. Prior to 2009, Fairy Walk was home to the highest known population density of mountain chickens on Montserrat (Young 2008) and, at the commencement of this study, it contained the last remaining intact population following the emergence of chytridiomycosis on the island in 2009.

3.2.2 Study design

The field experiment took place between August 2009 and January 2010. Fairy Walk was visited three times a week for 24 weeks and surveyed a predefined 800 m transect along the stream (Fig. 3.1) at a slow walking pace in a team of five. On each occasion, all mountain chickens seen within 5 m of the transect were caught and any dead animals recovered. All captured frogs were individually marked using a Passive Integrated Transponder (PIT) (11 mm x 2 mm, ID-100A Microtransponder, Trovan Ltd.), which was subcutaneously implanted in the dorsum where retention rates are maximal (Blomquist, Zydlewski and Hunter, Jr. 2008). Each frog was skin-swabbed for Bd on every capture using a rayon-tipped swab (MW 100-100, Medical Wire and Equipment Co.) three times across each of the following sites: ventral abdomen, ventral thighs and calves, and plantar surfaces of both hind-feet. Frogs were assigned to one of three groups during the study: itraconazole treatment (IT), stream water control (SWC), and non-bath control (NBC). On each capture, after skin-swabbing, each animal in the IT group was immersed for 5 minutes in a 0.01% aqueous solution of itraconazole (Sporanox, Janssen Pharmaceuticals, Inc.), prepared using stream water on site. Frogs in the SWC group were treated similarly, but in stream water without itraconazole in order to test for effects of handling during the treatment process. Each frog was immersed within a new, disposable food-grade plastic bag. Frogs in the NBC group were released after swabbing with no further intervention.

During the first 2 weeks of the study, animals were randomly assigned to the IT and SWC groups at the time of first capture, with a 2:1 bias towards treatment. From week 3, all further captures were assigned to the NBC group. In order to examine any treatment-specific long term effect on survival or infection rate, treatments were discontinued after 15 weeks, but re-sighted animals continued to be captured and skin-swabbed. Monitoring was continued until week 24 when the study was prematurely ended by a major volcanic eruption.

3.2.3 Laboratory methods

Skin-swabs were refrigerated until transport to the laboratory where DNA was extracted using methods adapted from Hyatt *et al.* (2007) (explained in Appendix E). Extracted DNA was diluted 1:10 in molecular grade water and examined for the presence of Bd DNA using a Bd-specific TaqMan real-time PCR as described by Boyle *et al.* (2004) modified by the inclusion of bovine serum albumin to reduce PCR inhibition (Garland *et al.* 2010). Samples were tested in duplicate, incorporating two negative control wells containing laboratory grade distilled water and four positive controls (100, 10, 1, and 0.1 zoospore equivalents) in duplicate on each plate. A sample was considered positive if PCR amplification occurred in both duplicates. If duplicates generated conflicting results, the samples were re-run up to three times until matching results were obtained. If there was no consensus on the third occasion, the sample was considered negative.

Quantification of Bd DNA in each well was determined as Bd genome equivalents (GEs) by multiplying the real time PCR result by 120 (4 µl of 60 µl total elute used to make up the dilution (x15) and 5 µl of 40 µl 1:10 dilution used in qPCR (x8) [15 x 8=120]).

3.2.4 Bd infection intensity comparison

In order to test whether itraconazole treatment significantly reduced Bd infection intensity, a linear mixed effects model was used, with Bd infection load (zsp. equivalents) as the response variable, treatment group (control vs. IT) and time as fixed effects and frog ID as a random effect. Infection intensity was log transformed prior to analysis as values ranged over many orders of magnitude. Models were compared using AIC corrected for small sample size (AICc) and if no model was overwhelmingly supported (Akaike weight > 0.95), models with a ΔAICc<7 were considered for inference. Summed Akaike weight evidence ratios were used to assess variable importance (Burnham and Anderson 2002).

3.2.5 Capture-mark-recapture analyses

The capture-mark-recapture (CMR) data were analysed using the software program Mark (White and Burnham 1999) in a multi-state CMR framework (Lebreton *et al.* 2009). Multi-state CMR models are an extension of Cormack-Jolly-Seber (CJS) which are used to model the probability of transition between states alongside estimating state dependent survival and recapture rates. These transitions were modelled as first order Markov processes in which the state at time t+1 is dependent only on the state at time t. The states were defined as 'uninfected' (U), 'infected' (I), and 'dead' (D).

Data were converted from daily to weekly capture histories using weekly bins to generate weekly parameter estimates. Although grouping data in this way has been shown to produce biased parameter estimates of survival rate in a CJS model when survival rate is time-dependent (Barbour, Ponciano and Lorenzen 2013), fixed estimates of survival and transition rate were best supported by our data. Where different states were detected during a single weekly bin (n=32) frogs were assigned to whichever state the individual was most commonly caught in, unless one of those states was dead, which superseded other states. In the majority of cases (n=17) the different states recorded within a week reflected a transition between the state recorded in the previous week and the state in the following week, meaning there was no loss of transition in the weekly data. Where the individual was caught in two different states in the same week, the individual was assigned randomly to either state. As this might have hidden capture heterogeneity an ANOVA was used to test for a difference in the mean number of captures per week in each group.

Infection state (inf), treatment group (gr), sex and time dependence (time) were examined in estimates of survival, recapture, and transition probabilities. Recovery rates of dead frogs were modelled as a function of treatment group, and sex. Models were also used in which survival, recapture and transition rates were a function of group, but with two estimates for the IT group; one estimate during treatment with itraconazole (weeks 1-15), and one after this treatment had ended (week 16-24) (gr[split T]). This enabled testing for any post-treatment effects. An effect of the immersion process was tested for by comparing models with one estimate for both control groups combined (gr[C]) and one where SWC and NBC were estimated separately (gr). No occasion-specific environmental variables were available. Juveniles were excluded from the analysis due to low sample size.

In order to reduce the potentially very large number of candidate models, a two-step process modified from Lebreton *et al.* (1992) was used to estimate parameters in the CMR analysis. In step one, the top model for survival and recapture probabilities from a preliminary Burnham dead recoveries analysis (Burnham 1993) was used to model dead recovery and transition rates. In step two, the best estimates of dead recovery and transition rates from step one were used to model survival and recapture probabilities. This led to the generation of a model set of 128 models.

3.2.6 Model selection and goodness of fit

Model selection was based on AICc. To account for model selection uncertainty, robust estimates of the parameters were computed using weighted model averaging (Burnham and Anderson 2002).

A preliminary diagnostic goodness of fit test for the multi-state models was performed in program U-CARE (Choquet *et al.* 2009) which detected slight over-dispersion and so the variance inflation factor was adjusted to 1.15 and the adjusted QAICc was used for model selection.

Summed Akaike weight evidence ratios were used to examine the support for dependencies in the models. The strength of the support provided by the evidence ratios was extracted from Table 3 in Lukacs *et al.* (2007).

3.2.7 Population modelling

In order to predict how treatment with itraconazole would have affected the entire sampled population had it been applied across all frogs in this study, a deterministic population model was produced in a susceptible–infected–susceptible (SIS) framework using the transition and survival rate estimates from the CMR modelling. Recruitment to the adult population was excluded as no nests have been recorded on Montserrat since the onset of the chytridiomycosis epidemic. Population extinction is defined as population size below 1.

Two versions of this model were produced for a population of 228 frogs (the number of unique captures in this study). The first assumed that all frogs were treated at the same rate as the treated frogs in this study using the model averaged CMR transition and survival rate estimates for the IT group. The second population was modelled as untreated, using the model averaged CMR parameter estimates for the control groups. The simulation was initiated with one infected individual. The number of frogs in each state at each time step was calculated using the matrix below, following the notation in Lebreton *et al.* (2009) in which $\varphi(1,2)$ indicates the rate of transition between state 1 and state 2.

$$\begin{pmatrix} nI \\ nS \\ nD \end{pmatrix}_{t+1} = \begin{pmatrix} \varphi(I,I) & \varphi(U,I) & 0 \\ \varphi(I,U) & \varphi(U,U) & 0 \\ \varphi(I,D) & \varphi(U,D) & 1 \end{pmatrix} \begin{pmatrix} nI \\ nS \\ nD \end{pmatrix}_{t}$$

where: $\varphi(I,I) = 1 - \varphi(I,U) - \varphi(I,D)$ and: $\varphi(U,U) = 1 - \varphi(U,I) - \varphi(U,D)$

In order to include model-averaged parameter uncertainty from the CMR models, two further models were made for each group, the shortest and longest times to extinction. To make the lowest time to extinction model the lower 95% CI estimate for the rate of loss of infection and the upper 95% CI estimates for infection and mortality rates were used. The opposite 95% CIs were used to make the longest time to extinction model. Only the mean model is presented graphically.

3.3 Results

In total we made 1735 captures of 228 frogs. We caught frogs assigned to the IT group (841 captures of 80 frogs) more often in both absolute terms and relative to the group size than frogs from the SWC group (326 captures of 42 frogs) and the NBC group (482 captures of 106 frogs). The sex ratio was circa 1:1 in each treatment group. Frogs with clinical signs of chytridiomycosis were found throughout the study and in all groups.

By the end of the study, 22% (n=50) of the frogs had been found dead (SWC=21% (n=9), NBC=18% (n=19), IT=28% (n=22)). The proportion of animals known to be extant was greatest in the IT group throughout the study, and this was especially evident towards the end of the study period (Fig. 3.2).

Across the study we captured, and therefore treated, frogs in the IT group an average of 0.98 (SE=0.06, min=0.16, max=2.50) times per week.

3.3.1 Skin swab diagnostic data

During the study 67% of the 1735 skin swabs taken tested positive for Bd (SWC=84% (n=317), NBC=80% (n=463), IT=64% (n=819)). Until the itraconazole treatment ended at week 15, frogs in the IT group were more likely to test negative for Bd than frogs in the control groups, after which the likelihood of testing negative became the same across all groups (Fig. 3.2). We captured only 13 frogs which never tested positive for Bd. Eleven of these were in the NBC group and were captured only once (n=8) or twice (n=3). The remaining two were in the IT group and were captured 3 and 16 times. Bd infected animals in the IT group had a lower infection intensity during treatment than animals in the control group (IT: naïve mean=5666 GE, SE=1879; Control: naïve mean=71 607 GE, SE=24 218). The top linear mixed model for the treatment period contained a group-time interaction and received overwhelming support (Akaike weight=0.9997). This provided evidence that although the Bd infection intensity of infected animals was similar in the IT and control groups at the start of the study (IT=168.81 GE, SE=1.63; Control=87.46 GE, SE=1.44), the rate at which the infection intensity increased

was much greater in the control group (on the log scale: IT=0.015 GE/week, SE=0.03; Control=0.138 GE/week, SE=0.02; Appendix F). In the post-treatment period, the infection intensity of infected animals in the IT group increased (IT: naïve mean=47 002 GE, SE=19 169) and there was very weak evidence (summed Akaike weight =0.4041, evidence ratio=0.7) of a difference with the control group animals in the same period (Control: naïve mean=69 480 GE, SE=57 678) suggesting the benefit of treatment were lost after treatment ended (see Appendix F).

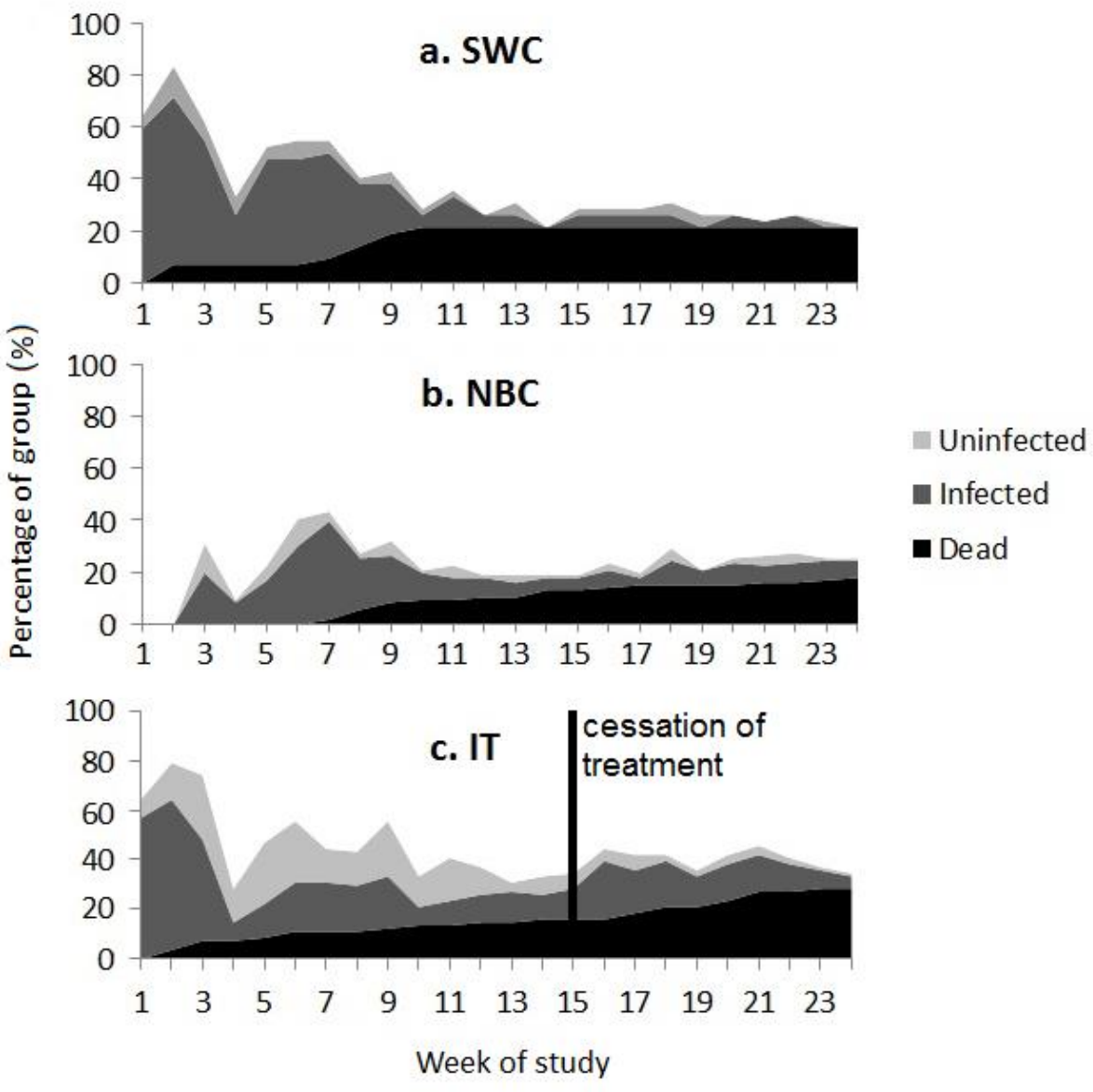

Figure 3.2 Weekly states of captured mountain chickens by proportion of total number in the (a) stream water control group, (b) non-bath control group and (c) itraconazole treatment group. Higher levels of uninfected individuals are visible throughout the study in the itraconazole treatment group and a larger number of known extant individuals persist in that group at the end of the study.

3.3.2 Multi-state mark-recapture models

The top models (ΔQAICc<7) are listed in Table 3.1. As no model received overwhelming support (top model Akaike weight 0.293), model averaging was used to generate robust parameter estimates to account for model variation. Grouping captures into weekly bins may have hidden heterogeneity in the capture rate between groups, but we found no evidence for a significant difference in the mean number of captures per week between groups (ANOVA: SWC: mean=1.12, SE=0.08; NBC: mean=0.97, SE=0.04; IT: mean= 0.98 SE=0.06; $F(2,225)=1.598$, MSE=0.236, $p=0.205$).

All of the most parsimonious models (Akaike weight > 0) contained a difference in survival between the IT and control groups, and between Bd infected and uninfected animals. There was moderate support for no difference in the SWC and NBC groups (summed Akaike weight = 0.969; evidence ratio = 31.3) and so, only one estimate of survival for the two control groups is presented. Model averaged parameter estimates showed that itraconazole treatment increased the weekly survival rate of Bd infected animals by 11.6% compared to animals in the control groups (IT = 0.903, 95% CI = 0.860 - 0.934; Control = 0.809, 95% CI = 0.764 - 0.841; Fig. 3.3). All of the most parsimonious models, however, included a second estimate for the IT group when treatment ended: the estimate decreased to a value similar to the control groups (0.795, 95% CI = 0.709 - 0.864). Uninfected animals had a higher weekly survival rate than Bd infected animals in both the IT (0.988, 95% CI = 0.972 - 0.995, effect size = 9.4%) and control groups (0.974, 95% CI = 0.939 - 0.987, effect size = 20.3%; Fig. 3.3).

Each of the most parsimonious models contained a difference in recapture rate between Bd infected and uninfected animals, and with time dependency. The top models also contained a difference in the recapture rate of the IT and control groups, with limited support for a difference in the NBC and SWC groups (summed Akaike weight = 0.877; evidence ratio = 7.1). There was very weak support for an interaction between infection state and treatment group (summed Akaike weight = 0.095, evidence ratio = 0.1). As time dependent recapture probability was best supported, mean estimates averaged across each occasion are presented (Fig. 3.4 - full results). Model averaged parameter estimates showed that Bd infection increased recapture probability by a mean of 99.1% in the IT group (Uninfected (U) = 0.354, Infected (I) = 0.711), 120% in the SWC group (U = 0.310, I = 0.686), and 136% in the NBC group (U = 0.270, I = 0.637). Based on these estimates, the recapture rate of Bd infected animals in the IT group was 3.6% greater than the SWC group and 11.6% higher than the NBC group. The recapture rate of uninfected animals in the IT group was 14.1% greater than the SWC group and 31.1% higher than in the NBC group. The recapture rate of Bd infected animals in the SWC group was 7.2% higher than in the NBC group and 14.8% higher than uninfected animals.

Table 3.1 Multi-state mark recapture model selection table showing the top models (ΔQAICc<2), the next best models (ΔQAICc<7) and the general model.

Abbreviations: no group or time variation (.), infection state (inf), all group difference (gr), difference between treatment and control groups (gr[C]), with two estimates for the treatment group: one during and one post-treatment gr[splitT], time, sex, difference in AIC between selected model and top model (ΔQAICc), the QAICc weight (W), the number of parameters (K). The AIC score is corrected for small sample size (AICc) and an adjusted variance-inflation factor to account for slight-overdispersion (QAICc).

Survival	Recapture	Dead recovery	Transition	QAICc	ΔQAICc	W	K	QDeviance
Inf + gr[C + splitT]	Inf + gr + time	.	Inf * gr[C + split T]	5057.429	0.000	0.296	37	4978.787
Inf + gr[C + splitT]	Inf + gr + time	gr	Inf * gr[C + split T]	5057.937	0.508	0.230	38	4981.433
Inf + gr[C + splitT]	Inf + gr + time	.	Inf * gr[C + split T] + sex	5059.732	2.274	0.095	38	4981.061
Inf + gr[C + splitT] + sex	Inf + gr + time	.	Inf * gr[C + split T]	5059.857	2.429	0.088	38	4981.216
Inf + gr[C + splitT]	Inf * gr + time	.	Inf * gr[C + split T]	5060.888	3.459	0.052	39	4980.104
Inf + gr[C + splitT]	Inf + gr + time	.	Inf * gr[split T]	5061.289	3.861	0.043	39	4980.506
Inf + gr[C + splitT]	Inf + gr[C] + time	.	Inf * gr[C + split T]	5061.697	4.268	0.035	36	4985.193
Inf + gr[C + splitT]	Inf + gr[C] + time	gr	Inf * gr[C + split T]	5062.263	4.834	0.026	37	4987.893
Inf + gr[C + splitT]	Inf * gr + time	.	Inf * gr[C + split T] + sex	5062.526	5.097	0.023	40	4979.598
Inf + gr[C + splitT] + sex	Inf * gr + time	.	Inf * gr[C + split T]	5062.776	5.347	0.020	40	4979.847
Inf + gr[C + splitT]	Inf + gr[C] + time	.	Inf * gr[C + split T]	5063.183	5.754	0.017	37	4986.679
Inf + gr[splitT]	Inf + gr + time	gr	Inf * gr[C + split T]	5063.433	6.004	0.015	42	4976.202
Inf + gr[splitT]	Inf + gr + time	.	Inf * gr[C + split T] + sex	5063.483	6.055	0.014	41	4978.406
Inf + gr[C + splitT]	Inf + gr[C] + time	.	Inf * gr[C + split T] + sex	5064.112	6.683	0.010	37	4987.608
Inf * time	Inf * gr + time	gr + time	Inf * gr + time	5186.711	110.834	0.000	124	5645.1061

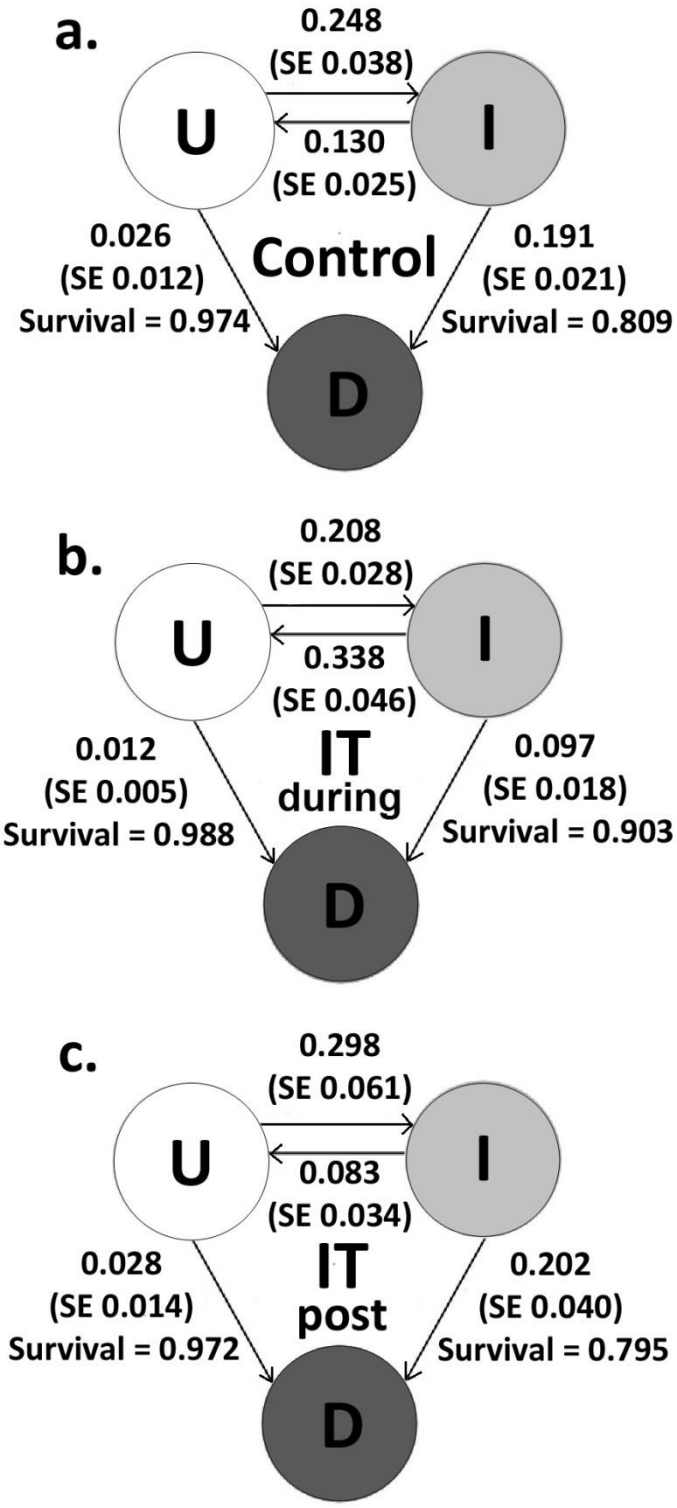

Figure 3.3 Model averaged weekly multi-state mark recapture parameter estimates with unconditional standard errors. Estimates are shown for control groups and itraconazole treatment group (IT) during and after treatment. Abbreviations: uninfected state (U), infected state (I) and dead (D).

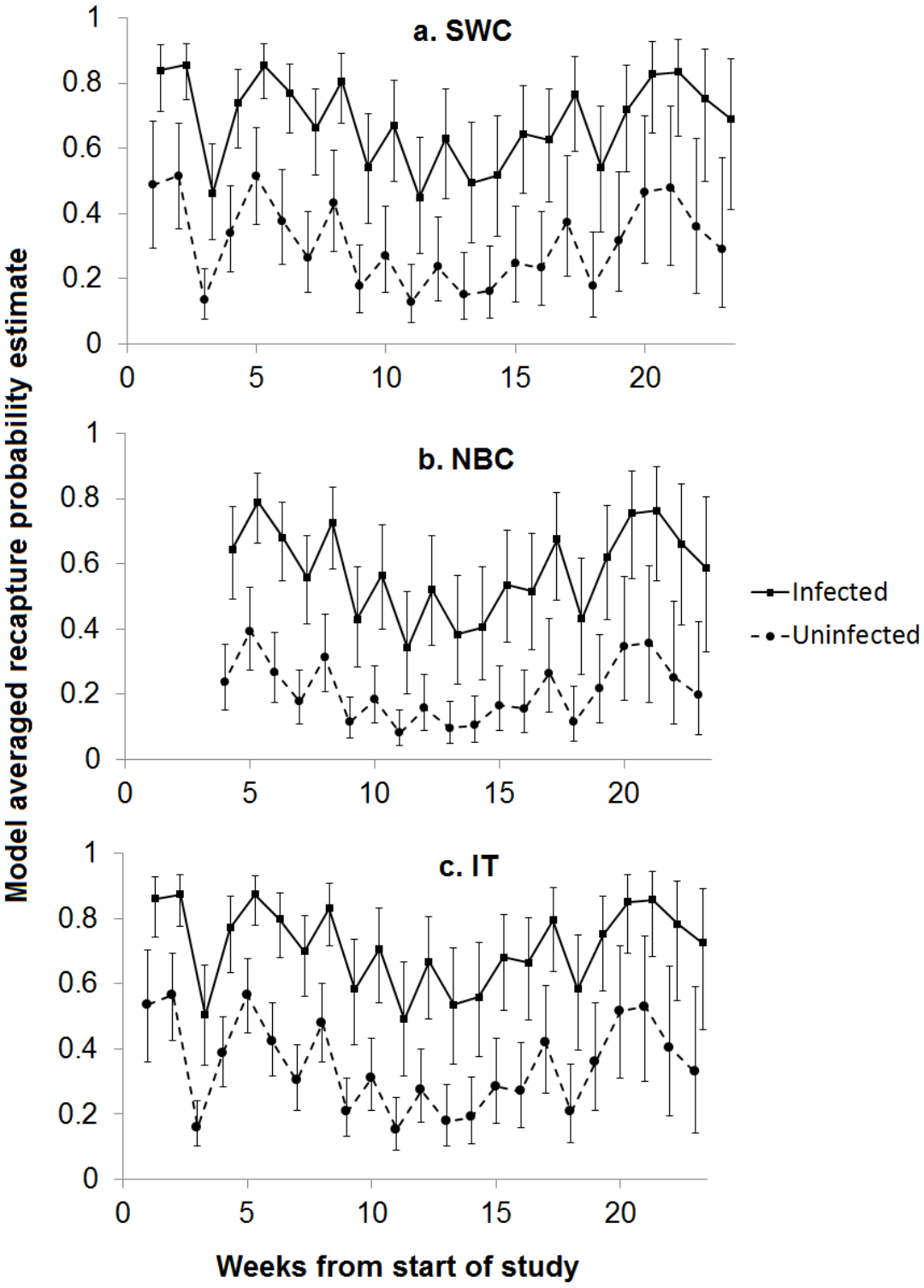

Figure 3.4 Model averaged weekly estimates of recapture probability from the multi-state capture-mark-recapture model for Bd infected and Bd uninfected mountain chickens. Estimates shown for the (a) stream water control group, (b) non-bath control and (c) itraconazole treatment group. No animals were allocated to the NBC group for the first two weeks of the study and so no estimates are shown.

All of the most parsimonious models contained a difference in state transition rates (infection and loss of infection) between the itraconazole treatment and control groups. There was very weak support for a difference in the transition rates of the two control groups (summed Akaike weight = 0.043; evidence ratio < 0.1), and in the different sexes (summed Akaike weight = 0.142; evidence ratio = 0.2). As a result one estimate for both control groups and sexes is presented. Itraconazole treatment reduced the weekly infection rate of uninfected animals by 19.3% compared to the control groups (IT = 0.208, 95% CI = 0.158-0.269: Control = 0.248, 95% CI = 0.185-0.330; Fig. 3.3). Itraconazole treatment also increased the weekly rate of loss of Bd infection of infected animals by 161% compared to the control groups (IT= 0.338, 95% CI = 0.254-0.433: Control = 0.129, 95% CI = 0.088-0.177). All top models included a second estimate for transition rate for the itraconazole treatment group when treatment ended, when infection rate increased to a similar level to the control groups (IT= 0.298, 95% CI = 0.194-0.430) and rate of loss of infection declined to levels similar to the control groups (IT = 0.083, 95% CI = 0.036-0.178; Fig. 3.3).

There was weak evidence for a treatment group difference in dead recovery rate (summed Akaike weight = 0.271; evidence ratio = 0.3). The model averaged parameter estimate was 0.241 (95% CI = 0.163-0.340) across all three groups.

3.3.3 Population models

The deterministic SIS models indicate that if the entire sampled population had been treated with itraconazole at the rate applied to frogs in the IT group, it would have survived an estimated 124 weeks (min = 79, max = 236) compared to 49 weeks (min = 33, max = 73) if no drug treatment had been given. Consequently, treatment would have increased time until extinction by an estimated 75 weeks (min = 6, max = 203) (Fig. 3.5). This represents an estimated weekly survival of 95.7% for the treated population compared to 89.4% for the untreated population.

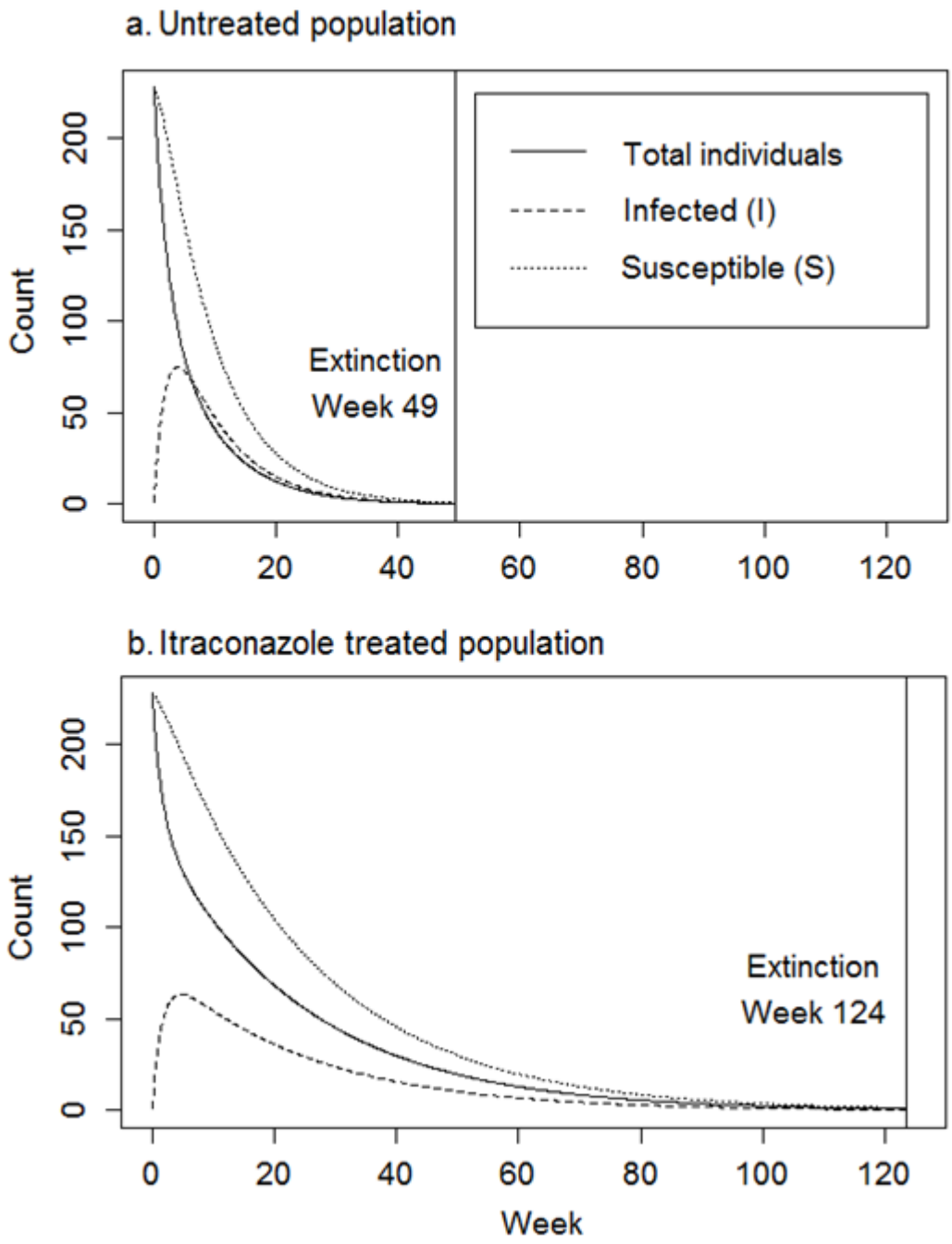

Figure 3.5 Deterministic SIS models (with mortality) of the total individuals in each infection state (and total live) using model averaged parameter estimates generated by the multi-state mark-recapture modelling for the (a) control group (untreated population) and (b) itraconazole treatment group (Itraconazole treated population).

3.4 Discussion

We used the emergence of amphibian chytridiomycosis in the mountain chicken on Montserrat as a model system to investigate the feasibility and impact of in-situ treatment of the disease using the antifungal drug, itraconazole. Our study shows that in-situ treatment of wild amphibians with itraconazole in the face of epidemic chytridiomycosis decreased the mortality rate of infected animals and increased their rate of loss of infection during the treatment period. Itraconazole treatment also reduced the infection rate of animals in the IT group during the treatment period, providing evidence of a short term prophylactic effect. On cessation of treatment, the benefits were lost and the rate of survival and loss of infection regressed and the infection rate increased to those of untreated individuals. It also suggests that, at least in the mountain chicken, repeated exposure to Bd and anti-fungal treatments does not facilitate resistance through the development of an immune response.

McMahon *et al.* (2014) reported that relatively small numbers of repeated exposures to Bd followed by clearances using heat treatment in captivity were sufficient to stimulate an immune response in *Osteopilus septentrionalis* resulting in a reduced mortality rate. Other studies have presented contradictory findings (Stice and Briggs 2010; Cashins *et al.* 2013; Fites *et al.* 2013), and it appears unlikely that this immuno-protective effect, if it does occur, can be stimulated in all species.

The decreased mortality rate conferred by itraconazole treatment in our study is encouraging considering each frog was treated on average just once a week. This is a substantially lower treatment rate than the once-daily treatment used in laboratory studies and recommended for captive animals (Pessier and Mendelson 2010).

There was no difference in survival or infection state transition rates between the two control groups, providing assurance that the physical action of handling and immersing frogs did not cause stress sufficient to contribute to mortality or infection. This is important as there are limited methods for the targeted delivery of antifungal compounds for mountain chickens or for the application of this technique to other amphibian species (Scheele, Hunter, *et al.* 2014). Hardy *et al.* (2015) recorded a prolonged decrease in Bd prevalence and an increase in overwinter survival in *Rana cascadae* treated with itraconazole in captivity prior to release into the wild. Although the pharmacokinetics of the drug have not been studied in amphibians, these authors proposed that the itraconazole might have persisted in the skin long enough for another mechanism of resistance to develop, but there is no evidence for this (e.g. Cashins *et*

al. 2013). In our study, itraconazole provided no prophylactic protection from Bd infection beyond the treatment period.

During the post-treatment period, the infection rate in the IT group increased from that seen in the treatment period to that seen in the control groups. The Bd infection intensity also increased in the IT frogs from the levels found during the treatment period to those found in the control animals. When Cashins *et al.* (2013) treated experimentally infected frogs (*Litoria booroolongensis*) with itraconazole and then re-exposed them to Bd, they found higher infection prevalence and intensity in frogs post-treatment than in frogs exposed only to Bd. These authors proposed an immunosuppressant effect of itraconazole treatment although this is not a recognised side effect of this drug in amphibians (Pessier and Mendelson 2010) or any other species (NOAH 2015). Itraconazole at concentrations of up to 0.08 µg/ml has been shown not to inhibit the growth of multiple symbiotic bacteria isolated from *Rana sphenocephala* skin (Holden *et al.* 2014). However, this is a low concentration compared to the treatment used in our study and the study described by Cashins *et al.* (2013) (0.1 mg/ml). At higher concentrations itraconazole solutions are lower in pH which might result in skin irritation or osmotic dysfunction (Baitchman and Pessier 2013). Modifications such as reducing the itraconazole concentration (Jones *et al.* 2012; Brannelly 2014) or using an alkalising buffer (Brannelly, Richards-Zawacki and Pessier 2012), might help to reduce any such side effects. The similarity in infection rate estimates in post-treatment and control group animals in this study suggests that any post-treatment impact was not associated with changes to immune function or skin microflora and was not ecologically important.

Using the mean parameter estimates from the CMR analysis, our population models predict a delay of 75 weeks to population extinction for an itraconazole-treated population compared to an untreated population; i.e. an approximated 60% increase in time to extinction. Whilst in-situ itraconazole treatment at the intensity conducted in our study would not prevent population extinction, it would prolong the period until extinction, thus allowing time to implement other conservation measures, such as the establishment of an ex-situ conservation breeding population. The prevalence of - and the risk of contracting - Bd infection have been repeatedly shown to vary seasonally in response to environmental conditions (Kriger and Hero 2006; Longo, Burrowes and Joglar 2010). The increased time until extinction predicted by our population model for populations treated in-situ with itraconazole has the potential to maintain a susceptible population through seasonally high risk periods.

In the current study, itraconazole treatment was applied for only 15 weeks, which was insufficient time for the epidemic phase to come to an end and therefore high infection loads likely persisted in untreated syntopic animals throughout this period. Should treatment have continued beyond the epidemic phase, it is possible that a longer term benefit from itraconazole treatment, such as the prevention of population extinction, could have occurred as exposure rates and inoculation doses decreased and this would be worth investigating in other systems.

Previous studies have predicted the importance of Bd infection state in species detectability (Jennelle *et al.* 2007), with reduced recapture probability of infected animals in populations where Bd is endemic (Murray *et al.* 2009). Other studies have provided no evidence for a difference in recapture rates of infected vs uninfected animals (Phillott *et al.* 2013), therefore this effect is likely species- and infection-load- specific. In our study, we found infection state to be an important predictor of detectability, but with higher recapture rates for infected animals. A possible reason for this difference from previous studies is that the mountain chicken is a large bodied and highly territorial species (Martin *et al.* 2007), thus sick animals will be more easily detected than cryptic species such as tree frogs. Our field observations showed that mountain chicken frogs with clinical chytridiomycosis were lethargic, active during the day, aggregated in ponds, and displayed decreased capture avoidance (authors' unpublished observations). It is possible that the increased recapture probability of infected animals may have increased the efficacy of itraconazole treatment, by increasing the likelihood of capture and, hence, treatment of infected animals. This is unlikely to be the case for all amphibian species.

We found that animals in the IT group had higher recapture probabilities than those in the control groups. At first, this seems to contradict our finding that infected animals were more likely to be recaptured than uninfected animals (with a higher proportion of the IT group being uninfected than the control groups). This result, however, appears to be due to a higher recapture probability of uninfected animals in the IT group compared to the control groups (Fig. 3.4). Itraconazole treatment has been reported to cause lethargy of some amphibians under laboratory conditions (Brannelly, Richards-Zawacki and Pessier 2012), but in these cases the drug doses were higher as they were administered daily compared to on average weekly in this study. Importantly, the apparent behavioural differences of animals in the IT and control groups did not impact survival sufficiently to negate the increased survival resulting from itraconazole treatment.

The NBC group also had lower recapture probabilities than either the IT or the SWC group. This could be because animals were assigned to the NBC group after the other groups and the first animals caught and assigned to the IT and SWC groups might have been more territorial and, hence, more-easily detected, and recaptured.

There has been little research into the potential for the development of antifungal resistance by Bd. Such resistance has been widely reported in human fungal pathogens, including to triazoles, the group of fungicides which includes itraconazole (e.g. Kanafani and Perfect 2008). It is possible, therefore, that in-situ treatment with itraconazole could enhance the development of resistance to this drug in Bd, especially if, as is the case in the field, treatment protocols cannot be conducted rigorously and treatment regimens are suboptimal with Bd survival within the treated population.

Our study has shown that in-situ treatment of individual animals by immersion in an aqueous solution of itraconazole is an effective tool for reducing the chytridiomycosis-induced mortality rate in the mountain chicken in the short term. This treatment, however, is highly labour intensive and limited to amphibian species for which recapture rates are relatively high.

A lack of capacity for captive assurance colonies for the large number of amphibian species at risk of decline should Bd reach naïve amphibian hotspots (Bielby *et al.* 2008) means alternative responses to the mitigation of Bd in-situ, such as anti-fungal treatment, are urgently required. The concurrent in-situ treatment of multiple endemic and sympatric species, such as those in Madagascar (where there is now evidence for Bd presence (Bletz *et al.* 2015)) and Sri-Lanka, could provide a more cost-effective treatment regimen and justify the high effort required.

Further work is urgently required to test the efficacy of new and existing treatments for chytridiomycosis in field settings. Field-trials such as ours should be replicated on species with different life histories and in systems where Bd infection is endemic. Modifications to the treatment protocol to include parallel electrolyte treatment (Baitchman and Pessier 2013; Brannelly, Skerratt and Berger 2015), and alterations in the concentration of itraconazole or the addition of pH buffers, should also be considered. New delivery methods for antifungals, and the use of longer-acting drugs if they become available, should be investigated to enable larger numbers of animals to be treated with lower effort over longer time periods.

4 Reservoir frogs: tree frog reservoirs of *Batrachochytrium dendrobatidis* infection, seasonality and implications for mountain chicken reintroductions

Funding

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Abstract

Infectious diseases do not impact all species equally, some can be driven to near extinction, whilst others can persist infected with a pathogen with no fitness costs acting as potential reservoirs with implications for the conservation of susceptible species. The mountain chicken (*Leptodactylus fallax*) suffered precipitous declines on Dominica and Montserrat, its entire global range, following the emergence of chytridiomycosis, whilst other amphibian species on the islands persisted. As part of a series of experimental reintroductions of mountain chickens, we conducted surveys of two important Bd reservoir species, cane toads (*Rhinella marina*) and tree frogs (*Eleutherodactylus spp.*). We conducted repeated tree frog surveys across three sites between 2009 - 2013 in order to identify seasonal patterns in Bd infection risk and elucidate long-term trends in Bd infection prevalence and load. There was significant seasonality in Bd infection prevalence in tree frogs, correlated with both decreasing temperature and rainfall. Bd infection load was also correlated with decreasing temperature. Bd infection prevalence in tree frogs was up to 35% in the cooler, drier months and was repeatedly undetectable in tree frogs during the warm, wet summer months. Cane toads were found to be infected at low prevalence. A decline in tree frog Bd infection prevalence was identified between the Bd epidemic (2009) and surveys in later years (2011-13), but no decline was recorded between 2009 - 2013. Understanding the epidemiology of disease threats such as chytridiomycosis is key to planning conversation measures for the species that they impact. These results have great implications for reintroductions of amphibians threatened by chytridiomycosis such as the mountain chicken, which could be timed to coincide with periods of low Bd infection pressure, reducing the potential impact of Bd infection of reintroduction success.

4.1 Introduction

Emerging infectious diseases are a growing threat to wildlife, causing declines and extinctions globally (Aguirre and Tabor 2008; Daszak, Cunningham and Hyatt 2000). Traditional epidemiological theory suggests that diseases are unlikely to cause extinctions: when a pathogen causes a host decline, host density declines below a threshold at which further transmission and resulting mortality is limited (Anderson and May 1979). However, transmission dynamics are altered in favour of the likelihood of extinction when pathogens are able to persist in environmental or species reservoirs (McCallum 2012; Mitchell *et al.* 2008; De Castro and Bolker 2005; Brunner *et al.* 2004). Neutralisation of threats has long been considered an essential precursor to interventions such as reintroduction (Caughley 1994). Therefore, an inability to eradicate pathogens that persist in reservoirs poses a problem to conservation managers, who must find alternative methods to mitigate disease impact (Harding, Griffiths and Pavajeau 2015).

Captive-breeding and reintroduction is often the last intervention available to prevent the extinction of highly threatened species with very small populations (Seddon, Armstrong and Maloney 2007). Without the ability to remove threats such as specific pathogens from the environment prior to release, imaginative approaches are required to reduce the impact of disease on reintroductions. One such suggestion is modifying the timing of reintroductions in relation to known disease processes and cycles (Phillips and Scheck 1991; Fentzloff 1984). This might provide a period of time for the reintroduced animals to acclimatise to their environment, reducing stress when pathogen levels increase and increasing survival. It might also allow them to disperse, reducing contact rates and the spread of disease through the population.

Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd), threatens hundreds of species of amphibian (Fisher, Garner and Walker 2009; Skerratt *et al.* 2007). It is a generalist pathogen which does not cause disease in all species infected (Gervasi *et al.* 2013; Stockwell, Clulow and Mahony 2010). The possibility that it could persist outside of a host (Walker *et al.* 2007; Johnson and Speare 2005; Johnson and Speare 2003) or in non-amphibian hosts (McMahon *et al.* 2013) have not been ruled out, meaning it has many potential natural reservoirs. Many of the species worst affected by this disease are now extinct in the wild or have extremely small remnant populations (Skerratt *et al.* 2007). These species likely require reintroductions to ensure their long-term survival, as the continued presence of the disease supresses the ability of affected populations to recover (Longo and Burrowes 2010; Pilliod *et al.* 2010).

The risk of infection with Bd has been shown to vary seasonally (Ruggeri *et al.* 2015; Berger *et al.* 2004) and is hypothesised to be driven predominantly by variation in temperature (Whitfield *et al.* 2012; Forrest and Schlaepfer 2011) and rainfall (Holmes, McLaren and Wilson 2014; Terrell *et al.* 2014; Longo, Burrowes and Joglar 2010). Mechanisms for these drivers are well described, frogs have been shown to exhibit temperature dependent immunity (Rowley and Alford 2013; Raffel *et al.* 2006), antimicrobial activity of frog skin microbiota is temperature dependent (Longo *et al.* 2015; Daskin *et al.* 2014), and Bd is sensitive to high temperatures and desiccation (Piotrowski, Annis and Longcore 2004; Johnson *et al.* 2003). Despite this understanding, there is no consensus on the relative importance of these drivers and they appear to differ between sites and species. Consequently, surveillance of reservoir and target species to identify local Bd epidemiology is required as it may allow the optimisation of the timing of reintroduction events to coincide with low disease risk periods.

There is evidence of reduced Bd prevalence post-epidemic in in other systems including amphibian assemblages in Queensland, Australia (McDonald *et al.* 2005; Retallick, McCallum and Speare 2004) and Panama (Brem and Lips 2008). There are likely multiple mechanisms for this pattern including increased immune response to infection with Bd (McDonald *et al.* 2005), or a reduction in the number of susceptible hosts following chytridiomycosis driven declines resulting in reduced contact rates (De Castro and Bolker 2005). A reduction in Bd prevalence in surviving individuals might reduce the infection pressure on reintroduced individuals through reduced likelihood of contact with an infected host. Identifying long-term patterns in Bd infection prevalence in reservoir species could help guide the timing of reintroductions such that infection risk is reduced sufficiently for immunologically naïve captive-bred animals to be successfully released into sites from which Bd has not been eradicated.

The mountain chicken (*Leptodactylus fallax*) is a giant Caribbean frog which is critically endangered (Fa *et al.* 2010) and found only on Dominica and Montserrat in the eastern Caribbean. Chytridiomycosis caused the catastrophic decline and near-extinction of mountain chickens on Dominica in 2002 and on Montserrat in 2009 (Chapter 2). As a result, a captive population was established with the offspring of the founders being destined for reintroduction (Chapter 2). Tree frogs (*Eleutherodactylus* spp.) are the only sympatric amphibians on Dominica and are the predominant sympatric amphibian on Montserrat alongside cane toads (*Rhinella marina*). Following the demise of the mountain chicken, these animals continue to act as a reservoir for Bd infection on the islands, rendering its eradication if not impossible, challenging. To better understand the epidemiology of Bd on the islands, we

conducted repeat surveys of tree frogs on Dominica and Montserrat. We identify seasonal patterns in Bd infection risk and elucidate long-term trends in infection prevalence and load. We also carry out a small number of surveys of cane toads in order to establish whether they represent an important Bd reservoir. These results are presented here and we discuss how they might be useful in designing mitigation strategies for the reintroduction of the mountain chicken.

4.2 Methods

4.2.1 Focal species

Eleutherodactylus johnstonei is an invasive tree frog, found throughout the Caribbean (Kaiser 1997) and is one of three species of amphibian found on Montserrat, alongside the mountain chicken and the cane toad (*Rhinella marina*). It is thought to have originated in the Antilles and has been described as a potential native of Montserrat (Kaiser 1997). The cane toad was introduced to Montserrat in the 20th century but this species does not occur in Dominica (Lever 2001). *E. johnstonei* has been reported to have been inadvertently introduced to Dominica with aid consignments following Hurricane David in 1979 (Kaiser 1992). The species is, however, often confused with *Eleutherodactylus martinicensis* (Kaiser 1997), a regional endemic found throughout most of the island (Kaiser and Hardy, Jr. 1994), and there is no certainty about its occurrence on the island. Recent surveys in areas from where it has been reported previously, have failed to detect *E. johnstonei* on Dominica (Authors' unpublished observations). Each of these *Eleutherodactylus* species and the cane toad are carriers of Bd, in the absence of chytridiomycosis. There is no evidence that the infection causes population declines (Author's unpublished data). All of the amphibian species sympatric with the mountain chicken, therefore, are potentially important reservoirs of Bd.

4.2.2 Study sites

The study was carried out on Montserrat and Dominica in the Caribbean Lesser Antilles. The climate in the lesser Antilles is characterised by a warmer wetter season between May and October and a cooler drier season between November and April.

4.2.3.1 Montserrat

Tree frogs were surveyed approximately monthly between February 2011 and December 2013 at three sites within the Centre Hills National Park on Montserrat. The three sites chosen for the study were: Fairy Walk (FW, 16.752 °N, -62.176 °W, 600 m asl) on the east coast, Sweetwater (SW, 16.782 °N, -62.185 °W, 600 m asl), also on the east coast and Collins Ghaut (CL, 16.790 °N, -62.206 °W, 500 m asl) on the west coast. The three sites were chosen to

represent three conditions: a site which is home to two of the last known surviving mountain chickens on Montserrat (FW - Chapter 2), a site from which mountain chickens have been extirpated by Bd and in which experimental releases were carried out as part of the mountain chicken project (SW - Chapter 5) and a site which is not known to have previously contained a population of mountain chickens (CL). One survey of 52 tree frogs was also conducted in 2009 at SW at the time when Bd was thought to have first emerged on Montserrat.

4.2.3.2 Dominica

On Dominica, surveys were conducted approximately every 2 months throughout 2014 at three sites: Wallhouse (WH, 15.280 °N, -61.370 °W, 100 m asl), Colihaut (CH, 15.489 °N, -61.455 °W, 100 m asl) and Soufriere (SF, 15.242 °N, -61.349 °W, 150 m asl). These sites were selected as they are three of the last remaining strongholds of the mountain chicken on Dominica following the emergence of Bd (Chapter 2). In addition, 61 tree frogs were surveyed at WH in December 2011.

4.2.4 Field methods

For each survey, 60 tree frogs were caught and skin-swabbed in order to estimate the Bd infection prevalence at each site. Sixty frogs provided a compromise between prevalence estimate precision which increases with sample size (DiGiacomo and Koepsell 1986) and the time required to catch this number of frogs. Tree frogs were exhaustively sampled by a team of between three and five people within a 20 metre radius of a chosen 'station' centred at the start of established amphibian monitoring transects. If the first station did not yield 60 frogs, the team would move 50 m along the transect and repeat the process. Up to three stations were used each night until 60 captures were made. Each site was therefore a maximum of 140 m in total (to include the 20 m radius at each of the transect). New disposable latex gloves were used to handle each frog caught to prevent cross-contamination of Bd or Bd DNA between animals.

On Montserrat, cane toad surveys were conducted in October and November 2011 at the three study sites on between 3 and 5 occasions per site. For each survey, a team of 15 people slowly walked the length of the ghaut while evenly spread out to cover its width. Every cane toad seen was caught and skin-swabbed. This exhaustive sampling meant sample sizes for each occasion varied.

On capture, frogs were swabbed 5 times on the ventral abdomen, legs and feet with a rayon tipped swab (Medical Wire & Co. MW100), as described by Hyatt *et al.* (2007). Frogs were

examined for signs of chytridiomycosis, specifically red ventral skin, muscle tremors and skin sloughing. After swabbing each *Eleutherodactylus spp.* was held separately until all captures had been made to ensure there were no recaptures within the same sampling period. Frogs were then released as close to the site of capture as possible. Cane toads were immediately released at least 10 metres behind the survey team to ensure they would not be recaptured. Swabs were stored in a refrigerator until ready to be analysed.

Two temperature data loggers (Thermochron iButtons, DS1922L-F5) were placed at ground level in a shaded area on either side of the SW ghaut and two were placed at each of the sites in Dominica throughout the surveys in order to record the air temperature at hourly intervals. Temperature data loggers were also placed at both CL and FW between June and September 2012. Limited differences in temperature patterns were identified between the sites (Appendix G) allowing temperature data for SW was used to represent all sites.

Rainfall data were collected by the Montserrat Utilities department and the Dominica Forestry department as a part of routine monitoring. Rainfall data were obtained for the nearest gauge to each study site. On Montserrat: Blakes FIFA [16.783979N, -62.185641W] for SW, Ginger Ground [16.772867N, -62.214672W] for CL and New Windward [16.765874N, -62.168201W] for FW. No analyses were conducted using the Dominica data because of the low incidence of Bd during the study period.

4.2.5 Laboratory methods

Skin-swabs were refrigerated until transport to the laboratory where DNA was extracted from each swab as described in Appendix E. A Taqman qPCR was used to quantify the amount of Bd in each swab following the procedures described in Chapter 3 (section 3.2.3).

4.2.6 Data analysis

Infection prevalence during each survey occasion was calculated as the number of frogs testing positive for Bd DNA divided by the number of frogs sampled. Binomial 95% confidence intervals around this prevalence were calculated using Quantitative Parasitology software (Rózsa, Reiczigel and Majoros 2000). Monthly data were only included in the analyses where all three sites had been sampled in the same month to ensure differences between sites could be tested without bias.

Uneven time intervals between sampling occasions meant time series analysis to decompose the seasonality and trend were not possible without interpolation over gaps which would

result in artificial smoothing of the data. Each sampling occasion (separated by a minimum of 30 days) was therefore treated as independent. In addition, as the population sizes of *Eleutherodactylus* spp. are large on both islands, the recapture rate of individuals was likely negligible-to- low and so the assumption of independence would not have been broken. As a result of the inability to decompose long term trends, in order to test for an annual change in Bd infection prevalence and load, the peak value for each year was compared. Comparisons of prevalence between sampling occasions were carried out using the chi-squared test except where expected values were < 5 where Fisher's exact tests were used. Bd infection loads were compared between occasions using the Kruskal-Wallis test.

The mean temperature across all readings taken from the data loggers in the 30 days prior to each sampling occasion were used to describe air temperature. The mean temperature was used as opposed to minimum or maximum as all three showed very similar patterns and the mean is less susceptible to temporary extremes through, e.g. contact with rainwater or wild animals. Rainfall was calculated as the total rainfall occurring in the 30 days prior to each sampling occasion at the nearest rainfall gauge to the site. In vitro at 22 °C, Bd has a lifecycle from zoospore to zoosporangium of c. 5 days (Berger, Hyatt, *et al.* 2005), 30 days should, therefore, be a long enough period of time for the detection of any environmental influences on Bd prevalence (Kriger and Hero 2006).

A logistic regression was used to examine the relationship between the environmental variables and location with likelihood of infection of *E. johnstonei* on Montserrat. A quasi-binomial model was fitted to compensate for over dispersion. A likelihood ratio-test was performed to test the importance of each variable and only those significant at alpha = 0.05 were retained. No analyses were carried out on the cane toad or Dominica data due to the very low prevalence estimates observed.

Rainfall and temperature on Montserrat were highly positively correlated (Appendix H) and so rainfall was excluded from the multivariate analysis and run in an independent univariate logistic regression.

Linear regression was used to test for a relationship between the environmental variables and infection loads of Bd-positive *E. johnstonei*. Infection loads varied over several orders of magnitude and so were log transformed prior to analysis. Tukey's HSD was used to perform post-hoc analyses of differences between sites. Again, a separate regression was carried out for rainfall.

Unless stated otherwise, all analyses were performed in R (R core team 2015).

4.3 Results

4.3.1 Montserrat

B. dendrobatidis was detected from 12.8% of the 3674 tree frog swabs sampled between 2011 and 2013. The highest infection prevalence was recorded in the survey carried out in 2009 at SW (51.9%, 95% CI = 38.3 – 65.5). Between 2011 and 2013, the maximum prevalence recorded was 35% (95% CI = 24.0 - 48.3) in CL in December 2011 (Fig. 4.1), although this was not significantly lower than the 2009 survey (chi-sq=3.258, df=1, p=0.071). The maximum annual prevalence across the sites did not decrease over the duration of the study, recorded as 25.7% (95% CI = 15.3 - 35.7) at SW in 2012 and 28.1% (95% CI = 18.2 - 40.6) at CL in 2013. In total 132 cane toads were swabbed during the cane toad surveys with only two animals, both from FW, testing positive for Bd (Table 4.1). No tree frogs or cane toads were captured with signs of chytridiomycosis.

Table 4.1 Cane toad survey Bd infection prevalence results.

Date	Site	No. Bd pos.	Prevalence (%)	95% CI	Bd load (zsp. eq.)
31/10/2013	CL	0/7	0	0 - 37.7	
07/11/2013	CL	0/14	0	0 - 23.8	
14/11/2013	CL	0/15	0	0 - 22.2	
29/10/2013	FW	1/31	3.2	0.2 - 17.2	4592.8
04/11/2013	FW	0/10	0	0 - 29.1	
12/11/2013	FW	1/26	3.8	0.2 - 18.8	3718.0
23/10/2013	SW	0/4	0	0 - 52.8	
01/11/2013	SW	0/8	0	0 - 36.5	
08/11/2013	SW	0/5	0	0 - 50.0	
15/11/2013	SW	0/7	0	0 - 37.7	
20/11/2013	SW	0/5	0	0 - 50.0	

Infection levels in tree frogs varied significantly across seasons, with the highest infection prevalence occurring during the cooler, drier season (November to April) (Fig. 4.1). This trend was repeated in each of the three years of this study, with significant differences in prevalence between the high (dry / cool season) and low (warm / wet season) points within each year (Table 4.2). Infection prevalence from 2011 - 2013 was as low as 0% on 12 occasions (CL: 5, FW: 1, SW: 6). A prevalence estimate of 0% is not sufficient evidence to conclude that infection was completely absent from the population, as a sample size of 60 provides a 95% confidence

that prevalence was < 5% (DiGiacomo and Koepsell 1986). The fastest increase in prevalence across consecutive months was recorded between December 2011 and January 2012 at SW, where prevalence increased from 6.2% (95 % CI = 2.1 – 15.4) to 24.3% (95 % CI = 15.3 – 35.7) in 30 days. The fastest decrease in prevalence across consecutive months was recorded over the same period at CL, decreasing from 35% (95 % CI = 24.0 – 48.3) to 15% (95 % CI = 9.6 – 22.2).

Table 4.2 Comparison of yearly highs and lows of Bd prevalence in *E. johnstonei* at three sites across Montserrat. Chi-squared statistics from a comparison of prevalence, the highs and lows in each year at each site were significantly different providing strong evidence of seasonality.

Site	Year	High month	High prev.	95% CI	Low month	Low prev.	95% CI	Chi-sq	Df	p-value
FW	2011	Dec	28.3	18.2 - 40.8	Jun	1.7	0.09 - 8.73	16.732	1	<0.001
FW	2012	Jan	21.7	13.4 - 33.2	Sep	1.6	0.09 - 8.33	12.772	1	<0.001
FW	2013	Jan	28.1	18.2 - 40.6	Oct	0.0	0.00 - 6.30	19.741	1	<0.001
SW	2011	Apr	26.7	16.4 - 39.1	Sep	1.4	0.08 - 7.61	18.104	1	<0.001
SW	2012	Jan	25.7	16.7 - 37.1	Sep	0.0	0.00 - 6.40	17.951	1	<0.001
SW	2013	Feb	20.6	5.6 - 22.1	Oct	0.0	0.00 - 6.10	7.769	1	0.005
CL	2011	Dec	35.0	24.0 - 48.3	Jun	3.3	0.60 - 11.4	19.417	1	<0.001
CL	2012	Feb	22.6	13.5 - 34.6	Sep	0.0	0.00 - 6.30	15.305	1	<0.001
CL	2013	Feb	24.2	14.9 - 36.3	Oct	0.0	0.00 - 6.30	16.661	1	<0.001

There was no difference between the median infection load of infected frogs in the peak month of the three years at any site (CL: chi-sq (2) = 1.114, p = 0.573; FW: chi-sq (2) = 3.748, p = 0.154; SW: chi-sq (2) = 0.044, p = 0.978).

The mean air temperature during the study was 24.8 °C (range = 18.2 – 34.2 °C, SE = 1.7), a value considered within the optimal range of the growth of *Bd* (Piotrowski, Annis and Longcore 2004; Longcore, Pessier and Nichols 1999). Likelihood of Bd infection in *E. johnstonei* was inversely related to 30-day air temperature (OR = 0.496, 95% CI: 0.409 - 0.602, p <0.001) (Fig. 4.2), 30 of 32 sampling sessions with 30-day air temperatures above 25 °C had a prevalence less than 10% whereas 16 of 27 sampling sessions with 30-day air temperatures less than 25 °C had a prevalence greater than 10% (Fig. 4.2). Prevalence > 20% was only recorded below 24.5 °C and > 30% prevalence below 23.5 °C. A difference in the likelihood of infection with Bd between sites was also near-significant and was therefore retained in the analysis (p=0.052). The difference between sites was driven by a significantly lower likelihood of infection at SW compared with FW (OR=1.85, 95% CI = 1.08 – 3.17, p = 0.044, Fig. 4.2). Likelihood of infection

at CL was not significantly different from FW (OR = 1.29, 95% CI = 0.78 - 2.12, p = 0.573) or SW (OR = 1.43, 95% CI = 0.836 – 2.46, p = 0.390). The interaction term between temperature and site was not significant (p = 0.369).

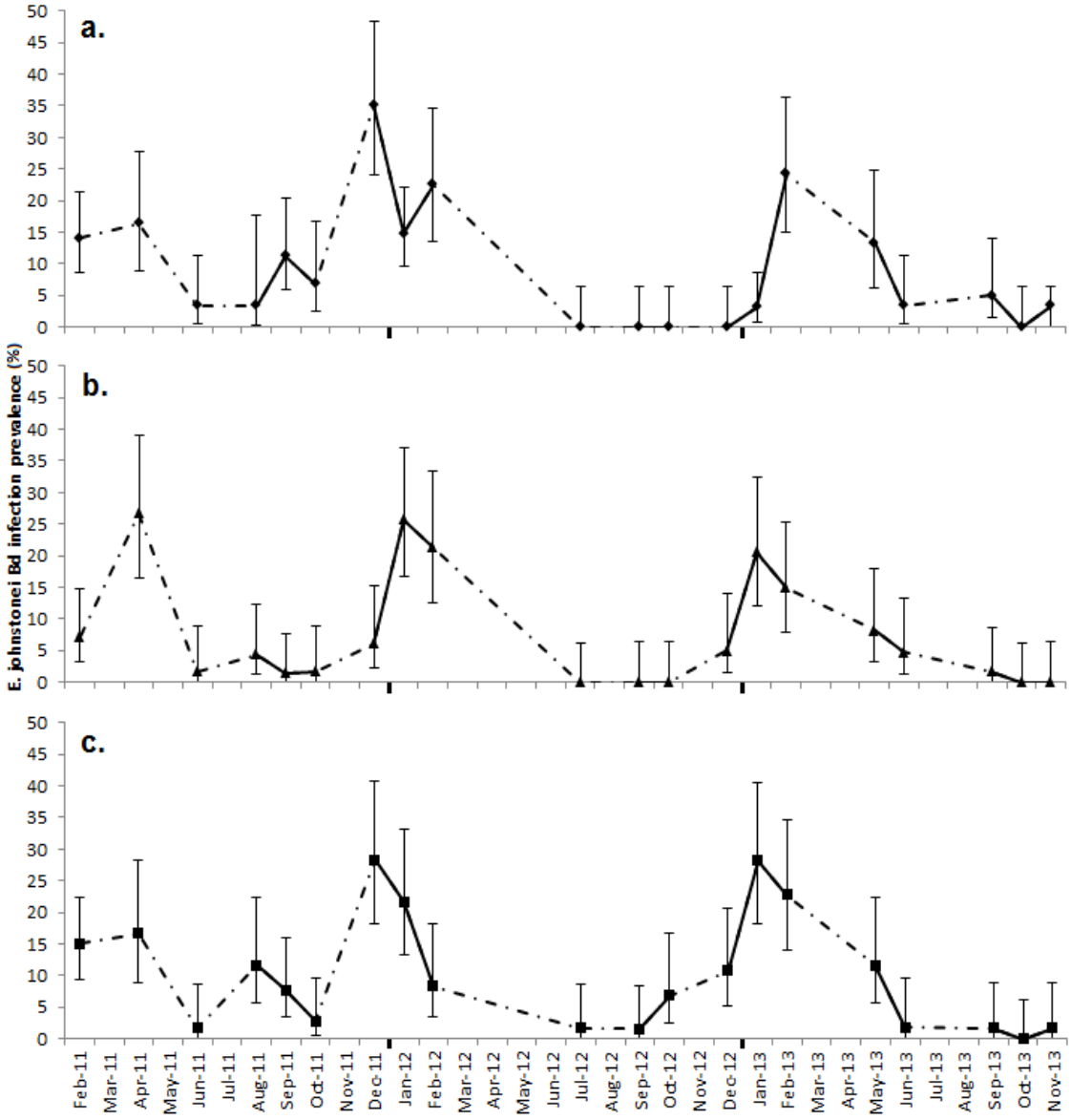

Figure 4.1 *Eleutherodactylus johnstonei* Bd infection prevalence for a. Collins, b. Sweetwater and c. Fairy Walk on Montserrat. Solid lines indicate connections between sampling occasions in successive months and broken lines indicate connections between sampling occasions separated by more than one month. 95% binomial confidence intervals are presented based on a sample size of 60 frogs at each occasion.

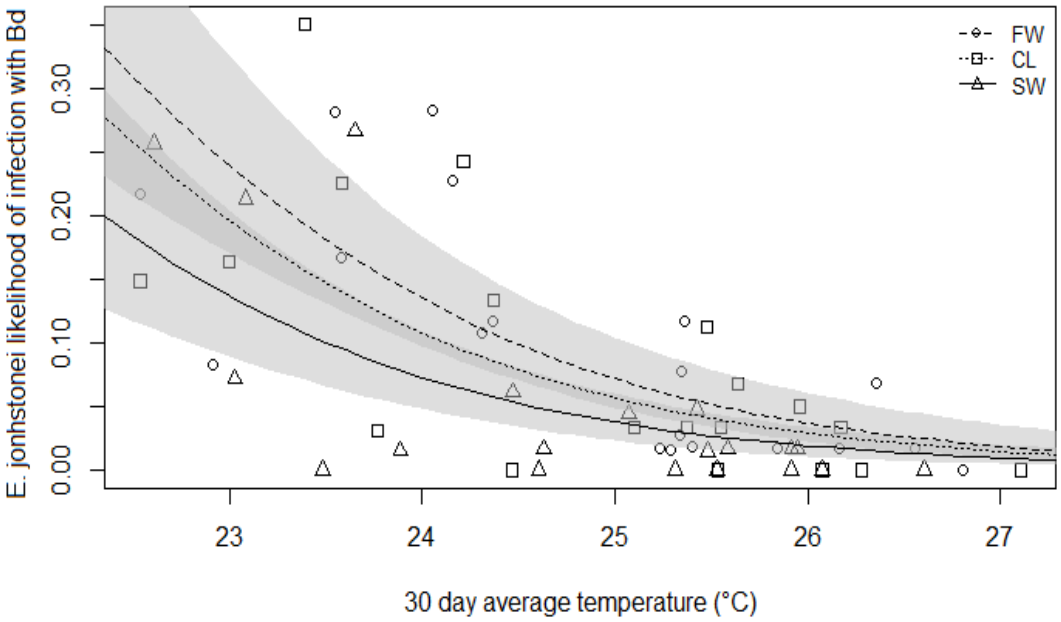

Figure 4.2 Logistic model of relationship between 30-day average temperature, site and likelihood of *E. johnstonei* infection with Bd on Montserrat. 95% CI are plotted in grey for FW and SW which were predicted to be significantly different. No confidence interval is plotted for CL as it was not significantly different from either site.

Independently, in a univariate analysis, mean daily rainfall (mm) was also found to be significantly inversely related to likelihood of *E. johnstonei* being infected with Bd (OR = 0.153 , 95% CI = 0.049-0.477, p = 0.001). Seven of eight sampling sessions with 30-day mean daily rainfall above 0.3 mm had a prevalence of less than 15%. Prevalence over 20% (n = 10) occurred only in months with less than 3 mm of rain / month with the exception of two months where rainfall was c. 9 mm.

Mean air temperature was inversely related to the Bd load of infected tree frogs (beta on the log scale = -0.7439, SE = 0.14, p < 0.001) (Fig. 4.3). The load of infected frogs was significantly different between sites, with infected frogs at FW and SW having significantly lower loads than at CL (SW beta = -1.067 SE = 0.377, p = 0.005; FW beta = -1.095, SE = 0.332, p = 0.001) (Fig. 4.3). There was no difference in infection load between FW and SW (beta = 0.028, SE = 0.378, p = 0.940). The interaction term between temperature and location was not significant (p = 0.112). The final model accounted for c. 12% of the variation in infection loads of infected frogs (R^2 = 0.1185).

In a separate univariate analysis mean daily rainfall was not found to be a significant predictor of infection load (p = 0.789).

4.3.2 Dominica

Batrachochytrium dendrobatidis was detected from 1.3% of the 900 tree frog swabs sampled during 2014. The highest infection prevalence was recorded in the December 2011 survey carried out in WH (39.3%, 95% CI = 27.8 – 52.5). The highest prevalence recorded during 2014 was in February at SF (8.3%, 95% CI = 3.4 – 18.1, Fig. 4.4), significantly lower than the 2011 survey in WH (chi-sq = 15.963, df = 1, p < 0.001). Tree frogs testing positive for Bd infection were only identified in SF and WH during February and April, with none detected during the rest of the year (Fig. 4.4). At these two sites, the February 2014 prevalence was significantly higher than at any other sampling period that year (chi-sq = 5.217, df = 1, p = 0.022). No positive samples were detected at any time at CH.

The mean air temperature during 2014 at the study sites on Dominica was 25.5 °C (min = 18.63, max = 39.1), which is within the optimal temperature range for Bd. As the number of positive tree frogs in Dominica was so low, no analysis was undertaken to examine the relationship with the environmental variables, however the seasonal peak in prevalence did match that on Montserrat, and the climatic variables are similar for both islands.

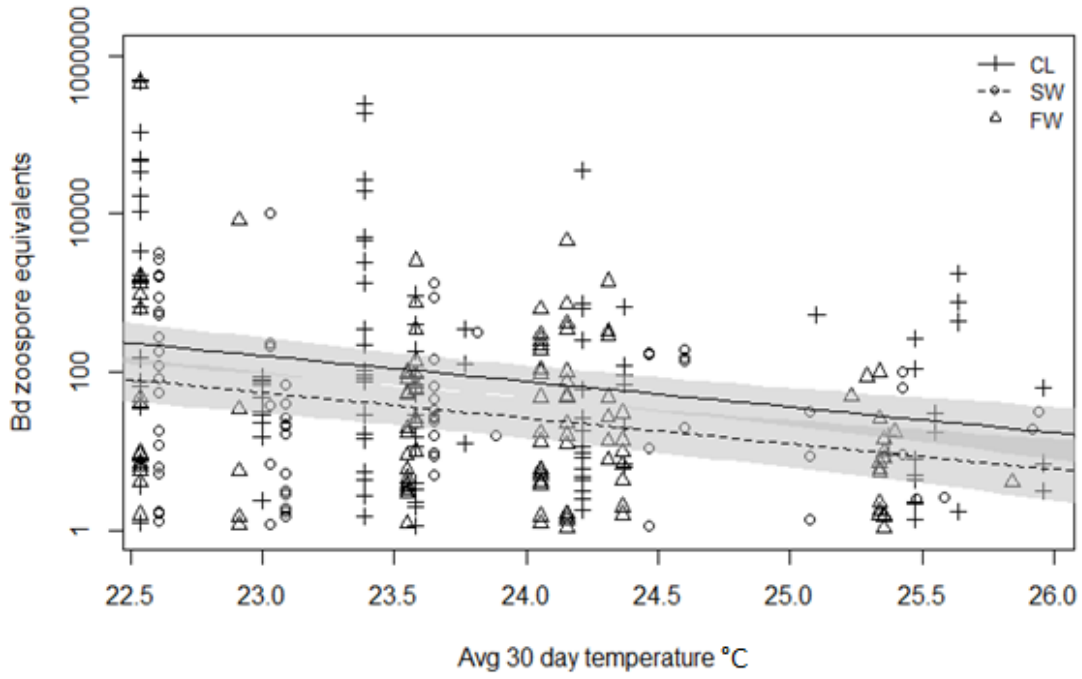

Figure 4.3 Linear model prediction of relationship between 30 days average temperature, site and infection load of infected *E. johnstonei* on Montserrat. Bd zoospore equivalents were log transformed prior to analysis. 95% CI are plotted for CL and SW. No model prediction is plotted for FW as it was very similar to the prediction for SW.

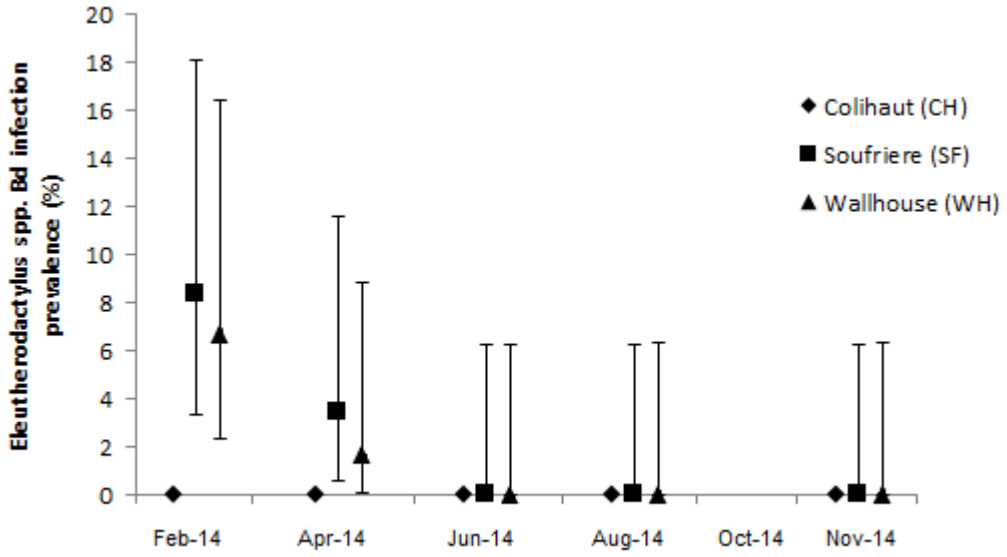

Figure 4.4 *Eleutherodactylus* spp. Bd infection prevalence for Colihaut, Soufriere and Wallhouse on Dominica. 95% binomial confidence intervals are presented based on a sample size of 60 frogs at each occasion.

4.4 Discussion

A growing number of species are threatened by emerging infectious diseases and understanding their epidemiology is key to the design of conservation initiatives. Amphibian chytridiomycosis is one such disease which threatens many hundreds of species world-wide. Our results show that there is significant seasonality in Bd infection prevalence in an important reservoir species of tree frog on Montserrat with strong evidence of an inverse relationship with temperature. Although seasonality was similar at each of the three study sites on this island, there were significant differences in the likelihood of infection with Bd between sites. Whilst rainfall was too strongly correlated with temperature to be included in a multi-variate analysis, a separate analysis showed an inverse relationship between rainfall and Bd infection prevalence. Temperature was an important predictor of Bd infection load on Montserrat with significant variation between sites. Although fewer data were available and Bd infection prevalence in tree frogs was much lower on Dominica than on Montserrat, a similar pattern of seasonal variation was observed on both islands.

It has previously been hypothesised that rainfall and Bd prevalence would be positively correlated (Kriger 2009), but on Montserrat, we found the opposite to be the case, with both decreased Bd infection prevalence and decreased Bd infection load during the wetter months. This reflects the findings of chytridiomycosis driven mortality being greatest during the cooler drier season in other parts of the Caribbean and in South America (Ruggeri *et al.* 2015; Longo *et al.* 2013). From these results, it appears easy to dismiss the relationship between increasing Bd prevalence and reduced rainfall as much of the Bd lifecycle is dependent on water, the infectious stage (the zoospore) is water-borne (Kriger 2009; Berger, Hyatt, *et al.* 2005) and the correlation between rainfall and temperature allows for a satisfactory explanation from temperature alone. However, the behavioural adaptations of frogs to dry conditions could result in this phenomenon (Burrowes, Joglar and Green 2004).

Eleutherodactylus johnstonei on Montserrat and the *Eleutherodactylus* spp. on Dominica have been observed aggregating on the forest floor during the dry season, possibly in search of water (Author's unpublished observations) with anecdotal evidence of tree frogs being found in remnant pools. In addition to these behaviours increasing contact rates amongst tree frogs, they increase contact rates with, and infection probability to/from the terrestrial mountain chicken frogs and cane toads (e.g. Chapter 4, Fig. 4.5). This aggregation in damp refugia has been reported elsewhere (Roznik and Alford 2015) and has been shown experimentally to lead to increased Bd prevalence (Longo, Burrowes and Joglar 2010).

Figure 4.5 Direct contact observed between a reintroduced mountain chicken and a Bd reservoir species of tree frog (*E. johnstonei*) on Montserrat.

We did not identify a relationship between rainfall and Bd load. Variation in Bd load is likely the result of individual immunological, behavioural and habitat use differences rather than that of wide scale variation in water availability. We did find a significant inverse relationship between Bd load and temperature which may be the result of supressed immune responses due to the lower temperatures (Rowley and Alford 2013), lower antifungal activity of skin microbiota (Daskin *et al.* 2014) or the sensitivity of Bd to temperature (Johnson *et al.* 2003).

Between the study sites on Montserrat, there were small but significant differences in Bd infection load and likelihood of infection in tree frogs. Although small, these differences could be important for optimising site selection for the reintroduction of mountain chickens. The highest likelihood of infection with Bd on Montserrat was recorded at FW, with significantly lower likelihood of infection at SW. In contrast, the highest Bd infection loads were recorded at CL. There are several key differences between the sites which might explain this variation. It is possible that there are some temperature differences between the sites which are not represented in the data. The comparison between the sites in 2012 (Appendix G), showed limited temperature differences, but FW was the site with the lowest temperature which may have driven the increased Bd infection prevalence. Secondly, FW has a permanently flowing stream whereas CL has a stream that flows for several months a year and the stream in SW only flows after heavy rain and the site can dry up completely. Bd is known to be sensitive to desiccation (Johnson *et al.* 2003) which might explain why tree frogs at SW had the lowest likelihood of infection and lower infection loads than at FW or CL. The final notable difference is the presence of mountain chickens at FW, their absence at CL, and their reintroduction at SW. The mountain chicken is very sensitive to fatal Bd infection and it seems unlikely that the few mountain chickens present at FW could explain the increased prevalence in tree frogs. The reintroductions of mountain chickens do not appear to have affected the infection prevalence or load at SW as the seasonal patterns observed at SW are so similar to those at the other sites. Although not studied, it is possible that there was variation in the population density in tree frogs between the sites, although no differences in capture efforts were required to sample 60 frogs across the three study sites (Author's unpublished data).

The tree frog surveys carried out at WH on Dominica in 2011 and at SW on Montserrat in 2009 showed higher Bd infection prevalence rates compared with the 2014 surveys on Dominica and the 2011-2013 surveys on Montserrat. The 2009 Montserrat survey took place during the epizootic phase, shortly after the first record of Bd infection on the island. The observed reduction in prevalence from the epizootic stage to the enzootic stage reflects similar patterns recorded elsewhere (Brem and Lips 2008). A similar reduction in the Bd infection load from the

epidemic sample in 2009 to the samples in 2011-2013, was seen in the tree frogs on Montserrat.

Taking into account the seven year difference in time in the emergence of chytridiomycosis on the islands (Dominica 2002, Montserrat 2009 - Chapter 2), and assuming the Bd infection prevalence was similar on both islands at the height of the epizootic phase, the low prevalence recorded across Dominica in 2014 might represent a long-term decline. We did not, however, identify a downward trend in maximum annual prevalence at each site on Montserrat, or a significant reduction in Bd infection load, but three years is a relatively short study period and may have failed to detect those trends. Montserrat is also home to a population of cane toads which do not occur on Dominica. Whilst the Bd prevalence in the cane toads was very low, the survey was carried out at a time when prevalence in tree frogs was low across the sites. The Bd infection loads detected on the cane toad swabs were moderate (3000-4000 zsp. equiv.; Table 4.1) suggesting cane toads are an important reservoir that might serve to reduce the rate of decline of Bd on Montserrat. The 2011 Dominica survey in WH, which appears not to fit with the long-term decline hypothesis, was carried out in response to the discovery of the first small population of mountain chickens for 7 years. Ad-hoc sampling of this population was undertaken between August 2011 and January 2012, with Bd positive mountain chickens first detected in December (Appendix J). High Bd infection loads were detected from December until January and most of this population then disappeared likely as a result of chytridiomycosis. It is possible there was an increase in Bd prevalence at the end of 2011 as expected as temperatures decreased, but in this case, spillover from susceptible mountain chickens may have exacerbated the prevalence in the tree frogs through a positive feedback mechanism.

The temperature difference between the islands might provide a mechanism for the long-term decline on Dominica. The highest maximum temperature recorded on Dominica was over 39 °C which is greatly above the temperature at which Bd mortality is expected (Young, Berger and Speare 2007), and this might explain why the prevalence is so much lower in the tree frogs on Dominica. Under these conditions, it is likely only small pockets of Bd could survive the warmest periods of the year (in low temperature microcosms), increasing the likelihood of decline of the pathogen.

Although no tree frogs or cane toads were recorded as exhibiting signs of chytridiomycosis, there were a small number of very high Bd-infection loads recorded on tree frogs during the study, with nine over 100,000 zsp. equiv. (Fig. 4.3). Despite these high loads, there has not

been a noticeable decrease in the number of tree frogs on Montserrat, or in the time taken to capture 60 frogs (L. Martin pers. comm. and Authors' unpublished data). However, due to their small body size and cryptic colouration, there is a very low chance of encountering dead tree frogs. Behavioural changes resulting from the development of chytridiomycosis may also reduce detection of severely affected individuals (Murray *et al.* 2009; Jennelle *et al.* 2007) meaning the impacts of Bd may be missed. Chytridiomycosis has been implicated in the decline of closely related *Eleutherodactylus spp.* on Puerto Rico (Longo *et al.* 2013; Longo, Burrowes and Joglar 2010) and whilst this might not be considered a conservation issue with regards to the potentially invasive *E. johnstonei*, it could be of concern for the endangered Dominican endemic *E. amplinympha* which occur only at high elevations, at which temperatures are cooler and chytridiomycosis is likely to have the greatest impact.

Understanding the epidemiological patterns of Bd infection in reservoir hosts is key to decision making for mountain chicken conservation. Reintroductions of mountain chickens were first performed in the cooler drier season in early 2011 (Chapter 5), both to reduce logistical issues regarding severe storms, and because it was thought the reduced moisture levels might reduce Bd prevalence in reservoir hosts. We have shown however, that this is not the case and consequent to these results the month in which reintroductions were conducted was altered to July, providing a period of 4 months when the mean temperature exceeded the 25 °C threshold at which Bd prevalence dropped below 10% (Fig. 4.2 and Appendix H). Early observations suggest this has had a positive effect on short-term infection risk of reintroduced animals (Chapter 5). Combining optimising the timing of reintroductions with in-situ treatments such as antifungals during high risk periods could allow reintroduced populations to persist long term until immunity could develop, although there is currently no evidence for this in mountain chickens (Chapter 3 / Hudson *et al.* 2016). Of the three study sites on Montserrat, SW was identified as the site with the lowest Bd risk (based on prevalence and infection load in extant amphibians) and therefore the best release site for mountain chicken reintroductions.

Long term monitoring of Bd infection loads and prevalence trends is required to understand the drivers of seasonal variation in Bd infection risk. This information is essential to inform the timing of reintroductions of Bd threatened amphibians for which no other sustainable, long-term conservation intervention is available. We have also shown that Bd-infection prevalence decreases post-epidemic. Despite no evidence of a short-term continuation of this decline, it is possible that it will continue in the long term reducing infection risk for susceptible species. Although expensive, long term monitoring would facilitate the capture of these long-term

reductions in Bd infection risk allowing reintroductions to be postponed until a time at which they would be successful. Once site-specific drivers of seasonality are understood, monitoring efforts could be reduced and targeted at high risk periods, reducing the high economic and resource costs associated with monitoring and facilitating long term implementation. Finally, we have shown that Bd infection prevalence and load vary by site, and determining the site with the lowest infection pressure might increase the success of reintroductions.

5 Impacts of seasonal risk in *Batrachochytrium dendrobatidis* infection on success of mountain chicken frog reintroductions

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Abstract

Amphibian chytridiomycosis, caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd), has driven the greatest disease driven loss of biodiversity ever recorded, with many species close to extinction in the wild, including the mountain chicken (*Leptodactylus fallax*). The persistence of Bd infection in sympatric reservoir species often prevents its eradication, long considered a pre-requisite for successful reintroduction of threatened species. In the face of these irreversible threats, novel reintroduction strategies are required. We carried out four experimental reintroductions of captive bred mountain chickens to test for the effect of seasonality of Bd infection in an important Bd reservoir species (*Eleutherodactylus johnstonei*) on reintroduction success. We intensively monitored reintroduced mountain chickens over a six-month period in a multi-state mark recapture framework to estimate Bd infection rates, and Bd infection state dependent survival. Chytridiomycosis driven mortality resulted in reintroduction failure in every release, except for the wet season release when Bd infection prevalence and load in *E. johnstonei* was lowest and no mountain chickens were recorded with signs of chytridiomycosis. The first Bd infection of a mountain chicken in the wet season did not occur until 12 weeks post-release compared with the first and second weeks of the dry season releases. Mountain chickens survived at the release site for over 10 months in three releases, but were not recorded after 11 months, in part due to dispersal. Optimising the timing of reintroductions of amphibians threatened by chytridiomycosis to coincide with periods of low Bd infection risk represents a potential method for reducing the impact of the disease. In combination with environmental modification to reduce the environmental suitability for Bd, or in-situ treatment regimes, this method could facilitate successful reintroduction and long-term persistence.

5.1 Introduction

Compared to the other vertebrate classes, there are relatively few reports of amphibian reintroductions documented in the IUCN reintroduction case study reports (Soorae 2016; 2013; 2011; 2010; 2008). However, the Amphibia are currently experiencing the greatest decline of all vertebrate taxa (Stuart *et al.* 2004), meaning reintroductions are becoming increasingly important and widely used (Harding, Griffiths and Pavajeau 2015; Gascon *et al.* 2007). Despite the recent increase in their use, there is little research on the effectiveness of amphibian reintroductions (Harding, Griffiths and Pavajeau 2015; Griffiths and Pavajeau 2008). Despite many amphibian reintroductions being described as successful, most are not assessed against fundamental criteria linked to population viability, and if they were, many would likely be deemed failures (Ewen, Soorae and Canessa 2014). The most commonly cited reasons for amphibian reintroduction failure are failure to mitigate against the original drivers of decline (Stockwell *et al.* 2008; Fellers *et al.* 2007; Rickard 2006), emigration away from release sites (e.g. Matthews 2003), and poor release site / habitat choice (White and Pyke 2008). There have, however, been notable successes in amphibian reintroductions, such as in the natterjack toad (*Bufo calamita*; Beebee *et al.* 2012) and the Majorcan midwife toad (Buley and Garcia 1997). As a result, small, intensively monitored pilot studies are required to examine the potential of reintroduction as a conservation strategy for each target species (e.g. Bodinof *et al.* 2012).

The use of these small scale 'experimental' reintroductions might also facilitate the testing of multiple release strategies in order to adapt protocols and optimise reintroductions. Caughley (1994) advocates the use of experimental reintroductions to test whether the threats that led to the original declines continue to pose an extinction threat to the target species. By varying reintroduction strategies experimentally it is possible to explicitly test hypotheses and provide recommendations for the optimisation of reintroduction strategy (Armstrong and Seddon 2008). Much of the focus of reintroduction monitoring to date has focussed on easy to measure components such as reintroduction technique (Seddon, Armstrong and Maloney 2007). Monitoring should, instead, be focussed on the collection of data that allows these hypotheses around long-term success to be tested. This is especially important in the face of low reintroduction success (Fischer and Lindenmayer 2000) and the likely increase in the use of reintroductions as the number of conservation reliant species increases (Scott *et al.* 2010; Morell 2008).

Alongside habitat loss and invasive species, emerging infectious diseases (EID) present one of the most important threats currently facing amphibians globally (Stuart *et al.* 2004).

Chytridiomycosis is one such EID which has caused the decline or extinction of over 200 species of amphibian (Skerratt *et al.* 2007). In response, the Amphibian Conservation Action Plan (ACAP) advocates the creation of Bd-free captive populations for eventual release and to buy time in which to research mitigation of the disease (Gascon *et al.* 2007). However, the IUCN reintroduction guidelines state that prior to reintroduction there should be confidence that the threats which caused past declines will not again be a threat to translocated populations (IUCN/SSC 2013). The persistence of Bd in the environment poses a problem for reintroduction programmes as eradication from natural habitats and reservoir species is problematical (Bosch *et al.* 2015). Irreversible threats such as chytridiomycosis therefore require the development of innovative techniques to facilitate reintroduction success.

Selection for tolerance to Bd infection prior to release has been suggested as a method for increasing the probability of amphibian reintroduction success (Scheele, Hunter, *et al.* 2014; Venesky *et al.* 2012). However, there are currently no known traits which offer tolerance to Bd infection that could be selected for. Reintroduction techniques are required, therefore, which allow the release of naïve animals which have not been exposed in captivity, to a pathogen by which they are threatened in the wild.

It is regarded as best practice to target reintroductions in seasons that would be beneficial for the target species (IUCN/SSC 2013). This might be to encourage breeding, reduce dispersal, or ensure there are sufficient resources such as prey and freshwater. However, targeting reintroductions to coincide with periods of low pathogen or parasite pressure is a less commonly utilised strategy (Phillips and Scheck 1991; Fentzloff 1984; Chapter 4). This might provide a period of time for the reintroduced animals to 'settle' reducing stress when pathogen levels increase, or disperse, reducing contact rates and the spread of the disease through the population. A long-period of low-level pathogen exposure may also facilitate the development of an acquired immune response (Viggers, Lindenmayer and Spratt 1993). Previous studies have identified strong seasonality in the infection prevalence and load of the causative agent of chytridiomycosis; *Batrachochytrium dendrobatidis* (Bd) in a wide range of locations (Ruggeri *et al.* 2015; Longo, Burrowes and Joglar 2010; Berger *et al.* 2004). Seasonality has also been confirmed in Bd reservoir species on Montserrat with reduced Bd infection prevalence and load, during the summer months (Chapter 4).

In 2009, epidemic chytridiomycosis emerged on Montserrat and rapidly swept through the mountain chicken population, causing a devastating decline and extirpating the species from many sites within months (Chapter 2). During this epidemic, 50 frogs were evacuated in order

to establish a safety net captive breeding population. These individuals have been held in strict bio-secure quarantine since this date at Durrell Wildlife Conservation Trust, Zoological Society of London (ZSL), Chester Zoo and Parken Zoo. Mountain chickens were successfully captive bred at Durrell, ZSL and Parken in preparation for experimental reintroduction. Biosecurity was an essential pre-requisite for reintroduction in order to prevent the captive mountain chickens contracting parasites from species from elsewhere and subsequently translocating these back to Montserrat (e.g. Walker *et al.* 2008), a process known as pathogen pollution (Cunningham 1996).

Here, we present an analysis of four experimental reintroductions of first generation captive bred mountain chickens to Montserrat. In all cases, the animals were released into Sweetwater ghaut (SW), a site from which they were previously extirpated by chytridiomycosis (Chapter 2). The reintroductions were carried out over a period of four years, at different times of year to assess the impact of seasonality on reintroduction success. The establishment phase of a reintroduction (Armstrong and Seddon 2008) can be assessed at three stages: 1. short-term survival and settlement of reintroduced individuals; 2. breeding of reintroduced individuals and survival of their offspring; and 3. long-term population viability. This study will assess the reintroductions against the first stage of this success spectrum.

5.2 Methods

5.2.1 Release design

Four experimental reintroductions were undertaken approximately annually between 2011 and 2014 (See Table 5.1 for details). The initial experimental design incorporated two dry and two wet season releases, over the course of the project. The first (REL 1) and second (REL 2) releases took place during the dry season. The third release (REL 3), which was scheduled to take place at the height of the wet season was delayed by several months due to an accident, resulting in it becoming a late wet/early dry season release, exacerbated by a wet season with low rainfall (Table 5.1). The fourth release (REL 4) took place in the middle of the wet season.

Table 5.1 Details of the four experimental reintroductions of mountain chickens to Montserrat.

REL	Release date	Season	No. dead on arrival	No. tracked	No. untracked	Institutional origin of cohort
1	27 & 28 Jan 2011	Dry	0	33	31	Durrell & Parken
2	28 & 29 Jan 2012	Dry	1	32	0	Durrell & Parken
3	9 & 10 Nov 2012	Late wet/dry	6 (+3)	25	0	Durrell
4	27 & 28 June 2014	Wet	3	32	20	Durrell & ZSL

The animals were released at between 18 - 24 months of age, i.e. as young adults, so that surviving frogs would be of breeding age at, or within one year of, release (Martin *et al.* 2007). Approximately 30 frogs from each release cohort were implanted with intracoelomic radio-transmitters (Holohil BD-2HX (REL 1 and 2); PD-2HX (REL 3 and 4) in an operation carried out at Jersey Zoo under license from the relevant authorities (full description of implant procedure - Appendix K). The procedure was undertaken approximately 10 days before the frogs were transported to Montserrat in order to allow wound healing prior to shipping. Radio-transmitters were used only to increase recapture probability of each frog and no radio-tracking data were analysed. Each frog destined for release was implanted with a PIT tag (11 mm x 2 mm, ID-100A Microtransponder, Trovan Ltd.) at approximately 10 months of age. The frogs were transported in individual cloth bags which were placed in polystyrene boxes with shredded newspaper, six frogs per box. The boxes were shipped in wooden crates. A temperature logger (Thermochron iButton DS1922L-F5) was included in each crate to monitor conditions during transport. A sustained drop in temperature was recorded in the aeroplane hold during shipment of frogs destined for the third release which resulted in the death of six frogs during transport and a further three on arrival, reducing the number of frogs available to release (Table 5.1). Mountain chickens were transported from the European institutions to Antigua and on to Montserrat via commercial airliners. On arrival in Montserrat, the frogs were removed from their crates, examined for injuries, weighed and placed in a bath of amphibian Ringer's solution for 20 mins to ensure they were fully hydrated. The frogs were then placed into four indoor enclosures in the Montserrat Botanical Gardens as an initial holding area to ensure that the frogs were healthy prior to release. Each enclosure contained baths of amphibian Ringer's solution and was lined with leaves collected from the forest floor at the release site. The frogs were held in these enclosures for two days prior to release and were offered 10 - 15 crickets each day.

For each release, half of the frogs were released on each of two consecutive days. On the day of release, each frog was weighed and skin-swabbed for Bd using a rayon-tipped swab (MW100-100, Medical Wire and Equipment Co.) three times across each of the following sites: ventral abdomen, ventral thighs and calves, and plantar surfaces of both hind-feet. At the release site, Sweetwater ghaut (SW), the frogs were initially placed in 2 x 2 m camping tents, five frogs per tent, set up at between three and five release points (depending on the number of frogs in the release) approximately 100 m apart along a predefined transect (Fig. 5.1). Each tent contained a water bath and a substrate comprising leaves from the forest floor. The release sites were chosen in order to establish a density of released frogs approximately the same as the encounter rate of mountain chickens at this site prior to the emergence of

chytridiomycosis (Authors' unpublished data). The frogs were kept in these tents for approximately one hour until 18:00 (dusk) when the tent flaps were opened to allow the frogs to disperse of their own accord.

5.2.2 Release site

The release site was chosen through a site comparison study conducted by Adams (2010). SW is on the north-east flank of the Centre Hills and had previously supported a medium density mountain chicken population which had been extirpated during the 2009 chytridiomycosis epidemic (Chapter 2). SW is a shallow sided ghaut, and contains a stream which flows only after heavy rainfall during the wet, warm season on Montserrat (May - October). When the ghaut stops flowing a series of large ponds remain for several months until they dry up completely during the dry, cool season (November - April).

5.2.3 Post-release monitoring

A fixed transect (consisting of one main transect including both forks of the upper ghaut and a series of secondary parallel transects up the shallower east bank (Fig. 5.1)) was monitored six times per week for the first three months post- release, when the implanted radio-transmitters were still active. After the initial post-release monitoring period was complete, surveys were reduced to once a week for an additional six months, or longer if captures continued to be made. Each night a team of three to five trained personnel walked the transect at a slow walking pace carrying out both visual surveys for non-tracked individuals and radio tracking for implanted frogs using two TRX-1000 receivers (Wildlife Materials Inc., USA). In REL 1, frogs were caught, examined for injuries and skin swabbed for Bd approximately once every fortnight. From REL 2 onwards, frogs were skin swabbed for Bd once per week. From the REL 2 onwards, one weekly day time search along the SW transect and in surrounding ghauts was also carried out.

During REL 1, frogs were not weighed for the first month, and subsequently weighed only once per month. This generated insufficient data to make useful conclusions and so in the subsequent releases, morphometric data were recorded on every swabbing occasion (once per week). Body mass measurements were rounded to the nearest 5 g and snout-vent lengths (SVL) to the nearest 5 mm.

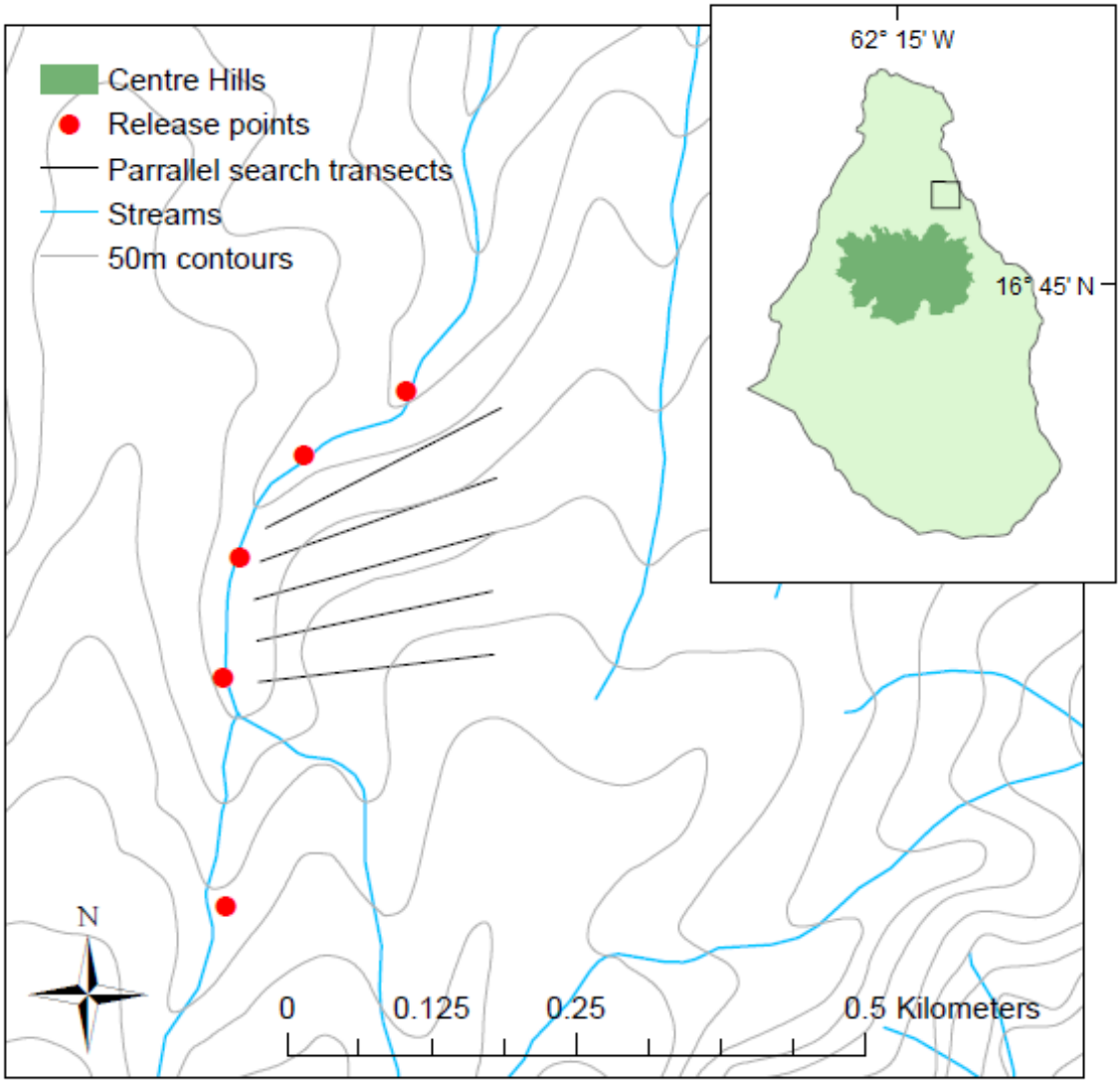

Figure 5.1. Map of the study site (Sweetwater ghaut) on Montserrat with parallel transect positions.

Reintroduced animals should exhibit behaviours that enable them to successfully breed and survive (Annex 7 - IUCN/SSC 2013). As a result, the behaviour of reintroduced mountain chickens was observed on an ad-hoc basis throughout the post-release monitoring period with a focus on the ability of the animals to feed, use natural refugia and exhibit breeding behaviours such as calling to attract mates and paired use of potential breeding burrows.

During the releases skin swab surveys of the Bd reservoir tree frog species, *Eleutherodactylus johnstonei* were carried out up to once per month to investigate whether there was a correlation between Bd infection prevalence in tree frogs and the Bd infection risk for mountain chickens. On each survey, 60 tree frogs were caught and skin swabbed as described in Chapter 4. For all comparisons the raw estimate of prevalence (number Bd positive / 60) was used.

5.2.4 Laboratory analyses

Skin-swabs were refrigerated until transport to the laboratory where DNA was extracted from each swab as described in Appendix E. A Taqman qPCR was used to quantify the amount of Bd in each swab following the procedures described in Chapter 3 (section 3.2.3).

5.2.6 Mark-recapture analyses

Capture-mark-recapture (CMR) data were analysed using RMark (Laake 2013), an extension of Program Mark (White and Burnham 1999) in a multi-state framework (Lebreton *et al.* 2009). Multi-state CMR models are an extension of Cormack-Jolly-Seber which are used to model the probability of transition between states alongside estimating state dependent survival and recapture rates. These transitions were modelled as first order Markov models in which the state at time $t + 1$ is dependent only on the state at time t. States were defined as uninfected (U), Bd-infected (I) and dead (D). The estimates of survival generated by this analysis are 'apparent' as they are confounded by migration out of the study site. From here on the term survival is used to mean apparent survival and the difference with true survival is discussed where appropriate.

Capture histories were converted from daily to weekly capture histories using weekly bins. Frogs were assigned to a state based on the presence or absence of Bd in the skin swab from each weekly swabbing occasion. The use of weekly bins for continuous CMR data can bias estimates of survival (and transition) if they are time dependent (Barbour, Ponciano and Lorenzen 2013), as might have been the case in this study. As a result, and to allow the inclusion of data from REL 1 in which frogs were only swabbed once per fortnight, only the

data from every second week was utilised in order to create intervals between the sampling occasions in REL 2 - 4. This resulted in the estimation of fortnightly parameter estimates. Whilst this might not entirely satisfy the requirement for a sampling occasion to be instantaneous in time relative to the interval between occasions (Pollock *et al.* 1990), it was considered the best compromise between fulfilling this requirement and retaining sufficient data to draw useful conclusions whilst allowing the comparison of REL 1 with the other releases. As weekly bins might have hidden capture heterogeneity in between groups, an ANOVA was used to test for a difference between releases in the number of captures per week between first and last capture using only implanted frogs to ensure comparability between releases.

In order to limit the otherwise extremely large number of possible models, a three-step process was used to create the CMR model set adapted from Lebreton *et al.* (1992). In step one, survival and recapture probabilities were modelled as saturated (infection state x release x time) whilst transition parameterisation was varied. In step two, the parameterisations of transition from the models with a ΔAICc < 6 in step one were used alongside saturated survival whilst recapture parameterisations were varied. In the final step, the parameterisations of recapture and transition from the top models (ΔAICc < 6) in step one were used whilst survival parameterisation was varied. Models were excluded from the top set at each step if the top model was nested within them and they contained two additional AICc points per extra parameter (the penalisation per parameter when calculating AICc), as this meant they had not improved model fit (Richards, Whittingham and Stephens 2011; Richards 2008; Burnham and Anderson 2002).

Survival, recapture and transition were modelled as release, infection state, time (independent estimates at each time point) and *Time* (a trend through time) dependent, both additively and interactively. Each parameter was also modelled as sex dependent (and sex x release) and recapture rate with separate estimates for radio-tracked and untracked frogs. Dead recovery was modelled using the same parameterisation as the other uninfected and Bd infected states, but with a separate intersect. Whilst inclusion of season in the CMR models would have allowed intrinsic testing of possible seasonal differences, REL 3 could not be decisively assigned to either season, and so post-hoc comparison of CMR parameter estimates was used to assess seasonal variation.

We based model selection on AICc. To account for model selection uncertainty, robust parameter estimates were calculated using weighted model averaging across the models with

ΔAICc < 6 (Burnham and Anderson 2002) as this provides a 95% chance of the final model set including the model with the best Kullback-Leibler distance (Richards 2008). We performed a preliminary diagnostic goodness of fit test for the multi-state CMR models using U-CARE (Choquet *et al.* 2009) with one test per release. No evidence of over-dispersion was detected in any release (REL 1 ĉ = 0.96, REL 2 ĉ = 1.03, REL 3 ĉ = 0.84, REL 4 ĉ = 1.06) and so AICc was used unadjusted. Support for different model parameterisations was calculated where necessary with evidence ratios (Lukacs *et al.* 2007).

5.2.7 Population modelling

In order to assess the impact of the survival and infection estimates from the CMR models, a deterministic population model in a susceptible-infected (SI) framework with mortality was constructed for each release. The number of frogs in each state at each time step was calculated using the matrix below adapting the notation of Lebreton *et al.* (2009) so that $\varphi(X,Y)_t$ indicates the rate of transition between states *X* and *Y in interval between t* and *t+1*. In this formula $\varphi(U,I)$ is equivalent to the 'force of infection' in epidemiological models (Begon *et al.* 2002).

$$\begin{pmatrix} nI \\ nS \\ nD \end{pmatrix}_{t+1} = \begin{pmatrix} \varphi(I,I)_t & \varphi(U,I)_t & 0 \\ 0 & \varphi(U,U)_t & 0 \\ \varphi(I,D)_t & \varphi(U,D)_t & 1 \end{pmatrix} \begin{pmatrix} nI \\ nS \\ nD \end{pmatrix}_t$$

Where: $\varphi(I,I)_t = 1 - \varphi(I,D)_t$

and: $\varphi(U,U)_t = 1 - \varphi(U,I)_t - \varphi(U,D)_t$

No recruitment was included in the model as the released frogs were too young to reproduce and none was recorded during the releases. All models were initiated with 50 uninfected individuals. One model was produced for each of the releases, using the robust-model averaged parameter estimates from the multi-state CMR models.

In order to assess the impact of Bd related mortality, the sum of the number of individuals that transitioned from Bd-infected to dead (I -> D) over the entire model period was calculated. Confidence intervals around this estimate were produced using the upper 95% CI estimate for uninfected survival, the lower CI estimate for Bd-infected survival and the upper CI for Bd infection rate.

The number of individuals remaining at the end of each release was calculated based on these models, with 95% CI calculated using the extreme 95% CIs of the robust model averaged CMR parameter estimates. For example, the lower CI estimate was calculated using the lower CI estimate of infection rate and the highest CI estimates of survival.

5.2.8 Body condition analysis

A scaled mass index (Peig and Green 2010; 2009) was created for each sex using pairs of SVLs and body masses from 266 unique wild adult Montserrat mountain chickens caught in 2005 prior to the chytridiomycosis epidemic (Appendix L). This scaled mass index was used to scale the body mass at each capture to that of an animal with a SVL of 130 mm (the mean SVL of the released frogs) using the following equation from Peig and Green (2009):

$$\widehat{M_i} = M_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}}$$

Where $\widehat{M_i}$ is the scaled mass, Mi is the mass of an individual, L_0 is the SVL at which the mass should be scaled to, L_i is the SVL of the individual, b_{SMA} is the slope of the standardised major axis regression between the natural log of the mass and the natural log of the SVL from the reference dataset.

The mean scaled mass at the start of each release was compared between releases using an ANOVA; post-hoc comparisons were made using Tukey's HSD where necessary. The same analysis was used to compare each release cohort with the 2005 wild sample.

The changes in scaled body mass were analysed using a Gaussian linear mixed effects model with frog ID as a random effect and release number, Bd infection load (which was log-transformed as these values were spread over several orders of magnitude), sex and time as fixed effects. Only interactions between time and the other fixed terms were included in the model as all models had a fixed intercept of zero, (change in scaled mass at $t_0 = 0$). Three way interactions between sex-release-time and sex-Bd infection load-time were also included. Where no model received overwhelming support (AICc weight > 0.9), weighted model averaging of models with a ΔAICc < 6 was used to generate robust parameter estimates. These analyses were carried out using R (R core team 2015).

5.3 Results

In total, 28 frogs were found dead across all four post-release monitoring periods (REL 1 n = 9 (14%); REL 2 n = 11 (34%); REL 3 n = 7 (28%); REL 4 n = 1 (2%)). The majority of the dead individuals were confirmed as displaying lesions consistent with chytridiomycosis, and almost all had high Bd infection loads at the time of, or in the days prior to, death (Appendix M). In REL 1 there was one death which did not appear to be a result of chytridiomycosis (Bd negative and absence of lesions) and in REL 2 there were four (Appendix M). The frog found dead in REL 4 could not be identified as no PIT tag or radio implant were found in or around the carcase. As a result, the death could not be assigned to the capture history of a specific frog so it was excluded from further analysis. In REL 1, 3 and 4, at least one frog continued to be captured between 10 and 11 months post-release with the majority of frogs being last captured between six and seven months post-release (Appendix M). In REL 2 the final captures were made in the fifth month post-release. In REL 4, the final captures coincided with the establishment of a prolonged period of drought on Montserrat, during which captures of all frog species including the very common tree frogs occurred at greatly diminished levels.

5.3.1 Adaptation to the wild

Mountain chickens were recorded eating wild prey, such as Montserrat tarantula (*Cyrtopholis femoralis*) within hours of release. The animals utilised naturally occurring burrows in every release; some of which contained pairs of males and females. Two foam nests were recorded, one following REL 3 and one following REL 4. Males were heard making mating calls following every release. Rat bites and broken bones were noted in a small number of otherwise apparently healthy mountain chickens, but in the majority of these cases, subsequent recaptures indicated that the lesions had healed rapidly and they did not appear to contribute to mortality (Appendix M).

5.3.2 Bd skin swabs

Skin wabs taken during the pre-release health check all tested negative for Bd indicating that the release cohorts were Bd free at the time of release. In total 38% of the 1367 skin swabs taken from the reintroduced mountain chickens tested positive for Bd DNA, but the proportion positive varied by release (REL 1 = 50% of 411 swabs; REL 2 = 67% of 349; REL 3 = 24% of 261; REL 4 = 4% of 346). The proportion of frogs with at least one positive Bd swab varied by release with far more recorded with at least one positive swab during the dry season releases (REL 1 = 49/64; REL 2 = 33/33; REL 3 = 15/25; REL 4 = 10/52). The timing of the first Bd positive swab also varied by release, but was relatively constant according to the season in which the release took place with earlier infection in the dry seasons releases (REL 1 = week 4, REL 2 = week 2)

and late wet / dry release (REL 3 = week 3). The first recorded infection in the wet season release (REL 4) did not occur until the 11th week post-release.

5.3.3 Mark recapture analyses

As no model received overwhelming support (AIC weight > 0.9, Table 5.2), model averaging was used to generate robust parameter estimates. All of the most parsimonious models contained a difference in apparent survival between (1) Bd-infected and Bd-uninfected frogs and (2) releases, and all showed a trend in survival through time. The interaction between release and infection state in apparent survival was very poorly supported (summed AICc weight = 0.266, evidence ratio = 0.344), as was the interaction between infection state and *Time* (summed AICc weight = 0.385, evidence ratio = 0.626), suggesting little difference between the time trend of survival for each Bd-infection state. There was limited support for a sex difference in survival (summed AICc weight = 0.485, evidence ratio = 0.942). Bd-positive frogs were found to have a reduced survival rate compared to uninfected frogs in all releases (Fig. 5.2). Apparent survival of Bd-infected individuals decreased through time at a constant rate across all releases (Fig. 5.2). Survival was greatest in REL 1 and 2 (dry season), and reduced in REL 3 (semi-set) and REL 4 (wet) (Fig. 5.2). The trend in apparent survival of Bd uninfected frogs was relatively constant through time across all of the releases, decreasing slightly in REL 3 and 4, but didn't fall below 88% (95% CI = 59 - 98; Fig. 5.2). Whilst females were found to have marginally greater survival probability compared to males, the model averaged effect size 95% CI included zero indicating no effect of sex.

There was strong support for a difference in transition rate between releases and for different trends through time for each infection state, independent of release (summed AICc weight = 0.910). No other models for transition had a ΔAICc < 6. The likelihood of losing infection (transitioning from I to U) was estimated to be approximately zero in all releases, with approximately zero - one CI as only a very small number of such transitions were recorded from which the probability could be estimated. The likelihood of becoming infected was greatest, and increased earliest in REL 2, with lower rates in REL 1 and 3 (Fig. 5.2). Estimates of infection rate were zero for the first c. 10 weeks in REL 4, with a small increase at the end of the monitoring period (Fig. 5.3). There was no support for a sex difference when modelling transition rate.

Table 5.2 Model selection table for the multi-state mark recapture analysis of the experiment reintroductions of mountain chickens. Model selection is based on AICc and table contains only models with a ΔAICc < 6. Also shown are the next best model, the full and the null models. Rn = Release number, Inf = infection state, Time = constant change in a parameter through time, time = separate estimates at each time interval, trk = separate estimates of recapture probability for radio-tracked and untracked animals.

Survival	Recapture	State transition	K	AICc	ΔAICc	AICc weight
Rn + Inf x Time	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	41	2158.303	0.000	0.198
Rn + Inf + Time	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	40	2158.487	0.184	0.181
Rn + Inf x Time + Sex	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	42	2158.522	0.219	0.177
Rn + Inf + Time + Sex	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	41	2158.780	0.477	0.156
Rn x Inf + Inf x Time + Sex	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	44	2160.172	1.869	0.078
Rn x Inf + Inf x Time	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	43	2160.224	1.921	0.076
Rn x Inf + Time	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	42	2161.063	2.760	0.050
Rn x Inf + Time + Sex	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	43	2161.234	2.931	0.046
Rn x Sex + Inf + Time	Rn x Inf + Rn x Time + Rn x Sex + trk	Rn x Inf + Inf x Time	44	2165.191	6.888	0.006
Rn x Inf x time	Rn x Inf x time + Rn x Sex + trk	Rn x Inf x time	243	2681.184	505.580	0.000
.	.	.	3	2826.250	650.646	0.000

All of the most parsimonious models contained a difference in recapture rate between Bd-infected and Bd-uninfected individuals, the magnitude of which differed between releases. No other models for recapture had a ΔAICc < 6. Infected animals had an increased recapture probability in comparison with uninfected animals in all releases, although the 95% CI around the model averaged estimates for the infection states in REL 3 overlapped on every occasion and this was also true towards the beginning and end of REL 1 (when numbers of either U or I individuals was low) (Fig 4.). The trend through time, although constant between Bd-infection states, also varied between releases, with the recapture rates of all frogs in REL 2 declining to almost zero by the end of the monitoring period (Fig. 5.3). The rate of decline in recapture rate was relatively consistent between each of the other releases (Fig. 5.3). Infected individuals in REL 4 had a very high probability of recapture as a result of the very low number of Bd-positive captures and therefore number of Bd-infected frogs predicted to be present during that release.

The number of captures per weekly bin differed significantly between the releases (ANOVA: $F(3,115)=33.01$, $p<0.0001$). Post-hoc Tukey's HSD tests revealed that frogs in REL 4 (1.18 captures/week, 95% CI = 1.00 - 1.35) were caught significantly fewer times per week than those in REL 1 (2.66, 95% CI = 2.15 - 3.16, $p = 0.0004$), REL 2 (4.20, 95% CI = 3.68 - 4.88, $p < 0.0001$) and REL 3 (3.66, 95% CI = 3.22 - 4.09, $p < 0.0001$). Frogs in REL 2 were also caught significantly more times per weekly bin on average than those in REL 1 ($p < 0.0001$) and 3 ($p = 0.0324$). There was no significant difference between binned recapture rates between REL 1 and 3 ($p = 0.1687$).

There was an increased rate of recapture for radio tracked individuals (model averaged effect size on the logit scale = 1.155, 95% CI = 0.818 - 1.491). The effect of sex on recapture rate varied with release, with females having a higher recapture rate in the first release (model averaged effect size on the logit scale = 0.626, 95% CI = 0.317 - 0.935), males in REL 4 (model averaged effect size on the logit scale = 0.916, 95% CI = 0.623 - 1.350), and no difference between the sexes in REL 2 and 3 (95% CI around model averaged effect size included zero).

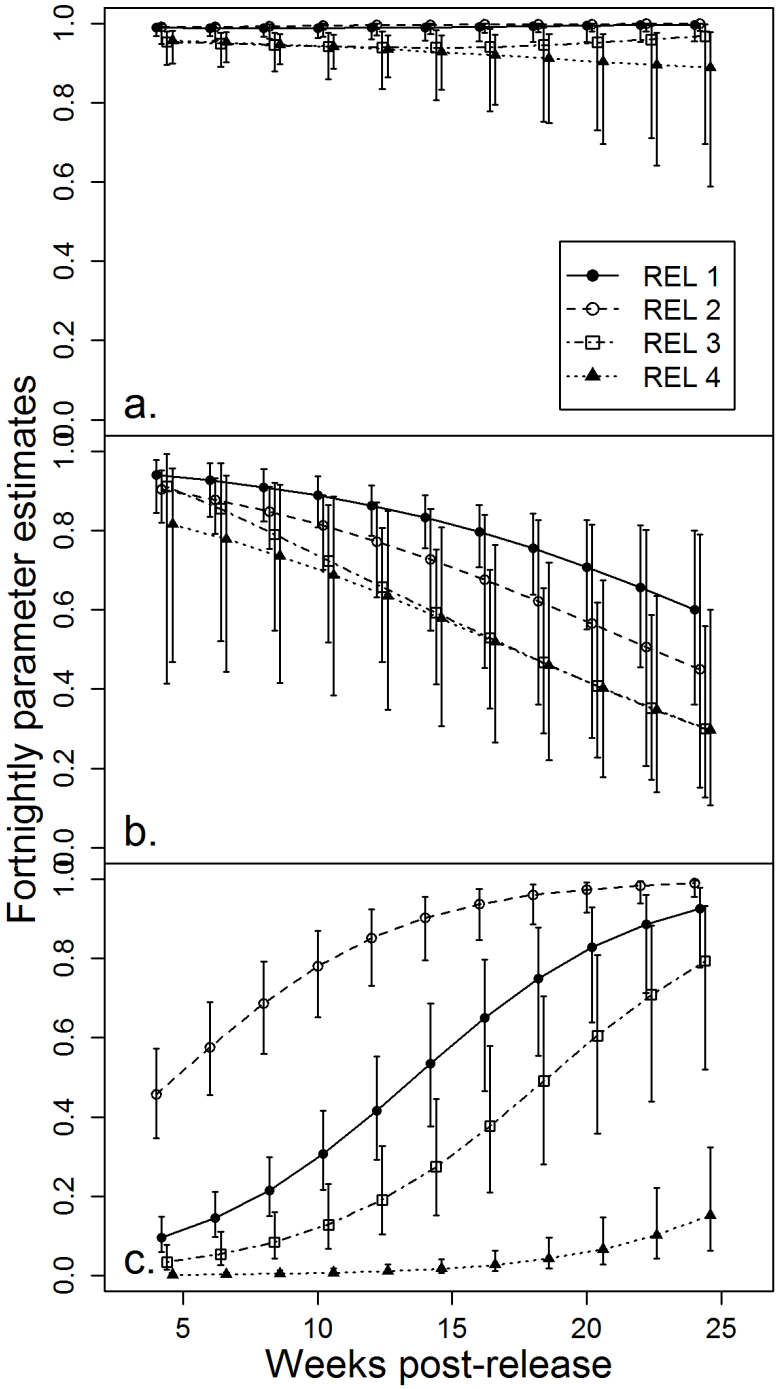

Figure 5.2. Model averaged fortnightly estimates from the multi-state mark recapture analyses of a. apparent survival of uninfected mountain chickens, b. apparent survival of Bd-infected mountain chickens and c. Bd infection rate (U -> I transitions) with 95% CI. There is no estimate of infected survival during the first interval as there were no mountain chickens in the infected state prior to the interval. The scale on the uninfected survival graph has been shortened to highlight inter-release variation.

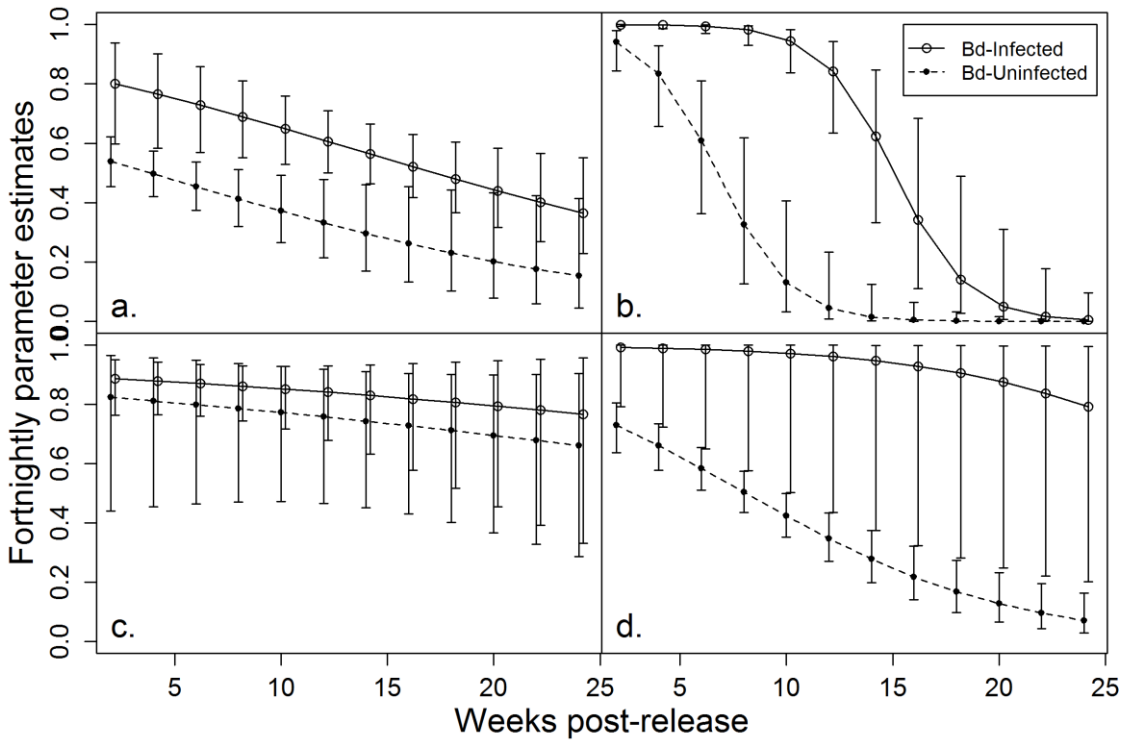

Figure 5.3. Model averaged recapture probability from the multi-state mark recapture models of a. Bd-uninfected, b. Bd-infected and c. dead mountain chickens during the four releases with 95% CI.

5.3.4 Population models

The population models show the proportion of the released animals that were infected with Bd was consistently higher throughout REL 1 and 2 than REL 3 and 4. Also, in the former two releases the peak in infection prevalence in released frogs occurred much earlier than in either of the latter two releases (Fig. 5.4). The Bd infection prevalence in tree frogs was also higher in REL 1 and 2 and the peaks earlier in the releases. Bd infection prevalence was 25.7% in the week prior to the release of frogs in REL 2 and in REL 1 the prevalence peaked at 26.7% 10 weeks post-release (Chapter 4). In REL 3 tree frog Bd infection prevalence peaked later at 20.6% 15 weeks post-release. The Bd infection prevalence in tree frogs during REL 4 was estimated to be near zero throughout (Appendix N), increasing to 16.6% in week 25 of the release when Bd infection was most prevalent in released mountain chickens in the wet season release (REL 4).

The population models predicted a large difference in the number of individuals that died whilst infected with Bd (transitioned from I -> D) during the 26 week monitoring period following each release. Nearly all 50 modelled frogs were predicted to have died whilst infected with Bd in REL 1 (rounded to the nearest whole individual: n = 41, 95% CI = 26 - 48) and REL 2 (n = 48, 95% CI = 34 - 50). Fewer mountain chickens were predicted to have died with Bd infection in REL 3 (n = 34, 95% CI = 14 - 45) and a greatly reduced number in REL 4 (n = 7, 95% CI = 1 - 20).

Based on the CMR parameters estimates, the population models predicted that the number of individuals alive and in the study area at the end of the 26-week post-release monitoring period was greatest in REL 4, but similar across the other releases (rounded to the nearest whole individual: REL 1 = 6, 95% CI = 1 - 19; REL 2 = 1, 95% CI = 0 - 11; REL 3 = 1, 95% CI = 0 - 13; REL 4 = 16, 95% CI = 1 - 35; Fig. 5.4)

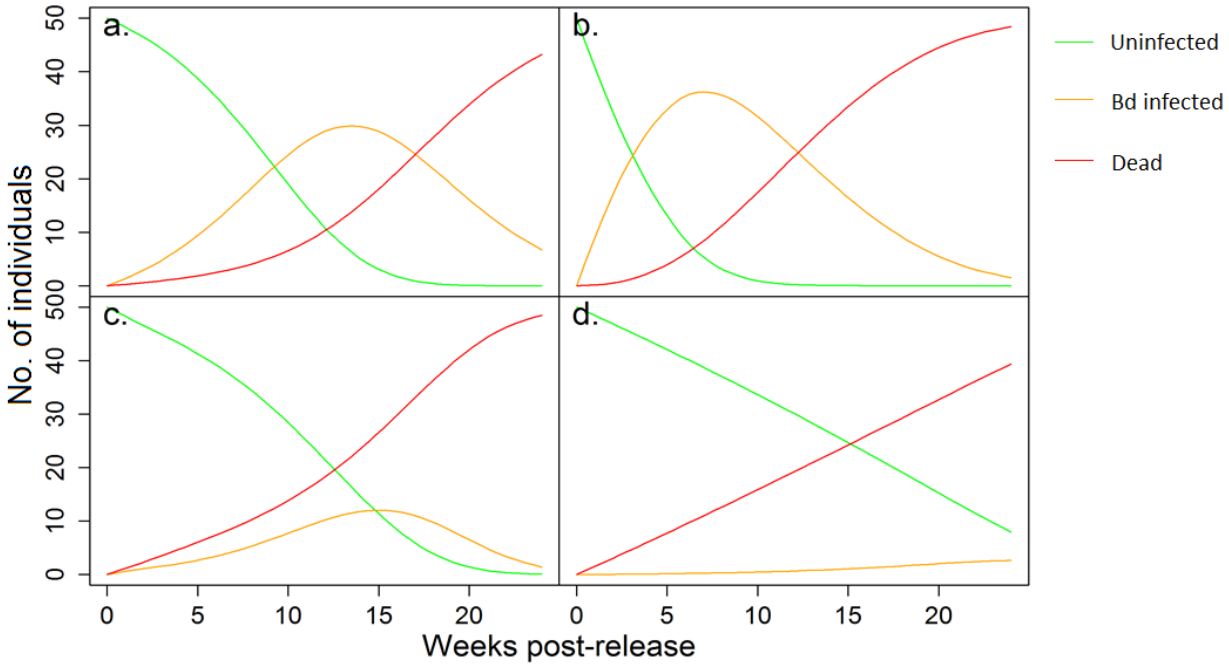

Figure 5.4. Deterministic susceptible- infected population models with mortality of mountain chicken reintroductions. Graphs show the total number of individuals in each Bd infection state using model averaged parameter estimates generated by the multi-state mark recapture models for a. REL 1 (dry), b. REL 2 (dry), c. REL 3 (semi-wet) and d. REL 4 (wet).

5.3.5 Body condition analyses

The mean scaled mass of individuals from the wild dataset (214, 95% CI = 209.9 - 218.4) was significantly lower than for animals at the time of release in REL 1 (238.6, 95% CI = 225.6 - 251.7, p = 0.0007), 2 (256.6, 95% CI = 245.2 - 267.9, p < 0.0001) and 4 (235.6, 95% CI = 223.7 - 247.6, p = 0.0134 - Appendix O). Although the mean scaled mass was higher for animals in REL 3 (238.3, 95% CI = 227.8 - 248.7) than in the wild, there was no significant difference between the two (p = 0.0715). There was no significant difference between the mean scaled mass at the start of each release (ANOVA; F(3,148) = 2.361, p = 0.0738 - Appendix O).

As no model for the change in scaled mass over time in the releases received overwhelming support, model averaging was used to generate robust parameter estimates. The top models contained a variation in the rate of change of scaled mass between releases and with Bd infection load (Table 5.3). There was moderate support for a variation in the rate of change of scaled body mass between the sexes (summed AIC weight = 0.732). There was no support for an interaction between sex, release and time or sex, Bd load and time (summed AICc weights = 0).

Table 5.3 Model selection table for the weight change comparison ranked by AICc. REL = release, Time = weeks since release, BdL = Bd load (ln (GE)). Top models (ΔAICc < 6) are shown alongside the next best model and the null model.

Fixed effects	Random effect	K	AICc	Δ AICc	AIC Weight
REL : Time + Bdload : Time + Sex : Time	Frog ID	8	8334.469	0.000	0.732
REL : Time + Bdload : Time	Frog ID	7	8336.479	2.010	0.268
Bdload : Time + Sex : Time	Frog ID	5	8352.619	18.149	0.000
.	Frog ID	2	8671.549	337.080	0.000

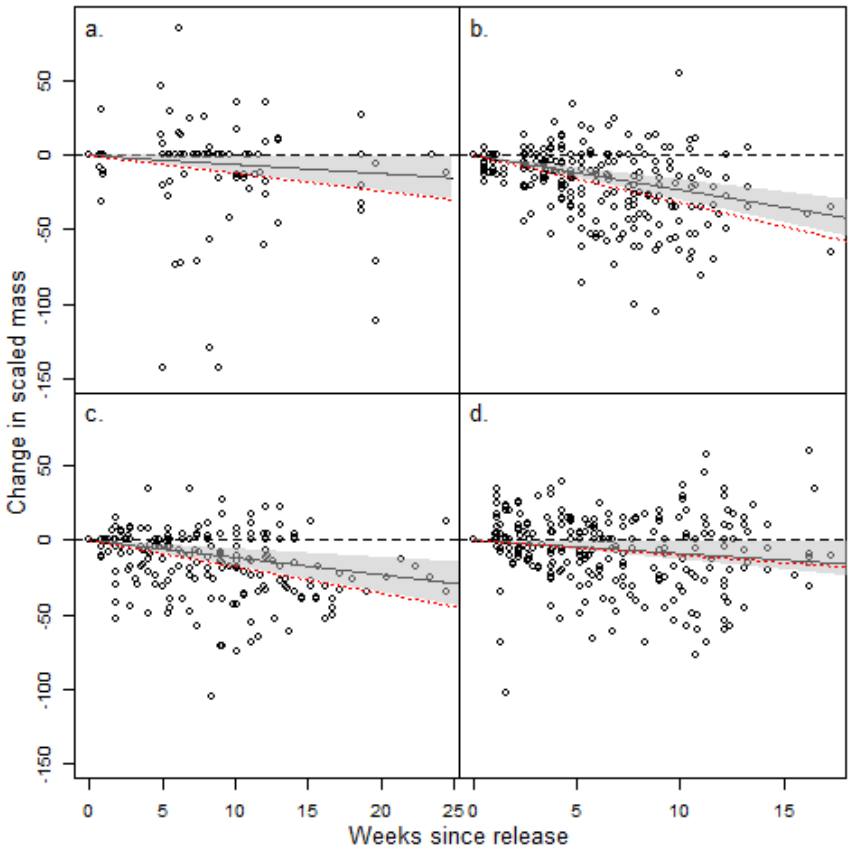

Figure 5.5. Linear mixed model of change in scaled body mass weeks post release for each of the four experimental releases of mountain chickens. Grey area indicates the 95% CI around the model prediction. The red line indicates the rate of change in scaled mass without accounting for Bd infection load indicating there is still some variation between releases that has not been explained.

Model averaged estimates controlling for Bd infection load and sex, indicated that individuals in REL 2 (-2.174 g/week, 95% CI = -2.817 - -1.531) and 3 (-1.155, 95% CI = -1.740 - -0.571) lost body condition at a greater rate than individuals in REL 1 (-0.578, 95% CI = -1.124 - -0.032) and REL 4 (-0.875, 95% CI = -1.315 - -0.065; Fig. 5.5). Individuals were found to lose condition at an increased rate with higher Bd infection loads (effect size = -0.183 / ln(GE), 95% CI = -0.299 - -0.065). Although the mean model averaged estimate of the rate of loss of scaled mass in females was 0.463 g / week greater than males, sex was not included in the second top model resulting in the model averaged 95% CI including zero (-0.897 - 0.219). Examination of the raw data suggested that some of the loss in body condition was the result of increasing SVL without an accompanying increase in mass.

5.4 Discussion

We carried out four experimental releases of mountain chickens into the same site on Montserrat. The species had been extirpated from the release site by epidemic chytridiomycosis and the releases were conducted to optimise the timing of reintroductions in the face of continuing endemic Bd infection in reservoir species. In total, 180 mountain chickens were reintroduced to Montserrat at different times of year in order to assess seasonal variation in reintroduction success. Following each release many of the mountain chickens were found to have contracted Bd infection, although the proportion infected varied by release. This variation was related to the pattern of Bd infection prevalence in sympatric tree frogs. Dead mountain chickens were found following every release and chytridiomycosis was confirmed as a major cause of death in all but REL 4, the only release in the wet season, when Bd infection prevalence in tree frogs was low to zero. Frogs from three releases survived over 10 months, although none were confirmed to have survived longer than 11 months.

Survival of Bd-infected frogs was lower than that of Bd-uninfected frogs. Although the survival estimates changed through time, the survival of Bd-infected frogs in the latter stages of the releases was comparable with the 80 % weekly (64% fortnightly) observed during the epidemic (Chapter 3 / Hudson *et al.* 2016). This suggests chytridiomycosis continues to be a major driver of mountain chicken mortality despite reduced Bd infection prevalence in tree frogs in comparison with the epidemic (Chapter 4). Estimates of survival in the current study appear strongly confounded by migration out of the study area. This would deflate apparent survival estimates, especially in the latter stages of the reintroduction. Whilst separating mortality and emigration is important for understanding the drivers of reintroduction outcomes, in relation to establishment at the release site, both processes contribute to failure (Armstrong and

Seddon 2008). This is reflected in the population models, in which a low number of individuals were predicted to be alive 26 weeks post-release. The lack of captures in any release post 11 months indicates a failure of mountain chickens to settle and survive at the reintroduction site, representing reintroduction failure. However, the prolonged dry period following REL 4 may have inhibited the detection of surviving frogs longer-term which may have taken refuge in damp refugia.

Reintroduction failure appears to have been driven predominantly by chytridiomycosis related mortality in REL 1 - 3 as the majority of dead animals were found to have high Bd infection loads at, or prior to, death. The majority of frogs not confirmed dead in REL 1 - 3 also had very high Bd loads prior to disappearance, when it is possible they died. In REL 4 no fatal case of chytridiomycosis was recorded (although some mortality whilst Bd-infected was predicted in the population models), along with only one incidental death. Some low-level Bd-infections were recorded in the latter stages of the post-release monitoring period, but none near the 10,000 zoospore equivalents at which three other amphibian species succumb to chytridiomycosis (Cheng *et al.* 2011; Vredenburg *et al.* 2010). This threshold is likely species specific, and the limited number of mountain chickens recovering from Bd infection in REL 1 - 3 suggests that Bd-infection will mostly result in eventual mortality. Despite the absence of observed chytridiomycosis related mortality in REL 4, apparent mortality was high and only 16 (95% CI = 1 - 35) of the 50 individuals in the population models were predicted to be alive, in the study area, 26 weeks post-release. Movement distance from the release site was greatest in REL 4 (Chapter 6), suggesting emigration contributed to apparent mortality most in REL 4.

Variation in apparent survival between releases was low compared to the variation in survival between the Bd-infection states. This is reflected in the number of individuals predicted to have died whilst Bd-positive in the population models. The Bd-infection rate (and number of Bd-positive dead animals) was higher in REL 1 and 2 (dry season) than REL 3 (semi-wet), and lower again in REL 4 (wet season). This reflects the patterns of Bd infection seasonality in tree frogs at the release site (Chapter 4). Inclusion of tree frog Bd infection prevalence estimates in the transition parameter of the CMR models would have facilitated direct testing of its importance in predicting mountain chicken Bd infection rate. However, tree frog prevalence estimates were not available for every CMR occasion, meaning inter-occasion variability would have been lacking, reducing its potential to improve model fit. Despite this, mountain chicken Bd infection rate and tree frog Bd infection prevalence were strongly correlated across each release.

The very low number of recovery transitions (I -> U) recorded during the current study contrasts with the recovery rate in the control group of the itraconazole treatment trial (13% weekly - Chapter 3 / Hudson *et al.* 2016). The greater time between sampling occasions in the current study (fortnightly vs. weekly in Chapter 3) may have driven a failure to detect short-term recoveries, likely to occur in the early stages of Bd infection, before innate immune responses were exhausted (Stice and Briggs 2010). Susceptibility to Bd infection might also have been increased (Viggers, Lindenmayer and Spratt 1993) by the stress of travel and release (e.g. Hartup, Olsen and Czekala 2005). Also, the lack of exposure to natural pathogens in captivity might have made the released mountain chickens susceptible to other wild pathogens to which wild individuals are immune (Viggers, Lindenmayer and Spratt 1993).

Whilst it is not possible to confirm transmission of Bd infection from tree frogs to mountain chickens during the releases, tree frogs were recorded in contact with mountain chickens (Chapter 4, Figure 4.5) especially during the dry season when they are often seen on the forest floor. Alternatively, both species could exhibit the same behavioural adaptations to drought, that lead to aggregations, increased contact rates and Bd infection prevalence (Burrowes, Joglar and Green 2004).

Reintroduction failure as a result of chytridiomycosis associated mortality has been recorded in green and golden bell frogs (*Litoria aurea*) (Stockwell *et al.* 2008). Brannelly *et al.* (2016) report reintroductions of *Litoria verreauxii alpina* as being sufficiently successful in the face of endemic chytridiomycosis to be considered a potential conservation strategy for this species. However, the proportion of released *L. v. alpina* caught six months post-release was limited to only 4.8% of the total release cohort. This is lower than the proportion of mountain chickens seen six months post-release in REL 3 (2/24 = 8%) and REL 4 (9/52 = 17%) and lower than the mean number predicted to be extant in the study area six months post-release by the population models for REL 1 (6/50 = 12%) and REL 4 (16/50 = 32%). The weekly survival rates of reintroduced frogs during the 10 week CMR study carried out by Brannelly *et al.* (2016) were similar to the survival of Bd-infected frogs during any of the releases in the current study after 10 weeks (c. 70%). Whilst this was similar to the estimates for wild *L. v. alpina* which are also susceptible to chytridiomycosis, it does not represent a sustainable strategy for species recovery. These results combined suggest reintroductions of Bd-naïve amphibians bred in captivity are problematic when Bd persists in reservoirs at the release site.

Recapture rates were higher in Bd-infected frogs in every release, as recorded by Hudson *et al.* (2016 / Chapter 3). This provides further support to the theory that the disparity between the

results of the current study and those of Murray *et al.* (2009) are the result of the differences in the biology of the amphibian species studied. The decline in recapture rate over time is likely a result of degradation of the radio-signal in tracked frogs, and dispersal of all frogs to the extremes of, and outside of, the study area. Frogs will also likely have established territories and found more permanent burrows towards the end of the study, from which they were less likely to move.

The scarcity of water in the dry season releases appears to have increased recapture rates, whilst the relatively widespread availability of water in REL 4 meant mountain chickens appeared less reliant on pools (L. Martin, pers. comm.). This is reinforced by the significantly lower number of captures per weekly bin in REL 4. The single dead recovery in REL 4 may be a consequence of this reduced recapture rate, rather than a true reduction in mortality, although this is not borne out in the population models. Seasonal variation in the sex effect on recapture rate was likely due to greater movement distances of males in the dry season (Chapter 6) and males calling during the wet breeding season (REL 4) (Daltry 2002). The lack of a sex effect in REL 2 and 3 is likely due to a reduction in male detection rates due to large distances moved, being cancelled out by mating calls.

Most of the released frogs lost body condition post-release. As the pre-decline wild mountain chickens had a lower scaled mass than the released frogs, it is possible that they were overweight and, therefore, returning to 'normal' post-release. This drop in body condition was expected as post-release activity was much greater than in captivity and mountain chickens needed to hunt prey. However, mountain chickens have lost weight during zoo-zoo translocations due to short-term appetite loss (B. Tapley, pers. comm.). The transportation of frogs from Europe to the Caribbean, therefore, likely resulted in loss of body condition, with a continuation of this loss during the stressful post-release period (Teixeira *et al.* 2007). Loss of body condition has occurred in other amphibian reintroductions (e.g. Brannelly *et al.* 2016) and if not part of a 'returning to normal' process, could represent a barrier to survival. There are limited data on post-release body condition available for amphibian reintroductions, but those with short-term success often do not report a loss in body condition (e.g. Zhang *et al.* 2016).

There was seasonal variation of loss of condition, with similar rates of loss in REL 2 and 3 (dry and semi-wet) and a reduced mean loss in body condition in REL 4 (wet). The lower rate of loss observed in REL 1 may be due to the low resolution of available data. Some mountain chickens might have lost condition and died within the first month when no morphometric data were

recorded. Reduced loss in the wet season may be related to greater food availability. During sustained dry periods, the number of observable invertebrates on the forest floor declines (Authors' observations). The reduced capture rate in REL 4 might have reduced handling stress which is acknowledged for many amphibian species (Homan, Reed and Romero 2003; Coddington and Cree 1995) including mountain chickens (B. Tapley, pers. comm.). Whilst increased Bd-infection load in released frogs likely increased the rate of loss of body condition, it is difficult to assign directionality to this relationship. Infection with Bd is known to cause weight loss (e.g. Bielby *et al.* 2015), but reduced body condition could result in / indicate a reduced ability to fight Bd infection, increasing Bd-infection load.

There were encouraging signs of adaption to the wild during the releases. Mountain chickens were observed feeding on large invertebrate prey such as the Montserrat tarantula, which wild individuals predate (Rosa *et al.* 2012). The reintroduced frogs were never exposed to this prey in captivity where their diet was limited to small commercially available invertebrates such as crickets. At present, therefore, rapid behavioural adaptation to captivity does not appear to represent a risk to the success of mountain chicken reintroductions. This is expected for a species with limited parental care (Snyder *et al.* 1996).

In the face of the global threat posed by chytridiomycosis, and in line with advice from the Amphibian Conservation Action Plan (Gascon *et al.* 2007), maintaining captive populations of amphibians threatened by infectious disease is becoming increasingly common. Our study illustrates the difficulties of reintroducing amphibians threatened by chytridiomycosis into sites which contain Bd-infected reservoir species. The low apparent survivorship due to both high levels of chytridiomycosis related mortality (in the dry season) and dispersal away from the study site, suggest that unmodified reintroductions are not a potential mountain chicken conservation strategy. However, some aspects of the releases were successful, such as the low Bd infection rate and absence of fatal chytridiomycosis in REL 4, the consumption of wild prey, use of burrows (sometimes in pairs), and the exhibition of breeding behaviours.

The reduced Bd infection rate and absence of fatal chytridiomycosis in REL 4 suggests that the chytridiomycosis risk to reintroduced mountain chickens could be managed by targeting releases to low Bd-risk summer periods. The Bd-risk increase associated with the onset of the dry season would then need to be managed to ensure longer term survival through, for example, habitat manipulation (Scheele, Hunter, *et al.* 2014) or short term in-situ treatment (Chapter 3 / Hudson *et al.* 2016). The individuals from REL 4 which migrated away from the study site may have survived long-term, and measures to facilitate long-term, wide-scale

monitoring should be investigated to ensure reintroduction management strategies can be adequately assessed (Seddon, Armstrong and Maloney 2007). Measures to delay dispersal such as temporary fencing (e.g. Hardman and Moro 2006) could be tested to counteract the impact of higher dispersal recorded during this season, although this might result in high Bd infection rates due to the increased density of susceptible hosts.

6 Post-release movement and spatial determinants of *Batrachochytrium dendrobatidis* infection risk in reintroduced mountain chicken frogs

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Abstract

Targeting mitigation against infectious diseases of wildlife is essential to maximise efficacy, reduce cost and minimise non-target impacts. At present, little is known about the fine-scale spatial variation in infection risk for the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* (Bd). To provide recommendations for optimising spatial targeting of chytridiomycosis mitigation, we radio-tracked reintroduced mountain chickens in different seasons and diagnosed Bd infections. Bd infection data from each individual was used with geographic profiling to predict sources of Bd infection in the environment. We examined seasonal variation in distance moved from the release site and home range overlap to determine how mountain chicken habitat usage impacted Bd infection risk and how population coverage of mitigation measures could be maximised. Standing pools of water were found to be likely sources of Bd infection, acting as environmental reservoirs or as focal points for the aggregation of mountain chickens and Bd reservoir species, facilitating the transmission of Bd infection. Following one release, Bd infection was detected at a distinct location before spreading across the release site over a period of weeks. During the wet season, the reliance of standing water was lower, resulting in higher dispersal and lower range overlap which may have contributed to the lower Bd infection rates recorded. Population coverage of mitigation measures could be maximised by targeting pools of water where mountain chickens were most likely to become Bd infected and most likely to contact other individuals. Combining real-time diagnostics with geographic profiling could help to identify sources of infection in wild and reintroduced populations of disease threatened species, targeting treatment at primary sources to prevent the spread of infection.

6.1 Introduction

Emerging infectious diseases EIDs are now widely accepted as a proximate cause of species declines and extinctions (Daszak, Cunningham and Hyatt 2000). Conservation in the face of EIDs, which in many cases cannot be eradicated, requires an understanding of the patterns and processes which drive infection (Smith, Acevedo-Whitehouse and Pedersen 2009). Risk of infection with a pathogen, and the outcome of host-pathogen interactions are recognised to vary both temporally and spatially, often interactively (Hess *et al.* 2001). For example, inter-social group transmission of Ebola virus in lowland gorillas (*Gorilla gorilla gorilla*) has been shown to be driven by aggregation around seasonally fruiting trees (Walsh *et al.* 2007; Caillaud *et al.* 2006).

Resource availability drives host aggregation in many other scenarios, including around the most important resource for all life: water. Water becomes a scarce resource during dry seasons around the globe and drives increases in host density around remaining water sources, increasing contact rates between infected and uninfected hosts, and increasing infection prevalence (e.g. Roznik and Alford 2015; Longo, Burrowes and Joglar 2010). Many pathogens are transported predominantly by infected hosts, through dispersal (Russell *et al.* 2005; Hess 1996) or migration (Altizer, Bartel and Han 2011; Reed *et al.* 2003). Understanding the habitat usage and movement patterns of hosts, and how these vary seasonally is, therefore, key in understanding the spatial dynamics of infectious disease.

Most pathogens are able to persist outside the host for varying periods of time. Where the correct environmental conditions exist and where these conditions coincide spatially with host habitat usage, environmental transmission of infection can occur. For example, bird feeders can act as fomites facilitating indirect transmission of pathogens such as *Mycoplasma gallisepticum* in the house finch (*Carpodacus mexicanus*) (Dhondt *et al.* 2007). Similarly, contaminated water sources can directly transmit water-borne diseases to uninfected hosts; examples include, avian influenza (Roche *et al.* 2009) and avian cholera (Blanchong *et al.* 2006) in birds, and *Cryptosporidium* spp. in mammals (Fayer 2004). Mitigation measures, including draining or treating contaminated water bodies and the provision of pathogen free water sources, have been used to reduce infection prevalence in target species (Wobeser 2007; Wobeser 2002).

Non-targeted mitigation strategies which are effective while having little impact on non-target species are rare (Wobeser 2002). The ability to target mitigation measures is, therefore, essential as these reduce non-target impacts, while increasing efficacy and reducing cost

(Carter, Mendis and Roberts 2000). Identifying habitat features around which hosts aggregate, or where host-pathogen interactions occur, facilitates the implementation of targeted interventions to reduce infection risk. This knowledge is also key in maximising the treatment coverage of infected hosts which is necessary to ensure treatment efficacy (Wobeser 2002). For example, in-situ anti-fungal treatment of amphibian chytridiomycosis, which requires multiple daily treatments (Nichols and Lamirande 2001), has been inhibited due to logistical difficulties such as low recapture rates and correctly dosing animals (Chapter 3 / Hudson *et al.* 2016).

Reintroductions of the critically endangered mountain chicken were carried out on Montserrat, 2009 - 2014 into a site from which they had been extirpated by chytridiomycosis (Chapter 5). This disease is caused by the waterborne fungus *Batrachochytrium dendrobatidis* (Bd) and has caused the decline or extinction of over 200 species of amphibian world-wide (Skerratt *et al.* 2007). The reintroduced mountain chickens did not survive beyond 11 months at the release site, predominantly as a result of continued chytridiomycosis driven mortality (Chapter 5). Attempts to immunise individuals, which could be used in reintroductions, has generated mixed results in other species (McMahon *et al.* 2014; Cashins *et al.* 2013; Stice and Briggs 2010) and the failure to stimulate a protective effect through repeated Bd infections and clearances with itraconazole during the chytridiomycosis epidemic (Chapter 3 / Hudson *et al.* 2016) suggest it is unlikely in captive mountain chickens. Whilst selection for tolerance in captivity prior to release has been suggested as one method for increasing reintroduction success (Scheele, Hunter, *et al.* 2014; Venesky *et al.* 2012), there are no known Bd tolerance traits to select for. As a result, at least in the near future, mitigation of the disease at the reintroduction site might be the only available method for reducing the impact of chytridiomycosis on reintroduction success.

Wide scale mitigation of chytridiomycosis in the form of, for example, anti-fungal treatment of every reintroduced individual throughout the release could be effective, but would be time consuming and expensive (Chapter 3 / Hudson *et al.* 2016). It would also be impractical as treatment would be indefinite, would require sufficiently frequent captures over the life-time of each individual to ensure efficacy and therefore be disruptive to breeding behaviours with welfare implications. Disinfection has been used to eradicate Bd from an isolated pond system containing *Alytes muletensis* in Majorca (Bosch *et al.* 2015), but the potential non-target impacts are not acceptable in a biodiverse forest such as those on Montserrat. The high density of organic matter in such an environment would also likely rapidly deactivate most disinfectants. Identifying and neutralising likely sources of Bd at the release site could allow

the infection threat to be mitigated without requiring widespread application of chemicals or other measures. It would also facilitate the targeted application of treatment, such as in-situ anti-fungal drugs, to Bd-infected individuals, that are most important for onward pathogen transmission. Currently, however, little is known about the fine-scale spatial variation of Bd infection risk meaning targeted measures cannot be utilised.

Water availability at the reintroduction site on Montserrat (SW) varies seasonally. The site comprises a ghaut (narrow valley) in which there is a stream which flows only after heavy rainfall. Outside the wet season pools of water persist for months before eventually drying completely. These ephemeral pools are the only water source in the ghaut during the dry season and so are likely visited by both mountain chickens and Bd reservoir species, tree frogs (*Eleutherodactylus johnstonei*) and cane toads (*Rhinella marina*) (Chapter 4). Frogs infected with Bd might shed large numbers of zoospores into standing water, creating environmental reservoirs (Reeder, Pessier and Vredenburg 2012) from which Bd could be indirectly transmitted to new hosts, as recorded experimentally (Longo, Burrowes and Joglar 2010; Rachowicz and Vredenburg 2004). Under sterile conditions, Bd zoospores have been shown to survive in sterile water and moist substrate for up to seven and twelve weeks respectively (Johnson and Speare 2005; 2003). Release mountain chickens displaying severe signs of chytridiomycosis have been observed occupying standing pools in SW with released healthy mountain chickens. This represents a potentially important mechanism for the transmission of Bd infection.

The use of individual spatial data has a long history in epidemiology, such as the identification of the source of London's cholera epidemic by Snow (1855). However, since then, there has been little advance in methods to analyse spatial data on infected individuals to identify sources of infection. Recently, Le Comber *et al.* (2011) adapted criminal geographic profiling (GP), in which crimes are mapped in order to identify areas most likely to include the offender's location (Rossmo 2000), for epidemiological data. Whilst the original GP algorithms were designed to identify a single source (i.e. a single offender: O'Leary 2009), Verity *et al.* (2014) modified the implementation of GP such that is can be utilised when the number of sources is unknown. This is important in the study of infectious disease, as failure to identify every source might result in inadequate coverage of any mitigation strategy. Using GP to identify likely sources of infection could allow targeted mitigation to prevent further contagion.

To investigate whether pools of water were likely sources of Bd infection in reintroduced mountain chickens, for each mountain chicken release conducted, we compared a geoprofile of the likely sources of Bd infection with maps of standing water bodies during the post-release monitoring periods. Combining this with temporal information, allows us to determine the likely spread of Bd infection within the reintroduced population. We also examined home range overlaps between radio-tracked mountain chickens and the distance moved from the release site in order to understand where transmission of Bd to and between mountain chickens was most likely to occur. These results enable us to provide recommendations to maximise the population coverage of in-situ mitigation measures.

6.3 Methods

6.3.1 Field data collection

A summary of the reintroduction of mountain chickens can be found in Table 5.1 of Chapter 5. Briefly, four experimental reintroductions of mountain chickens were carried out at the same site called Sweetwater ghaut (SW) on Montserrat. The timing of the releases was varied in order to establish whether there was seasonal variation in release success. The first (REL 1) and second (REL 2) took place during the cool, dry season. The third (REL 3) took place at the end of the warm, wet season and the fourth (REL 4) at the start of the wet season. Of the mountain chickens reintroduced to SW on Montserrat (see Chapter 5), only animals that were radio-tracked during the post-release monitoring period were used for the current study (REL 1 n = 33; REL 2 n = 32; REL 3 n = 25; REL 4 n = 32). This is because there was reduced detectability of animals that were not radio-tracked especially away from the centre of the release site which would bias the data. Up to five night time surveys were carried out weekly for 12 weeks. During each survey, two radio-tracking receivers (TRX-1000; Wildlife Materials Inc. and HR2600 Osprey; Habit Research and Locator Systems Corporation; both with three element yagi antennae) were used, one each by two teams, to locate radio-tracked mountain chickens along a transect. This transect included the ghaut, the two ghaut forks and five parallel transects up the East bank (Fig. 5.1 in Chapter 5). When a frog was detected, radio-tracking was used to estimate its location to within 5 m and the coordinates were recorded using a GPS (Garmin eTrex 10). In REL 2 - 4, one day search of neighbouring ghauts was also carried out once weekly.

To determine whether released mountain chickens were infected with Bd, frogs were caught and skin swabbed for Bd DNA up to once per fortnight in REL 1 and once per week in REL 2, 3 and 4 (see Chapter 5, Section 5.2.3 for more detail).

6.3.2 Laboratory methods

Skin-swabs were refrigerated until transport to the laboratory where DNA was extracted from each swab as described in Appendix E. A Taqman qPCR was used to quantify the amount of Bd in each swab following the procedures described in Chapter 3 (Section 3.2.3).

6.3.3 Distance moved from release site

Maximum linear distance from release site for each capture was calculated using adehabitatLT (Calenge 2006) in R. ANOVAs were used to compare the maximum linear distance from the release site between release number (1 - 4) and the sexes, additively and interactively, at three time points; the first 30, first 60 and first 90 days post-release at which point the majority of radio implants had failed. Sex and release number were only retained in each model if they were found to be significant at alpha=0.05. Although there was sample size variation between the releases, ANOVA is considered robust if the homogeneity of variances assumption is still met which was confirmed using Bartlett's test at alpha = 0.05. Tukey's HSD tests were used to make post-hoc comparisons of groups if the initial ANOVA showed a significant difference between groups.

6.3.4 Home range overlaps

As a result of the failure to generate useful home range estimates using KDE methods (Appendix P), minimum convex polygon (MCP) home ranges for the 26 week post-release monitoring period were calculated using adehabitatHR (Calenge 2006) in R. In order to reduce the confounding effect of release site location, the first two weeks of data post-release were excluded to allow mountain chickens to disperse and settle. Frogs were only included if their location had been recorded on at least 20 occasions as this represented the best compromise between ensuring accurate estimation of home range size and the number of individuals which could be included. Isopleths containing 95% of the radio-fixes were selected to represent the home range as they appeared to exclude only extra-ordinary habitat usage which might represent foraging or exploratory forays (Burt 1943).

Home range overlaps were calculated by recording the number of home ranges which intersected each cell of a 10 m grid and covered the entire range of radio-fixes recorded. Ten metre grid cells were used as this is the diameter of a circle around each radio-fix at the accuracy of 5 m used to record mountain chicken locations. Home range overlaps were then visually compared to the locations of pools, and the distance between the cells with the greatest number of overlapping home ranges and the nearest pool, was calculated.

6.3.5 Predicting sources of infection

Geoprofiling was used to determine the potential importance of pools of water as sources of Bd infection due to their importance in the biology of mountain chickens, Bd reservoir species, and Bd. Individuals were included if they had been skin swabbed according to the relevant protocol in the two weeks prior to their first recorded Bd-positive skin swab. Hence, inclusion required at least one (REL 1) or two (all other releases) skin swabs from which Bd had not been detected prior to the first Bd-positive swab. Thus, the likelihood that Bd infection had occurred around the time of the first detection was high. For this analysis, the location of the frog at the time of the first Bd-positive skin swab was assumed to be the point at which Bd infection occurred. A GPS malfunction towards the end of REL 4, coincident with when the first Bd infections were recorded, meant location data were not available. Data from REL 4 were, therefore, excluded from this analysis.

As no complete map of standing water bodies in SW and the surrounding ghauts was available, a pool was included in the analysis if a mountain chicken had been caught in or near it and the pool persisted for more than one month in order to distinguish pools from temporary puddles which would be problematical to target with interventions. As REL 4 took place during the wet season, small bodies of standing water were common across the release site meaning it was difficult to ascertain the geographic and temporal limits of each water body and they are not identified in the results.

6.3.6 Implementation of geoprofiling

In order to predict sources of Bd infection, we utilised the Drichilet process mixture implementation of the GP model as described by Verity et al. (2014). This creates an ordered probability surface of the likely source locations through the following two-step process. Firstly, a clustering algorithm groups observations (first Bd infections) into clusters. Each observation within a cluster is assumed to originate from the same source, thus, observations that are close to one another are more likely to end up in the same cluster. No prior assumptions are made about the number of sources and therefore clusters. In step two, a probability surface of the likelihood of an infection occurring at a given location, is defined for each of the clusters defined in step one in the form of a bivariate normal distribution. Bayes' rule is then used to invert the problem, generating the posterior distribution of the unknown source locations based on the observed infections (O'Leary 2009). Finally, a Gibbs sampler (a multivariate Markov Chain Monte Carlo method) is used to alternate between step one and two in order to integrate over all possible groupings of the data into clusters in order to generate a solution.

The Drichilet process mixture model was implemented in R (R core team 2015) using the R package Rgeoprofile (Verity *et al.* 2014). Model parameters were set to default values and sigma, the distance representing one standard deviation of the bivariate normal distribution of observations centred at the source, was initiated at 0.001 degrees. This is equivalent to a distance of approximately 100 m at the reintroduction site (SW). Under this assumption, 99% of infections are expected to occur within 300 m of the source, which is in line with the greatest distance a mountain chicken is recorded moving within a week during the reintroductions (Authors' unpublished data).

The location of standing pools was then plotted onto the resulting geoprofile, and the hit score for the point of each pool, reported. The hit score is equivalent to the area covering the infection observations (plus a buffer of 5%) that would need to be searched before the pool was found, according to the order of search priority assigned by the geoprofile. For example, a hit score of 0.01 would be located after searching the first 1% of the study area. The lower the hit score, the greater the probability of that pool being a source of infection.

Geoprofiling does not include temporal information on the acquisition of infections. Temporo-spatial interactions in Bd-infection risk were, therefore, seperately examined in REL 3 as the time to Bd-infection varied most in individuals during this release. All of the radio-fixes of individuals which did not become Bd-positive until after radio-tracking had finished (i.e. on transplant failure, approximately 01/02/2013), or were never recorded as Bd-positive, were plotted over the geoprofile of likely source locations for REL 3 to test for spatial overlap with the risk hotspots. The first five recorded Bd infections were dated on the geoprofiles for REL 1 and 2 in order to examine any temporo-spatial variation in infection risk. In REL 3, every infection was dated in order to provide more detail of any temporo-spatial interaction and potential spread of Bd infection across the release site.

6.4 Results

In total, 122 mountain chickens were radio-tracked during the 26 week post-release monitoring periods of the experimental releases, 2011 - 2014 (REL 1 = 33, REL 2 = 32, REL 3 = 25, REL 4 = 32). Radio-implants were recorded as failing on average 70 days post-release. Locations for implanted mountain chickens were collected 3945 times in the post-release monitoring period across the four releases (REL 1 = 1123, REL 2 = 1587, REL 3 = 887, REL 4 = 348). There were more radio-fixes of animals outside SW during REL 3 (10 of 2 individuals) and 4 (12 of 3 individuals) compared with REL 1 and 2 in which there were none (Fig. 6.1).

6.4.1 Distance moved from release site

There was a significant difference between the releases in the maximum distance from release site after 30, 60 and 90 days (ANOVA: 30 days $F(3,84) = 7.158$, $p = 0.0002$; 60 days $F(3,83) = 5.212$, $p = 0.0024$, 90 days $F(3,83) = 4.119$, $p = 0.0024$). Frogs in REL 4 moved significantly further from the release site than those in all other releases after 30 and 60 days, and more than REL 2 after 90 days (Table 6.1). There was also a near-significant difference between the maximum distance from the release site between frogs in REL 3 and 4 after 60 days. There was no evidence for any other significant differences between releases in the distance from release site (Table 6.1). There was no significant difference in the maximum distance moved between males and females after 30 days (effect size 95% CI = -6.40 - 105.80; $F(1,83) = 3.418$, $p = 0.0817$), but males were found to have moved further from their release sites than females after 60 (effect size = 80.26 95% CI - 21.42 - 139.09; $F(1,83) = 8.106$, $p = 0.0081$) and 90 days (effect size = 83.76, 95% CI = 23.20 - 144.31; $F(1,83) = 8.333$, $p = 0.0073$). There was no significant interaction between sex and release in any time period (30 days $F(3,80) = 0.017$, $p = 0.9968$; 60 days $F(3,80) = 0.099$, $p = 0.9603$, 90 days $F(3,80) = 0.311$, $p = 0.8175$).

6.4.2 Home range overlap

The home ranges of reintroduced animals overlapped to the greatest extent near the centre of the ghaut in every release except for REL 4 where overlap was more widespread (Fig. 6.2). The maximum number of overlapping home ranges was high in REL 1 (14 of 27 home ranges overlapped), 2 (15/29) and 3 (15/17), with a lower maximum number of overlapping ranges in REL 4 (4/14), although this might be, in part, due to the lower sample size. All of the grid cells with the highest number of overlapping mountain chicken ranges were in close proximity to a source of standing water (REL 1 = 15 m, REL 2 = 10 m, REL 3 = 0, 10 & 15 m).

Not all cells containing a standing water source had high values for home range overlap. Those furthest from the release site were within the 95 % MCP home range of only one or two mountain chickens.

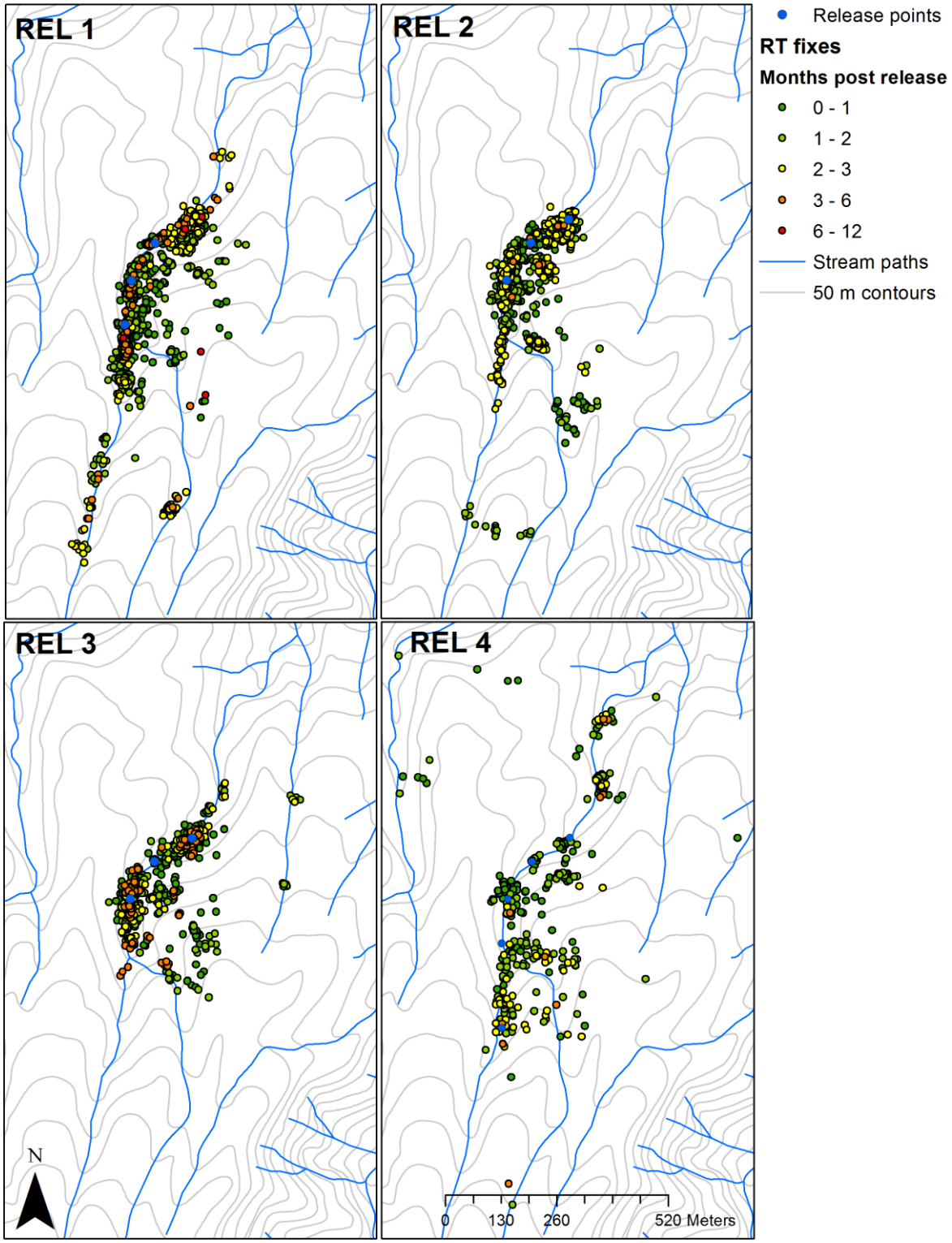


Figure 6.1 All captures made during the four mountain chicken reintroductions to Sweetwater ghaut on Montserrat. All captures were estimated to within 5 m of the frog location using a GPS.

Table 6.1. Summary of the mean maximum distance moved by each frog from release site 30, 60 and 90 days after release for each of the release cohorts. Also presented are pairwise comparisons between each release via Tukey's HSD, p-values significant at alpha = 0.05.

After 30 days	Raw mean maximum distance moved from release site (m)	SE	REL 1	REL 2	REL 3	REL 4
Release 1	128.02	19.20		0.9460	0.9683	0.0002
Release 2	147.68	27.61			0.9999	0.0008
Release 3	147.10	25.96				0.0032
Release 4	320.32	45.21				
After 60 days						
Release 1	219.70	22.24		0.5803	0.9992	0.0315
Release 2	172.61	26.70			0.7750	0.0010
Release 3	214.08	33.09				0.0426
Release 4	348.50	50.83				
After 90 days						
Release 1	263.92	28.87		0.2904	0.9568	0.2199
Release 2	196.36	26.12			0.7251	0.0046
Release 3	241.28	29.51				0.1261
Release 4	355.69	49.64				

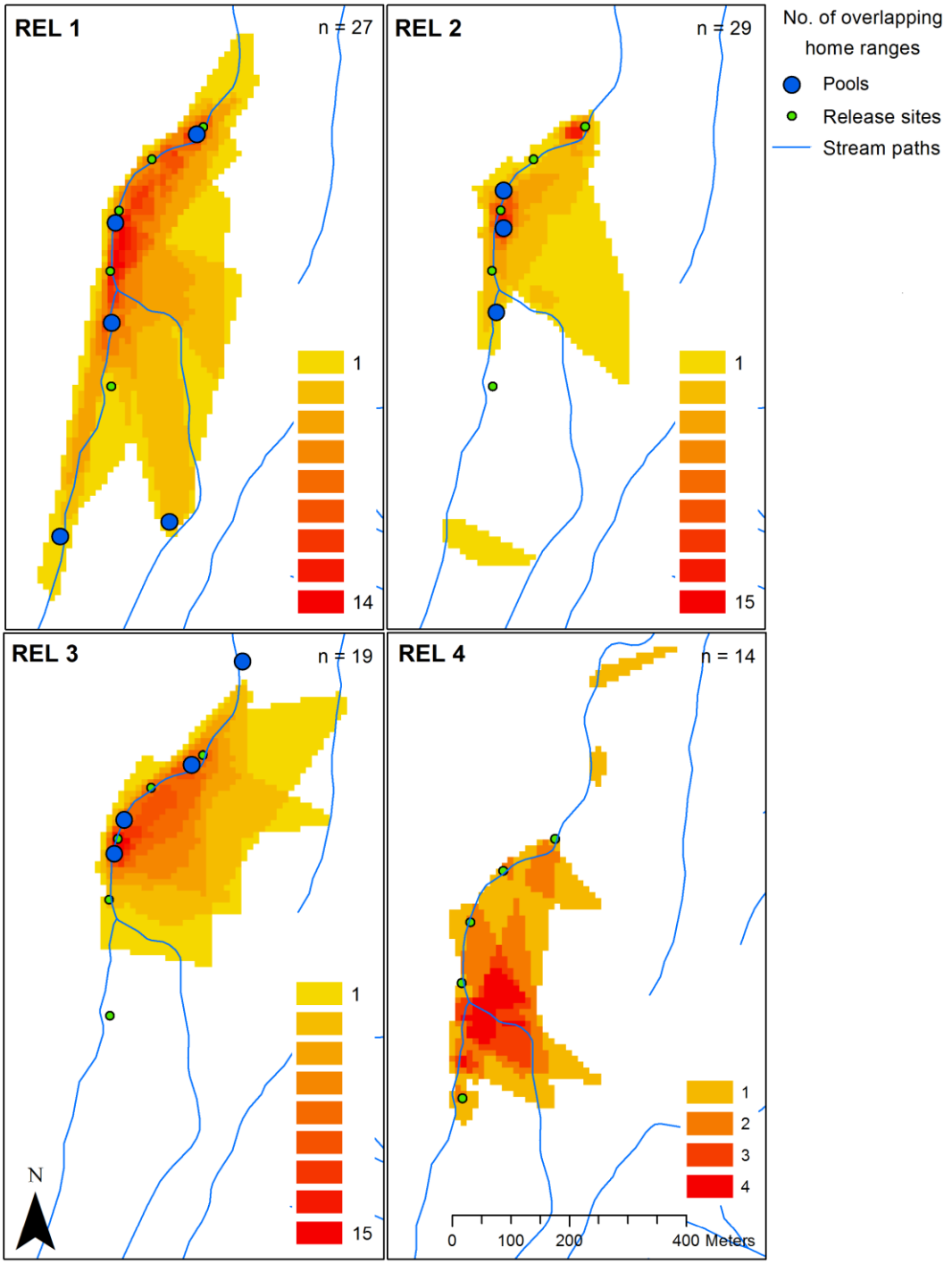


Figure 6.2 Map of mountain chicken MCP range overlaps between 3 - 26 weeks post-release for each of the experimental reintroductions. The first two weeks were omitted to allow time for the released animals to settle.

6.4.3 Geoprofiling

The pools close to the release site were located in areas of high search priority according to the ordered probability surface (geoprofile) in every release, with at least one pool in the top 1% of search (i.e. a hit score of <0.01) (Fig. 6.3 and Table 6.2). This suggests at least one pool was a likely source of Bd infection in every release. The further from the release site, the lower the hit score of the water body as a potential source. For each release, the model predicted multiple source sites, with at least three distinct clusters of infections.

Table 6.2 Geoprofiling hit scores for each standing body of water identified during the reintroductions. The hit score is equivalent to the area covering the infection observations (plus a buffer of 5%) that would need to be searched before the pool was found, according to the order of search priority assigned by the geoprofile. For example, a pool with a hit score of 0.01 would be located after searching the first 1% of the study area. The lower the hit score, the greater the probability of that pool being a source of infection.

Release	Pool number (Fig. 6.3)	Latitude	Longitude	Hit score
REL 1	1	16.78328	-62.18418	0.01773
REL 1	2	16.78191	-62.1854	0.00137
REL 1	3	16.78037	-62.18555	0.02682
REL 1	4	16.77729	-62.18462	0.11155
REL 1	Not in frame (left fork)	16.77706	-62.18637	0.51837
REL 2	1	16.78327	-62.18431	0.01011
REL 2	2	16.78241	-62.18539	0.2034
REL 2	3	16.78183	-62.18539	0.58644
REL 2	4	16.78052	-62.1855	0.1277
REL 3	1	16.78454	-62.18344	0.0869
REL 3	2	16.78325	-62.18426	0.04397
REL 3	3	16.7824	-62.18534	0.02465
REL 3	4	16.78187	-62.1855	0.005411

Six individuals in REL 3 remained Bd-uninfected until the end of the radio-tracking period. When the locations of radio-fixes corresponding to these individuals were plotted over the geoprofile of likely infection sources, two patterns emerged. Firstly, frog 112, which was never recorded as Bd-positive post-release, was caught within 10 m of Pool 4 (the pool with the lowest hit scores in REL 3) only once (Fig. 6.4). Secondly, the majority of captures of the frogs which did not become Bd-infected until late January, were near Pool 2 (Fig. 6.4). All of the early infections (November - early January) took place in the proximity of Pool 3 with none near Pool 2 where many of the Bd-uninfected individuals were found (Fig. 6.3 and 6.4). The latest Bd-infections that were detected (February onwards), occurred uniformly across the release site.

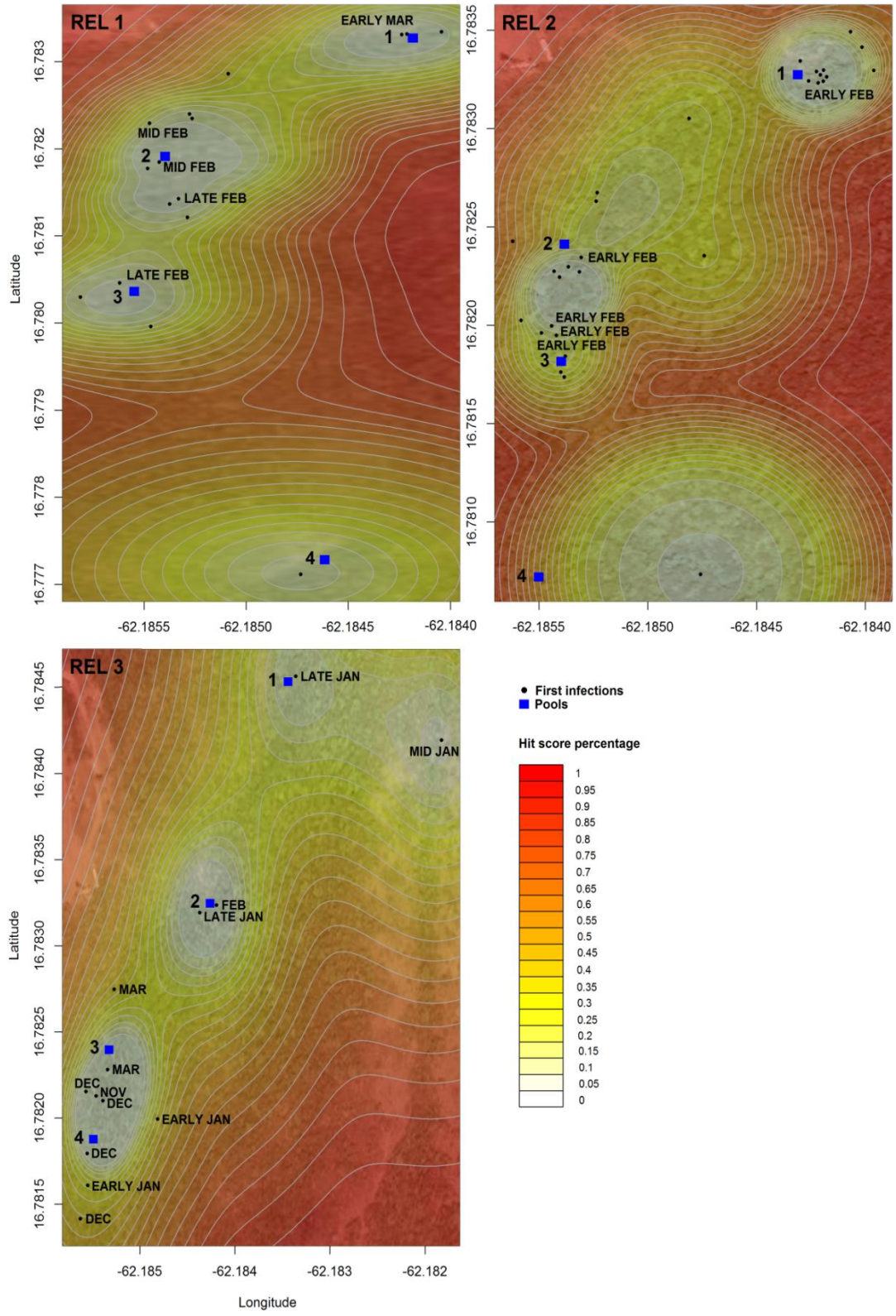


Figure 6.3 Geoprofiles of predicted sources of Bd infection during the reintroductions. The hit scores (akin to the inverse of the likelihood of being the source) for each body of standing water are reported in Table 6.2 according to the numbering of pools in this figure. The first five Bd positive mountain chickens detected are dated in REL 1 and REL 2, and all Bd infections are dated in REL 3 to indicate temporal spread through the population.

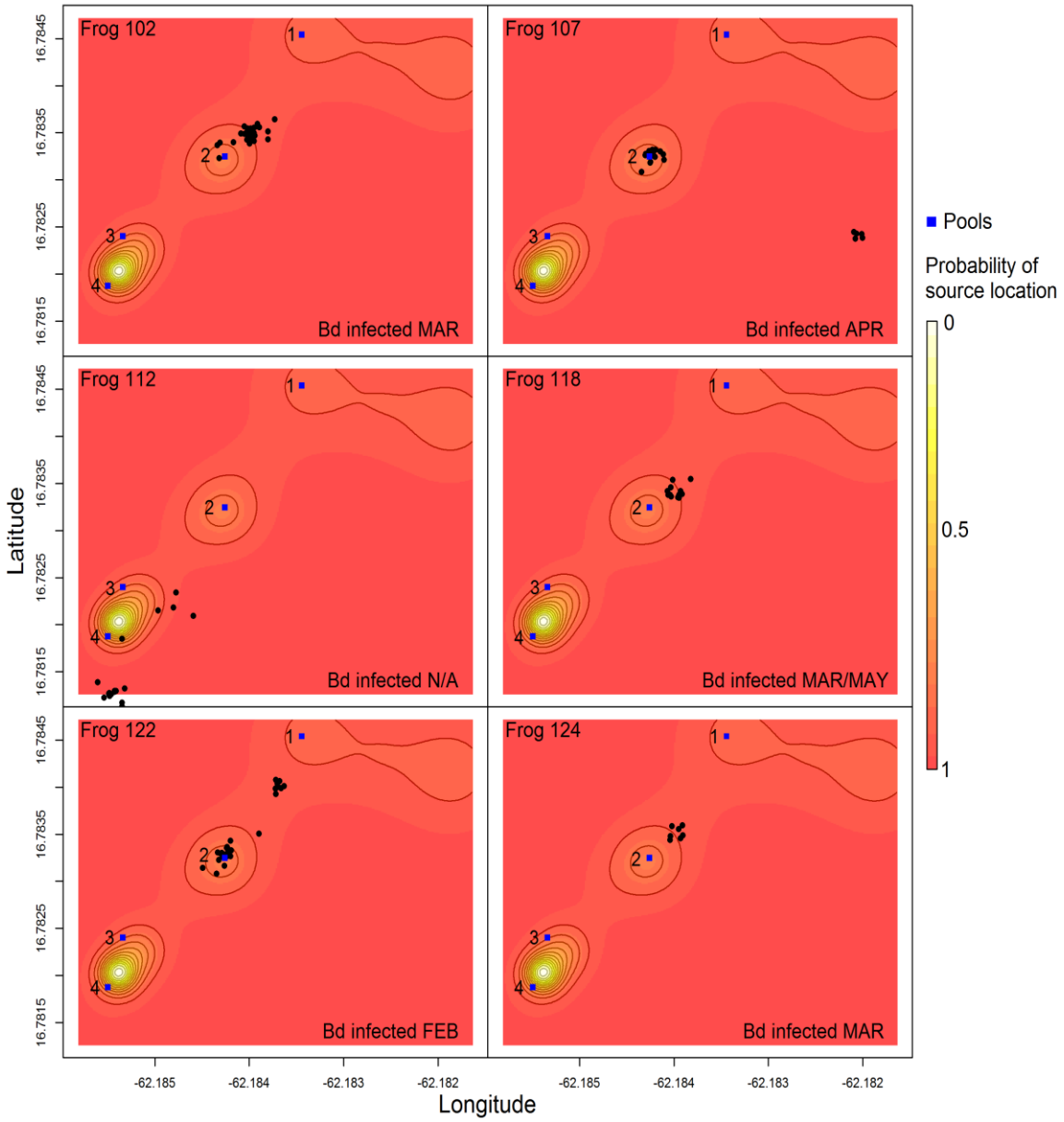


Figure 6.4 Comparison of the radio-fixes of late Bd infected / uninfected frogs during REL 3 with the geoprofile of Bd infection source locations. Each pool considered a potential Bd source in this analysis is numbered corresponding to those in Table 6.2.

In REL 1 and 2, where Bd infection prevalence in tree frogs was high throughout (Chapter 4 and 5), there is less evidence for a temporal pattern in the emergence of Bd infection in the reintroduced population. In REL 1 the first Bd infections were recorded near Pool 2, with Bd infections occurring within 2 weeks at nearby Pool 3, and within 4 weeks at Pool 1, much further north. The first Bd infections in REL 2 occurred at Pool 1, 2 and 3, simultaneously (Fig. 6.4).

6.5 Discussion

We radio-tracked 122 mountain chickens during four experimental reintroductions into a site from which they had been previously extirpated by chytridiomycosis in order to identify spatial patterns in Bd-infection risk and home range overlap. Frogs released in the wet season moved further from the release site than in any other release and the radio-fixes were recorded at a lower density. During the post-release monitoring period, the ranges of mountain chickens overlapped most near sources of standing water, especially during the dry season, indicating the most likely areas of contact and therefore intra-species pathogen transmission. Geoprofiling identified standing water bodies to be likely sources of Bd infection at which the reintroduced mountain chickens became infected.

Pools may have been highlighted as potential sources by the geoprofiling as a result of being environmental reservoirs for Bd. Whilst Bd is known to persist in water or moist substrate for months under sterile conditions (Johnson and Speare 2005; 2003), little is known about the ability of Bd zoospores to persist in natural conditions and predation by other micro-organisms has been observed (e.g. Schmeller *et al.* 2014). Environmental sampling for Bd from standing water in SW was attempted in the latter stages of REL 4 following the procedure described by Walker *et al.* (2007), but by this time, a prolonged period of drought had set in and the water had dried up. Whilst the presence of Bd DNA in the water would have provided evidence of Bd in the water, it would not have determined the viability of zoospores, if present. A laboratory method has been developed to determine the viability of Bd zoospores in-vitro, but this cannot be used in field conditions (McMahon and Rohr 2014). The only practical way to prove zoospore viability in the wild would be to expose animals to the suspected water source under experimental conditions.

Alternatively, the scarcity of water in the dry season might be a reason that pools were highlighted as potential sources of infection by the geoprofiling. The reliance of amphibians on water could have resulted in increased amphibian densities, thus, increasing the potential for Bd transmission. For example, under experimental conditions, *Eleutherodactylus coqui* has

been shown to aggregate in moist refugia during drought conditions, resulting in increased Bd infection prevalence due to increased contact rates between hosts (Longo, Burrowes and Joglar 2010). This drought driven aggregation has also been observed in the wild in *Litoria rheocola* (Roznik and Alford 2015).

It is also possible that Bd infected mountain chickens were predominantly found near pools as this is where all mountain chickens (Bd-infected or not) were most likely to be found (Fig. 6.1) and home range overlaps were greatest (Fig. 6.2). However, mountain chickens with signs of chytridiomycosis are thought to preferentially sit in pools (Chapter 3 / Hudson *et al.* 2016). Whilst this behaviour would increase opportunities for Bd transmission within water bodies, it could be exploited to reduce transmission rates by targeting mitigation measures, such as treatment, at frogs (or water) within pools. In this way, both Bd infected individuals and point sources of infection would be preferentially targeted.

As only pools were compared with the geoprofile of Bd infection, this study provides no information on the importance of other environmental features in the transmission of Bd. For example, contacts during shared use of moist refugia could also result in the transmission of Bd infection, although this would be difficult to prove in the wild due to low spatial and temporal resolution of the tracking techniques currently available for use on this species in which external trackers have proven challenging (B. Tapley, pers. comm.).

Home ranges estimated using MCP are often considered flawed as they are heavily influenced by the locations of outlying fixes and therefore incorporate potentially large areas of unused habitat (Harris *et al.* 1990). Whilst KDE home ranges of a more realistic size could have been produced utilising the procedure outlined in Row and Blouin-Demers (2006) (scaling the bandwidth to a value which produces the same home range size as MCP), they would likely underestimate the area outside the ghaut used by the mountain chickens. The detectability of radio-tagged animals appeared to be much lower outside the ghaut, therefore the number of captures outside the ghaut may have been underrepresented. This is reinforced by the very small number of captures made outside the ghaut once radio-transmitters failed (Authors' unpublished data). Further from the ghaut, the vegetation density is greater, resulting in reduced detectability and lower number of captures, despite a number of captures indicating it was used by many individuals (Fig. 6.1). As a result, MCPs might have represented the home range more accurately in this study than by those generated through KDE methods.

Geographic profiling does not take into account the temporal aspects of infection dynamics. The first detected Bd infections in REL 3 occurred near the same pools (Pool 3 and 4), predicted to have the greatest probability of being the source of Bd (Fig. 6.3, 6.4 and Table 6.2). Bd infections near other identified pools, in the North of the site, were detected later (the first, nine weeks later), suggesting a spread of Bd infection from the South to the North of the release site. The first detections of Bd infections in REL 1 followed a similar pattern, although the spread was more rapid. The first Bd infections occurred near Pool 2, and spread south to Pool 3 within two weeks, and north to Pool 1 within four weeks. REL 2 followed this pattern to a lesser extent. The first Bd infections occurred simultaneously and more uniformly across the site. This reduction in apparent temporal-spatial interaction in predicting the location of Bd infections, compared to the 'wave' like infection spread in REL 3, suggests that Bd was present more uniformly across the release site in REL 2. This variation in pattern of emergence might be the result of the timing of the release. REL 1 and 2 took place in January when tree frog Bd infection prevalence and loads were high and when water was scarce (increasing the likelihood of sympatric species sharing pools), whereas the tree frog Bd infection prevalence was low at the start of REL 3 (Chapter 4 and 5). The spread across the release site during REL 3 is probably the result of a combination of two mechanisms. Firstly, newly Bd-infected mountain chickens might move around the release site in search of resources or mating opportunities, transporting the pathogen. Secondly, the majority of Bd infections in REL 3 that did not occur near the primary source (Pool 3 and 4) occurred from January onwards (9 weeks after the first detected Bd infection). This was during the cooler drier season and is when tree frog Bd infection prevalence and load is found to increased (Chapter 4). Thus, in the dry season, the spatial risk of contact between mountain chickens and infected reservoir hosts likely increased because of the higher Bd infection prevalence in tree frogs and because most species are more likely to share water sources.

The importance of pools of water in the movement patterns of mountain chickens is clear. The majority of captures occurred near the centre of the ghaut, and pools were within many of the estimated home ranges of mountain chickens. The higher environmental availability of water in REL 4 likely resulted in the greater movement away from the release site. Frogs in REL 4 were caught at much lower densities than in the other releases, with multiple captures in neighbouring ghauts where the likelihood of contact with conspecifics was much reduced (Fig. 6.1), and hence, intra-specific Bd transmission less likely. The increased temperature in REL 4 (Chapter 4 and 5) might also have affected the distances moved as anurans are known to take shelter in colder conditions in order to effectively thermoregulate (Wells 2010) which would not have been a common problem during REL 4. This decreased density of captures due to

increased dispersal may have contributed to the reduction in Bd infection rate recorded in the mark-recapture study of the reintroductions (Chapter 5).

Direct mitigation of Bd at the release site might represent the only method available for improving the conservation management of amphibians threatened by chytridiomycosis, where immunisation (Cashins *et al.* 2013; Stice and Briggs 2010) and selection for tolerance cannot be used successfully. In the future, combining GP methods with rapid in-situ diagnostics (e.g. Durrant *et al.* 2014), might allow the targeting of mitigation measures in real-time at likely sources of Bd. This could prevent the spread of Bd infection within a reintroduced or remnant population whilst reducing the effort involved for in-situ mitigation measures, such as the repeated treatment of individual animals (e.g. Chapter 3 / Hudson *et al.* 2016). The lower the number of sources of infection, the easier the disease is to mitigate (Wobeser 2002). Timing a reintroduction to occur during an initially low Bd-infection risk period (such as REL 3) and identifying the primary source in real-time, might allow early treatment when infection occurs, thus slowing or even preventing infection spread. Additional measures might be required during periods of high risk, such as periods of elevated infection prevalence in tree frogs during the cool, dry season (Chapter 4). The results of the current study, however, indicate that these could be targeted to remnant water sources, thus facilitating the treatment of large numbers of individuals due to range overlap in those locations. Should water be confirmed as an environmental reservoir of Bd, direct treatment of the water might also help to reduce Bd-infection rates. Models have shown that the greater the time period that Bd can persist in reservoirs, such as water, the greater the likelihood of host extinction (Mitchell *et al.* 2008). There are numerous measures which could be used to reduce the number of viable Bd zoospores in a water body, including disinfection (Bosch *et al.* 2015), anti-fungal treatments (Appendix 1 within Woodhams *et al.* 2011), salinisation (Stockwell *et al.* 2015), microscopic aquatic predators (Searle *et al.* 2013) and heat (e.g. Young, Berger and Speare 2007). Each should first be assessed for its potential impact on both target and non-target species, including ecologically important fungal components of the ecosystem (Woodhams *et al.* 2011).

7 General Discussion

This thesis documents the decline and near-extinction of the mountain chicken, along with the implementation and analysis of two in-situ conservation strategies for the mountain chicken, as a model for chytridiomycosis threatened amphibians globally. A world's first trial of a field-based anti-fungal treatment during a chytridiomycosis epidemic was conducted which was successful in reducing mortality and Bd-infection rates in the short term (Chapter 3). This has implications for future chytridiomycosis epidemics and outbreaks, where this treatment could be employed to reduce the immediate impact and buy time to enact further conservation measures. The environmental drivers of Bd infection dynamics in sympatric reservoir species on Montserrat were identified (Chapter 4) and used to optimise the timing of mountain chicken reintroductions so that they coincided with periods of low Bd infection pressure (Chapter 5). This resulted in a large reduction in the impact of chytridiomycosis on the reintroduction in the short term, where it had previously resulted in reintroduction failure. Using an adaptive management approach, this success could be built upon through the trial of other novel mitigation measures such as in-situ treatment to reduce seasonal Bd infection risk (Chapter 3), providing a platform for successful reintroductions of chytridiomycosis threatened amphibians. Finally, radio-tracking data from the reintroductions were used to identify how the spatial targeting of mitigation measures for Bd infection could be optimised, both by maximising the population coverage of such measures and investigating the potential role of pools as environmental sources of Bd (Chapter 6). Clearly, the mountain chicken programme emphasises the significant challenges of carrying out reintroductions in the face of threats that cannot be completely neutralised. In such cases the strategy may need to focus more on threat management than threat neutralisation

7.1 The decline of the mountain chicken

Chytridiomycosis emerged in the mountain chicken on Dominica in 2002 and Montserrat on 2009 and caused rapid, precipitous declines, with the species almost disappearing within two years on both islands (Chapter 2). This represents one of the fastest vertebrate declines ever recorded, highlighting the propensity of chytridiomycosis to drive devastating declines. The decline was associated with a significant loss of genetic diversity on Dominica, something that is also certain on Montserrat as only two individuals are now thought to remain (Author's unpublished data). Loss of genetic diversity, and increased inbreeding due to small population size, result in lowered evolutionary potential, reduced reproductive fitness and increased risk of extinction (Frankham 2005; Spielman, Brook and Frankham 2004). The extreme range decline recorded and resulting fragmentation of populations within islands will reduce or

prevent gene flow between populations, exacerbating the decline in diversity. In the long term, low-level assisted transfer of individuals between populations might be required to reduce the impact of inbreeding and maintain genetic diversity (e.g. Ewing *et al.* 2008).

The decline on Montserrat was potentially preventable, with likely routes of Bd introduction and mechanisms to prevent that occurrence being identified to the government. Preventing the arrival of a pathogen into a susceptible population represents the best way of preventing infectious disease driven declines (Wobeser 2002). However, political and financial barriers prevented the implementation of these measures, in part due to the existence of trade deals, and concerns about the impact on the very small island economy. Much of the world's biodiversity is concentrated in developing countries (Myers *et al.* 2000) where there is often limited capacity to prevent the introduction of threats such as EIDs. If we are to achieve any of the global biodiversity targets set in Aichi (www.cbd.int), including preventing the loss of known threatened species, mechanisms are required to facilitate the transfer of resources and capacity from richer countries to locations in which it will have the greatest impact. Since the description of Bd, *Batrachochytrium salamandivorans* has driven declines in European salamanders (Martel *et al.* 2013). It is very likely that more fungal pathogens, including further chytrid species, will emerge as threats to biodiversity as humans continue to contribute to climate change, widening the potential geographic range of fungal pathogens, and transport such pathogens to novel locations (Fisher *et al.* 2012).

The decline of the mountain chicken, a top predator on both islands, likely had important ecological and environmental consequences. For example, the import of pesticides to Dominica increased following the decline of the mountain chicken (R. Thomas, pers. comm.). Mountain chickens diet includes many common crop pests (Brooks 1982) and so a direct relationship between the decline and increased pesticide use is not unexpected, although it would be difficult to prove a causal link. Similar costs to agriculture have been posited as a result of white-nose syndrome driven bat declines in the U.S.A. (Boyles *et al.* 2011). The increased use of pesticides in place of natural control by pest predators not only has direct costs for farmers and consumers, but may also cause a reduction in biodiversity around farmland as non-target species are impacted (e.g. Geiger *et al.* 2010). Together with the loss of an iconic species, this should provide further justification for increasing the speed at which in-situ mitigation for EIDs, such as chytridiomycosis, are developed.

7.2 In-situ anti-fungal treatment

The speed of decline recorded in Chapter 2 highlights the importance of developing emergency mitigation measures to prevent imminent species loss in the face of epidemic chytridiomycosis. Woodhams *et al.* (2011) argue that the focus of conservation management for chytridiomycosis threatened species should be on the development of measures to facilitate long-term persistence rather than short-term strategies. However, if species extinctions are to be prevented in the current absence of such measures, parallel research into effective short-term measures is required (Scheele, Hunter, *et al.* 2014). In Chapter 3, the anti-fungal drug, itraconazole, which has been used successfully in captive and lab settings (Brannelly, Skerratt and Berger 2015; Tamukai *et al.* 2011; Forzán, Gunn and Scott 2008; Nichols and Lamirande 2001), was used to treat mountain chickens in the field during a chytridiomycosis epidemic. This was the first reported in-situ application of an anti-fungal treatment for chytridiomycosis. Logistical difficulties of recapture and correctly dosing animals have previously prevented the use of in-situ treatment (A. Cunningham, pers. comm.). The research presented in Chapter 3 shows that despite relatively low recapture rates (on average once per week), itraconazole treatment increased survival, clearance of Bd infection and reduced short-term Bd reinfection risk in the mountain chicken. Should epidemic chytridiomycosis emerge in countries with mega-diverse amphibian assemblages such as Sri-Lanka and Madagascar (Bielby *et al.* 2008), the number of species at risk of extinction would be too great for captive breeding alone. Emergency measures such as individual anti-fungal treatment might buy time in which further conservation strategies could be implemented. It could also be used to minimise mortality during seasonally high Bd risk periods which occur in Montserrat (Chapter 3) and elsewhere (e.g. Ruggeri *et al.* 2015; Longo, Burrowes and Joglar 2010; Berger *et al.* 2004).

The deterministic presentation of the multi-state CMR analysis results in Chapter 3 suggested that itraconazole treatment did not facilitate the long-term persistence of the mountain chicken population. However, this might not be the case. Recruitment was excluded from the population models as no breeding has been recorded on Montserrat since the emergence of chytridiomycosis. Under this scenario, a modelled population will always tend to extinction where survival is not 100%. The treatment was only employed during the first 15 weeks of the epidemic, when disease pressure was likely highest. The population models were then used to extend predictions from this period indefinitely. As the chytridiomycosis outbreak progressed, causing a decline in population size and contact rates, Bd infection rates might have dropped meaning treatment could have facilitated long-term population persistence. Whilst this treatment might not be practicable for cryptic species, especially those in which Bd infection

reduces capture rate (Murray *et al.* 2009; Jennelle *et al.* 2007), it does represent an important tool for the in-situ management of chytridiomycosis considering the current paucity of measures available (Scheele, Hunter, *et al.* 2014).

The analyses in this chapter might have been improved by the inclusion of a second Bd infection state (i.e. low and high infection load) in the mark-recapture models. Bd pathogenicity is dependent on the infection burden on the host (Vredenburg *et al.* 2010; Voyles *et al.* 2009). A two-state infection intensity model would, therefore, have provided insight into the progression of Bd infection within individuals, and variation in recovery from different stages of infection. However, skin swabbing has been shown to be an imperfect measure of true Bd infection load (Clare *et al.* 2016). The presence of Bd zoospores and DNA on the skin at the time of swabbing is dependent on the stage of the lifecycle of the majority of the Bd infecting the frog (i.e. embedded within the epidermis or zoosporangia discharging zoospores onto the skin) (Berger, Hyatt, *et al.* 2005). As it incorporates state uncertainty, multi-event modelling in programme E-surge (Choquet, Rouan and Pradel 2009) is a promising framework for mark-recapture modelling in chytridiomycosis studies, as it accounts for imperfect swab measurements. Furthermore, Bd infection burdens are often over-dispersed (Grogan *et al.* 2016), resulting in an intrinsically small amount of data available from hosts with high infection burdens. Multi-state mark-recapture models are dependent on large amounts of data meaning the inclusion of states with limited captures, would result in poorly fitting models with low confidence in the parameter estimates. For mountain chickens, in which recovery from Bd infection appears to be infrequent (Chapters 3 and 5), utilising multiple states may not provide additional information, and the priority should be preventing, or successfully treating, Bd infection.

7.3 Optimising reintroductions of the mountain chicken

The Amphibian Conservation Action Plan (Gascon *et al.* 2007) advocates the establishment of captive safety net populations of species likely to be driven to extinction in the wild by chytridiomycosis. Over 500 species are known to have been infected with Bd (Olson *et al.* 2013), and over 200 have declined or become extinct (Skerratt *et al.* 2007). Sustainable long-term conservation of species which are extinct in the wild and for which successful captive breeding programmes have been established, requires successful reintroduction into the wild. Thus far, reintroductions have not been successful when Bd persists in the environment (e.g. Stockwell *et al.* 2008). This is problematical as there are no realistic exit strategies for captive breeding programmes of Bd threatened amphibians and so novel strategies are required (Harding, Griffiths and Pavajeau 2015).

The experimental reintroductions of the mountain chicken onto Montserrat documented in Chapter 5 were not successful in establishing a population at the release site. This was due, in part, to the continued impact of chytridiomycosis (during the dry season) and dispersal away from the release site. If long-term sustainable conservation of mountain chickens on Montserrat is to be achieved, strategies to improve these reintroductions are required. These might include a combination of optimising timing releases to coincide with low Bd infection prevalence in sympatric reservoir species (Fentzloff 1984; Chapters 4 and 5) with environmental manipulation (Scheele, Hunter, *et al.* 2014) and targeted individual in-situ treatment (Chapter 3 and 6).

The wet season release (REL 4) which took place during a period of time with low Bd infection prevalence in tree frogs was more successful than in the dry, with no fatal cases of chytridiomycosis recorded, low Bd infection rates and only one incidental death (Chapter 5). This approach shows promise, but further modifications are required. These include measures to limit dispersal which appeared to contribute apparent mortality across the releases. Measures are also required to limit the impact of Bd during the cool, dry season in which Bd infection prevalence and load increases in tree frogs (Chapter 4 and 5). The level of long-term survival of reintroduced mountain chickens in REL 4 is unclear. Few individuals were recorded as Bd-positive prior to being lost and survival in uninfected individuals was high. It is likely some became infected as the infection prevalence in reservoir species increased, but others may have survived longer term.

Fencing the release site would eliminate dispersal and provide improved data on long-term survival which is essential for measuring reintroduction success (Seddon, Armstrong and Maloney 2007). However, the containment of a number of mountain chickens at artificially high densities might result in an increased rate of Bd infection transmission if it emerged within the reintroduced population (IUCN/SSC 2013). A fenced enclosure would allow the application of intensive management of both the reintroduced population and the enclosed environment akin to that employed in soft releases of birds (e.g. Jones and Merton, 2012). This would facilitate the testing of explicit hypotheses about the effect of different management strategies on survival of reintroduced mountain chickens. Robust recommendations for improving reintroductions of amphibians threatened by chytridiomycosis could then be made through further experimentation, or in an adaptive management framework (Canessa *et al.* 2016; IUCN/SSC 2013; Armstrong, Castro and Griffiths 2007).

Environmental manipulation is one measure which warrants testing in such a framework. Targeted environmental manipulation has been utilised in the management of many wildlife diseases both to remove sources of infection and reduce host densities in areas of high infection risk. For example, Wobeser (2007) reports the draining of standing water acting as a source of avian cholera (caused by infection with *Pasteurella multocida*) in the eider duck (*Somateria mollissima dresseri*). Water sources were also modified in California to reduce the densities of deer near muddy pools which were sources of necrobacillosis (Wobeser 2002). The targeting of free-living or at least persistent, environmental reservoirs of Bd is an important part of mitigation (Mitchell *et al.* 2008). Agricultural fungicides are the most common form of environmental control for fungal pathogens (Woodhams *et al.* 2011), however the potential effects on non-target species and the wider environment are catastrophic.

Water sources were spatially important predictors of mountain chicken Bd-infection during the reintroductions (Chapter 6). Environmental manipulation might, therefore, include the heating of water bodies at the release site, especially during the cooler, drier periods when Bd infection prevalence in tree frogs is high (Chapter 4) and mountain chickens aggregated and became Bd-infected near, pools (Chapter 6). Amphibians found in naturally hotter pools have reduced Bd infection prevalence and loads compared with those in cooler pools in assemblages in Arizona, USA (Forrest and Schlaepfer 2011) and in *Litoria raniformis* populations in Australia (Heard *et al.* 2013). Measures that reduce viable Bd zoospore count in pools would reduce the likelihood of target species exposure to Bd, reducing the infection rate. However, the recovery rate of mountain chickens from Bd infection in the reintroductions was low (Chapter 5), suggesting that once Bd infected, most individuals developed chytridiomycosis and died. Mitigation measures implemented during an intensively managed reintroduction therefore need to include treatment to aid clearance of Bd infection. This does not exclude the use of heated pools. As well as reducing the number of viable Bd zoospores, heated pools could also act as a treatment for Bd infection. Exposure to water or air of between 30 - 37 ° C for a minimum of 16 hours (in one or multiple sessions) is an effective treatment for Bd infection in captivity (Chatfield and Richards-Zawacki 2011; Geiger *et al.* 2011; Woodhams, Alford and Marantelli 2003). Pools occurred in areas of high mountain chicken home range overlap, meaning the population coverage of this treatment would be high. It is, however, unclear whether mountain chickens would respond to heat treatment and this should be trialled in advance.

The tree canopy cover at the release site could also be reduced to increase sun exposure and evaporation of water from substrate, decreasing the suitability of the release site for Bd

(Hossack *et al.* 2013; Becker *et al.* 2012; Forrest and Schlaepfer 2011). Some species of amphibian bask in sunlight or on hot rocks to moderate body temperatures (mountain chickens have been observed sitting in sunlight: Appendix M) which might reduce Bd infection load (Scheele, Hunter, *et al.* 2014; Puschendorf *et al.* 2011). Decreasing canopy cover would increase basking sites and contact with hot surfaces (e.g. rocks) which might be especially important as Bd often infects the ventral skin of amphibians (Berger, Hyatt, *et al.* 2005; Longcore, Pessier and Nichols 1999; Pessier *et al.* 1999). Should heated pools and canopy cover reduction have the desired effect of increasing mountain chicken persistence in the face of endemic chytridiomycosis, these measures could be employed across the species range. Creating a connected network of Bd refuge sites might facilitate long-term persistence of remnant or reintroduced populations (Heard *et al.* 2015; Daskin, Alford and Puschendorf 2011).

Canopy cover and heated pools might not be effective if mountain chickens choose to avoid them, and so choice experiments should be carried out during the intensive management phase. Canopy cover reduction could also have unintended consequences for non-target species and such changes should be monitored during any trial before wide scale implementation.

Mountain chicken habitat usage outside the centre of the ghauts was poorly documented during the reintroductions due to the low range of the radio-implants (S-L. Adams, pers. comm.). Radio-tags attached to belts have been successfully utilised for other amphibian species (e.g. Muths 2003) which would have increased the detectable range, however a brief trial on mountain chickens in captivity resulted in reddening of the skin and was terminated (B. Tapley, pers. comm.). Associations between reintroduced mountain chickens and important habitat features such as pools (Chapter 6), could be quantified using remote PIT tag detectors which have been used with salmon (e.g. Roussel, Haro and Cunjak 2000). The equipment used by Roussel, Haro and Cunjak (2000) facilitated detection up to a distance of 1 m. In a fenced experimental release enclosure, a ring detector could be placed around each treated pool in order to quantify the treatment rate of each PIT tagged mountain chicken.

For reintroduced populations to persist long-term, they must breed in the wild. During the reintroductions described in Chapter 5, no breeding was recorded despite the exhibition of breeding behaviours such as calling and shared use of burrows. The impact of Bd infection on mountain chicken tadpoles is unknown. In other species, Bd colonises the newly keratinised skin of a frog as it metamorphoses from tadpole to adult (Marantelli *et al.* 2004; Rachowicz

and Vredenburg 2004). The high level of maternal care in mountain chickens includes the provision of infertile eggs for food (Gibson and Buley 2004). In order to feed on the eggs, the tadpoles climb on the back of the mother providing sufficient contact for the transmission of Bd. It is unclear, therefore, whether breeding and recruitment would be successful in a reintroduced mountain chicken population. However, the mountain chicken breeding season occurs in the warmer, wetter season and the low Bd risk during this period might reduce any impacts on breeding. Whilst breeding has been recorded in the remnant population on Dominica, these individuals may have evolved resistance or tolerance to Bd infection.

7.4 Sympatric Bd reservoir species

Identifying periods when Bd infection prevalence and loads in sympatric reservoir species are low can help to optimise the timing of reintroductions of susceptible species (Chapter 4 and 5). There may also be long-term trends in Bd infection prevalence and load in sympatric reservoir species which, if identified, could allow reintroductions to be delayed until disease risk was sufficiently low (Griffiths and Pavajeau 2008). In Chapter 4, the uneven timing between tree frog surveys meant time-series analysis could not be utilised to separate seasonality from trends in Bd infection data. Now that the environmental drivers of changes in Bd infection prevalence and load in tree frogs have been identified, the focus of this monitoring should shift to identifying long-term trends. In order to reduce the resource costs of such a programme (each swab can cost £10 in addition to the time required to process them), the frequency of monitoring could be reduced, and monitoring continued long term, whilst ensuring even time intervals to permit long-term trends to be identified.

Site level eradication of tree frogs might increase the success of future reintroductions of mountain chickens. Whilst there is no direct evidence that tree frogs are responsible for transmission of Bd into the reintroduced mountain chicken population, they were seen in contact during the reintroductions (Chapter 4, Figure 4.5) providing a mechanism for infection. Citric acid and lime have been used to control the invasive tree frogs *Eleutherodactylus coqui* on Hawaii (Beachy *et al.* 2011; Tuttle, Beard and Al-Chokhachy 2008) and caffeine has also been shown to be effective (Pitt and Doratt 2006). If these treatments were carried out sufficiently far in advance of any subsequent mountain chicken reintroduction, there should be no adverse effects on that population. There are no other native amphibians which could be coincidentally impacted, and prior surveys to ensure the absence of other threatened species could reduce the non-target risks. It would, however, be extremely difficult to prevent the reinvasion of the site by tree frogs, which are very small bodied, arboreal and found in large numbers on Montserrat.

Cane toads are a potentially important reservoir of Bd on Montserrat, however the sample size collected in this study was small and more research is required to establish their true importance (Chapter 4). The life history of the cane toad is closer to a mountain chicken than that of tree frogs and so cane toads may be a more important Bd reservoir. Further cane toad Bd infection surveys should be undertaken in the cool, dry season in line with the high infection prevalence detected in tree frogs in order to determine their potential role in transmission of Bd to mountain chickens (Chapter 4). Cane toads could be more easily eradicated from the release site, being more conspicuous and larger bodied than tree frogs, and could be excluded relatively cheaply using fencing (e.g. Florance *et al.* 2011; Wingate 2010).

Whilst eradication of Bd reservoir hosts might be effective in reducing disease pressure on a mountain chicken population within a fenced release enclosure, it would be difficult across the entire range of the species. The difficulty in effectively managing disease with a widespread and abundant reservoir host is well documented, for example European badgers (*Meles meles*) are reservoirs for bovine tuberculosis which impacts cattle (Wilson, Carter and Delahay 2011). Under this scenario, mitigation measures are most successful when applied to both the reservoir hosts and target species which should be considered in chytridiomycosis systems.

7.5 Alternative reintroduction strategies for the mountain chicken

The identification of Bd-free refuges inside or outside of the species historic range into which threatened species could be reintroduced (Scheele, Hunter, *et al.* 2014) might allow long-term persistence of threatened species in the wild. These refuges are, however, likely becoming fewer as Bd continues to spread, and there is a danger that Bd might reach such refuges in the future. This is a realistic prospect, especially as human transport would be required for the reintroduction (e.g. Walker *et al.* 2008). On Montserrat, the eruption of the Soufriere Hills volcano led to a large area in the South of the island, called Roches Estate, being separated from the North of the island by vast strips denuded of vegetation by pyroclastic flows. These strips of ash and rock are sufficiently large that amphibians are unlikely to cross them, although cane toads may be capable (e.g. Phillips *et al.* 2007). As this took place prior to the first detection of Bd on Montserrat, and is no longer accessible to humans without a helicopter or difficult boat landing, it may be free of Bd. Tree frogs and small numbers of cane toads persist in Roches which have been surveyed for Bd on several occasions throughout the reintroduction phase of this study. As yet, no Bd has been detected on skin swabs taken from this site. However, the reliance of these surveys on helicopter access and the weather

conditions required for flight, means these surveys have taken place in the warmer, wetter season when Bd prevalence elsewhere is low (Chapter 4). Should Roches be identified as a Bd refuge, it could be used for reintroductions of captive bred mountain chickens. Before this can be considered at least one Bd-free tree frog survey during the cool, dry season is needed. Obtaining sufficient statistical power to detect Bd (DiGiacomo and Koepsell 1986) may be difficult as surveys are often undertaken in the daytime (for helicopter safety) when tree frogs vocalise less and are less active.

Selection for resistance or tolerance to infection prior to release has been proposed as another method for increasing reintroduction success in the face of infectious disease such as chytridiomycosis (Venesky *et al.* 2012; Woodhams *et al.* 2011). Woodhams *et al.* (2011) suggest that selection in captivity should focus on phenotypes known to be good predictors of resistance to Bd, such as the quantity and diversity of antimicrobial peptides on the skin (Tennessen *et al.* 2009). As Woodhams *et al.* (2011) admit, however, this would require both within-population variability and heritability of the phenotype, which might vary between species. Venesky *et al.* (2012), argue that it would be favourable to select for tolerance over resistance. Resistance, by definition, involves a reduction in pathogen fitness within a host. This, Venesky *et al.* (2012) argue, would drive evolution of the pathogen making the mechanism of resistance rapidly redundant. Pathogen lifecycles are much shorter than amphibians, offering greater opportunity to win such an evolutionary arms race. Tolerance traits might include, for example, behavioural driven exposure to warm microclimates resulting in repeated clearance of Bd infection (Venesky *et al.* 2012; Daskin, Alford and Puschendorf 2011; Puschendorf *et al.* 2011). Time to death experiments, post-Bd exposure, would provide insight into those individuals which exhibit such traits. This might be possible during an intensively monitored reintroduction in an enclosure as described above. Again, heritability of these traits is required for successful selection within a population.

Alternatively, genetic analyses might facilitate the identification of genotypes in the remnant wild population which have increased the ability of those individuals to persist alongside Bd (e.g. Savage and Zamudio 2016; May, Zeisset and Beebee 2011). Should similar genotypes be identified in the captive population, these individuals could be selectively bred to produce future release cohorts which might persist in the presence of Bd. Should any such genotypes identified in the wild not also be found in the current captive population, it might be necessary to either directly translocate those wild individuals into empty sites, or, use the wild individuals as the basis for a captive population (e.g. J. Bosch unpublished in Woodhams *et al.* (2011)).

There have been a small number of cases of individuals in the remnant mountain chicken populations of both islands clearing Bd-infection (Author's unpublished data). Whilst these animals could be extracted and used as founders for a potentially disease-tolerant / -resistant captive population, the wild populations are currently too small to consider such an action.

Wildlife reintroductions in the face of persistent infectious disease, which had previously driven declines, have most famously been overcome through pre-release immunisation. The black footed ferret (*Mustela nigripes*), which was driven to near extinction by canine distemper virus contracted from reservoir species including dogs (Thorne and Williams 1988), has been successfully reintroduced after vaccine mediated immunisation against the disease (Wimsatt *et al.* 2006). Trials of immunisation against chytridiomycosis through exposure to live and dead Bd have had variable success (McMahon *et al.* 2014; Cashins *et al.* 2013; Ramsey *et al.* 2010; Stice and Briggs 2010). Recent findings including the reduced pathogenicity of Bd following multiple in-vitro passages (Langhammer *et al.* 2013), provide promising developments for this strategy. Large numbers of individuals could be exposed to Bd that had been processed in this way with minimal risk, increasing the likelihood of success.

Bio-augmentation of amphibian skin with commensal bacteria which inhibit the growth of Bd have also been posited as a potential tool to increase resistance to chytridiomycosis (e.g. Bletz *et al.* 2013). Trials with probiotics have shown variable success (Muletz *et al.* 2012; Becker *et al.* 2011) and in the wild commensal bacteria may face competition from naturally occurring species. Whilst these longer term solutions to chytridiomycosis are developed, short term measures to protect amphibians populations from imminent decline are required (Scheele, Hunter, *et al.* 2014).

7.6 Management to perpetuity

The measures suggested in this thesis to help recover this species in the continued presence of Bd, require a great deal of investment. However, even in the absence of Bd it is possible that mountain chicken reintroductions might be difficult due to changing environmental conditions on Montserrat. The frequency of climatic events such as the El Niño Southern Oscillation, of which one of the strongest ever was recorded in 2015 / 2016 (Levine and McPhaden 2016), and which result in droughts in the Caribbean (Enfield and Alfaro 1999), is expected to increase (Timmerman *et al.* 1999). Alongside climate change, this is expected to result in an unprecedented level of aridity in the Lesser Antilles over the next century (Karnauskas, Donnelly and Anchukaitis 2016). This increased drying means environmental manipulation will likely be required even before considering measures to mitigate the impact of

chytridiomycosis. Measures to reduce the impact of extreme climatic events, such as those predicted to increase through climate change, include the installation of climatic refuges and manipulation of water levels at sites important for key parts of the amphibian lifecycle (Shoo *et al.* 2011).

The increasing list of threats which need to be mitigated in order for chytridiomycosis threatened amphibians to be conserved is alarming. Alongside climate change, the mountain chicken also faces threats from volcanic eruptions, which cannot be managed on such small islands, and hunting (Fa *et al.* 2010), which is now illegal on both islands (although anecdotal evidence suggests it does continue at a low rate on Dominica; Author's observations). When conserving species, conservation practitioners are often forced to maximise the 'bang for their buck' to ensure the limited money available confers the greatest benefit to global biodiversity (e.g. MacKenzie 2009). Expending a huge amount of resources conserving a single species such as the mountain chicken, albeit a culturally and ecologically important one, might not be seen as doing so.

Authors including Cafaro and Primack (2014), however, argue that there is a moral obligation to conserve species facing anthropogenic threats (Cafaro and Primack 2014). This includes Bd which was likely, at least in part, spread through human-mediated transport (Rosenblum *et al.* 2013; Weldon *et al.* 2004). The mountain chicken is also an incredibly important model for chytridiomycosis driven species declines and for the trial of mitigation measures with which to tackle this threat. The importance of model organisms in biology has long been recognised and the majority of journal articles published in conservation journals with the highest impact factors are single species focussed with wider implications inferred (Griffiths and Dos Santos 2012). The resources used on mountain chickens conservation should therefore be seen as an important contribution to the testing of mitigation measures for chytridiomycosis which are applicable to many of the over 200 species which have declined as a result of this disease (Skerratt *et al.* 2007).

The mountain chicken has been managed by man throughout its history. Amerindian's are believed to have transported it from mainland South America to the Lesser Antilles (Breuil 2009; Chapter 2), and anecdotal evidence of transport from the West to the East coast of Dominica as a source of food. Managing the species to perpetuity, therefore, does not seem an unreasonable prospect.

7.7 Concluding remarks

Whilst the immediate response to precipitous species declines and irreversible threats such as EIDs is often necessarily the creation of captive safety net populations, a focus on the conservation of wild populations must be maintained. Remnant populations which have survived disease epidemics represent an incredibly important source of information on mechanisms of persistence (Woodhams *et al.* 2011).

Where no viable wild populations of a threatened species remain, and a captive breeding programme has been established, sustainable long-term conservation is reliant on successful reintroduction of captive bred animals. Rapid genetic adaptation to captivity (Frankham 2008) and the emergence of further threats across the species range can make reintroduction increasingly challenging (IUCN/SSC 2013). Furthermore, not all species are suited to captivity so that maintaining a viable population in even the short term is difficult (Tapley et al. 2015). For example, mountain chickens have large space requirements, a voracious appetite and a complex breeding system making them difficult and expensive to maintain in captivity (Tapley et al. 2015). For these reasons, reintroduction protocols must be developed immediately so that these species can be sustainably saved from extinction.

The removal of threats has long been seen as an essential pre-requisite to successful reintroductions (Caughley 1994). However, the emergence of threats which cannot be easily mitigated, such as EIDs and climate change, mean this is no longer possible and novel reintroduction strategies are required. The management of EIDs in reintroduced populations requires a two-pronged approach. Firstly, ex-situ research is required to develop new technologies such as immunisation or probiotics which can be applied to captive populations to prepare them for reintroduction where pathogens persist. Secondly, an experimental approach should be applied to optimise the timing and conditions of reintroductions to minimise the impact of the EID. This might include optimising the timing of release to coincide with periods of low disease risk, or modifying the environment to reduce pathogen prevalence in both environmental and host reservoirs.

References

Adams, S.-L. (2010). *Centre Hills Ghaut Assessment for the Release Site of Mountain Chickens (Leptodactylus fallax)*. Jersey, Channel Islands: Durrell Wildlife Conservation Trust.

Aguirre, A.A. and Tabor, G.M. (2008). Global factors driving emerging infectious diseases. *Annals of the New York Academy of Sciences* **1149**:1–3.

Albert, E.M. *et al.* (2014). Genetic management of an amphibian population after a chytridiomycosis outbreak. *Conservation Genetics* **16**:103–111.

Allender, M.C. *et al.* (2015). The natural history, ecology, and epidemiology of *Ophidiomyces ophiodiicola* and its potential impact on free-ranging snake populations. *Fungal Ecology* **17**:187–196.

Allentoft, M.E. and O'Brien, J. (2010). Global amphibian declines, loss of genetic diversity and fitness: a review. *Diversity* **2**:47–71.

Altizer, S., Bartel, R. and Han, B.A. (2011). Animal migration and infectious disease risk. *Science* **331**:296–302.

Anderson, R.M. and May, R.M. (1979). Population biology of infectious diseases: Part I. *Nature* **280**:316–367.

Armstrong, D.P., Castro, I. and Griffiths, R. (2007). Using adaptive management to determine requirements of re-introduced populations: the case of the New Zealand hihi. *Journal of Applied Ecology* **44**:953–962.

Armstrong, D.P. and Seddon, P.J. (2008). Directions in reintroduction biology. *Trends in Ecology & Evolution* **23**:20–25.

Bai, C. *et al.* (2012). Global and endemic Asian lineages of the emerging pathogenic fungus *Batrachochytrium dendrobatidis* widely infect amphibians in China. *Diversity and Distributions* **18**:307–318.

Baitchman, E.J. and Pessier, A.P. (2013). Pathogenesis, diagnosis, and treatment of amphibian chytridiomycosis. *Veterinary Clinics of North America: Exotic Animal Practice* **16**:669–685.

Balmford, A., Mace, G.M. and Leader-Williams, N. (1996). Designing the ark: setting priorities for captive breeding. *Conservation Biology* **10**:719–727.

Barbour, A.B., Ponciano, J.M. and Lorenzen, K. (2013). Apparent survival estimation from continuous mark–recapture/resighting data. *Methods in Ecology and Evolution* **4**:846–853.

Barrios-Garcia, M.N. and Ballari, S.A. (2012). Impact of wild boar (*Sus scrofa*) in its introduced and native range: a review. *Biological Invasions* **14**:2283–2300.

Bd-Maps.net (2016). [Online]. Available at: http://www.bd-maps.net/ [Accessed: 1 March 2016].

Beachy, J.R. *et al.* (2011). Successful control of an incipient invasive amphibian: *Eleutherodactylus coqui* on O'ahu, Hawai'i. In: Veitch, C. R., Clout, M. N. and Towns, D.

R. eds. *Island Invasives: Eradication and Management*. Gland, Switzerland: IUCN, pp. 140–147.

Becker, C.G. *et al.* (2012). Disease risk in temperate amphibian populations is higher at closed-canopy sites. *PLoS ONE* **7**:e48205.

Becker, M.H. *et al.* (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proceedings of the Royal Society of London B: Biological Sciences* **282**:20142881.

Becker, M.H. *et al.* (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *EcoHealth* **8**:501–506.

Becker, M.H. and Harris, R.N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS ONE* **5**:e10957.

Beebee, T. *et al.* (2012). 40 years of natterjack toad conservation in Europe. *FrogLog* **101**:40–44.

Beebee, T. (1996). *Ecology and Conservation of Amphibians*. Springer Science & Business Media.

Begon, M. *et al.* (2002). A clarification of transmission terms in host-microparasite models: numbers, densities and areas. *Epidemiology and Infection* **129**:147–153.

Bellard, C., Cassey, P. and Blackburn, T.M. (2016). Alien species as a driver of recent extinctions. *Biology Letters* **12**:20150623.

Berger, L. *et al.* (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences* **95**:9031–9036.

Berger, L. *et al.* (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian veterinary journal* **82**:434–439.

Berger, L., Hyatt, A.D., *et al.* (2005). Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **68**:51–63.

Berger, L., Marantelli, G., *et al.* (2005). Virulence of the amphibian chytrid fungus *Batrachochytium dendrobatidis* varies with the strain. *Diseases of aquatic organisms* **68**:47–50.

Berger, L. and Skerratt, L.F. (2012). *Disease Strategy for Chytridiomycosis (Infection with Batrachochytrium dendrobatidis). Version 1.* Canberra, Australia: Department of Sustainability, Environment, Water, Populations and Communities, Public Affairs.

Bielby, J. *et al.* (2015). Host species vary in infection probability, sub-lethal effects, and costs of immune response when exposed to an amphibian parasite. *Scientific Reports* **5**:10828.

Bielby, J. *et al.* (2008). Predicting susceptibility to future declines in the world's frogs. *Conservation Letters* **1**:82–90.

van de Bildt, M.W.G. *et al.* (2002). Distemper outbreak and its effect on African wild dog conservation. *Emerging Infectious Diseases* **8**:212–213.

Blanchong, J.A. *et al.* (2006). Persistence of *Pasteurella asteurella multicoda* in wetlands following avian cholera outbreaks. *Journal of Wildlife Diseases* **42**:33–39.

Blaustein, A.R. *et al.* (2003). Ultraviolet radiation, toxic chemicals and amphibian population declines. *Diversity and Distributions* **9**:123–140.

Blaustein, A.R. and Wake, D.B. (1995). The puzzle of declining amphibian populations. *Scientific American* **272**:52–57.

Blehert, D.S. *et al.* (2009). Bat white-nose syndrome: an emerging fungal pathogen? *Science* **323**:227–227.

Bletz, M.C. *et al.* (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters* **16**:807–820.

Bletz, M.C. *et al.* (2015). Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild amphibian communities in Madagascar. *Scientific Reports* **5**:8633.

Blomquist, S.M., Zydlewski, J.D. and Hunter, Jr., M.L. (2008). Efficacy of PIT tags for tracking the terrestrial anurans *Rana pipiens* and *rana sylvatica*. *Herpetological Review* **39**:174–179.

Bodinof, C.M. *et al.* (2012). Survival and body condition of captive-reared juvenile ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) following translocation to the wild. *Copeia* **2012**:150–159.

Bosch, J. *et al.* (2015). Successful elimination of a lethal wildlife infectious disease in nature. *Biology Letters* **11**:20150874.

Boyle, D.G. *et al.* (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of aquatic organisms* **60**:141–148.

Boyles, J.G. *et al.* (2011). Economic importance of bats in agriculture. *Science* **332**:41–42.

Brannelly, L.A. *et al.* (2016). Chytrid infection and post-release fitness in the reintroduction of an endangered alpine tree frog. *Animal Conservation* **19**:153–162.

Brannelly, L.A. (2014). Reduced itraconazole concentration and durations are successful in treating *Batrachochytrium dendrobatidis* infection in amphibians. *Journal of Visualized Experiments: JoVE* **85**:doi: 10.3791/51166.

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

Brannelly, L.A., Skerratt, L.F. and Berger, L. (2015). Treatment trial of clinically ill corroboree frogs with chytridiomycosis with two triazole antifungals and electrolyte therapy. *Veterinary Research Communications* **39**:179–187.

Brem, F.M.R. and Lips, K.R. (2008). *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. *Diseases of Aquatic Organisms* **81**:189–202.

Breuil, M. (2009). The terrestrial herpetofauna of Martinique: past, present, future. *Applied Herpetology* **6**:123–149.

Briggs, C.J. *et al.* (2005). Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology* **86**:3149–3159.

Briggs, C.J., Knapp, R.A. and Vredenburg, V.T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences* **107**:9695–9700.

Brooks, G.R. jr. (1982). An analysis of prey consumed by the anuran, *Leptodactylus fallax*, from Dominica, West Indies. *Biotropica* **14**:301–309.

Brühl, C.A. *et al.* (2013). Terrestrial pesticide exposure of amphibians: an underestimated cause of global decline? *Scientific Reports* **3**:1135 doi:10.1038/srep01135.

Brunner, J.L. *et al.* (2004). Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. *Ecology* **85**:560–566.

Buley, K.R. and Garcia, G. (1997). Recovery programme for the Mallorcan midwife toad. *Dodo* **33**:80–90.

Burnham, K.P. (1993). A theory for combined analysis of ring recovery and recapture data. In: Lebreton, J.-D. and North, P. eds. *Marked Individuals in Bird Population Studies*. Basel: Birkhauser-Verlag, pp. 199–213.

Burnham, K.P. and Anderson, D.R. (2002). *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. New York: Springer-Verlag.

Burrowes, P.A., Joglar, R.L. and Green, D.E. (2004). Potential causes for amphibian declines in Puerto Rico. *Herpetologica* **60**:141–154.

Burt, W.H. (1943). Territoriality and home range concepts as applied to mammals. *Journal of Mammalogy* **24**:346–352.

Cafaro, P. and Primack, R. (2014). Species extinction is a great moral wrong. *Biological Conservation* **170**:1–2.

Caillaud, D. *et al.* (2006). Gorilla susceptibility to Ebola virus: the cost of sociality. *Current biology: CB* **16**:R489-491.

Calenge, C. (2006). The package adehabitat for the R software: a tool for the analysis of space and habitat use by animals. *Ecological Modelling* **197**:516–519.

Campbell, C.R. *et al.* (2012). Frog skin epithelium: electrolyte transport and chytridiomycosis. *The International Journal of Biochemistry & Cell Biology* **44**:431–434.

Canessa, S. *et al.* (2016). Adaptive management for improving species conservation across the captive-wild spectrum. *Biological Conservation* **199**:123–131.

Carey, C. and Alexander, M.A. (2003). Climate change and amphibian declines: is there a link? *Diversity and Distributions* **9**:111–121.

Carter, R., Mendis, K.N. and Roberts, D. (2000). Spatial targeting of interventions against malaria. *Bulletin of the World Health Organization* **78**:1401–1411.

Cashins, S.D. *et al.* (2013). Prior infection does not improve survival against the amphibian disease chytridiomycosis. *PLoS ONE* **8**:e56747.

Caughley, G. (1994). Directions in conservation biology. *Journal of Animal Ecology* **63**:215–244.

Ceballos, G. *et al.* (2015). Accelerated modern human–induced species losses: entering the sixth mass extinction. *Science Advances* **1**:e1400253.

Ceballos, G. and Ehrlich, P.R. (2002). Mammal population losses and the extinction crisis. *Science (New York, N.Y.)* **296**:904–907.

Chatfield, M.W.H. and Richards-Zawacki, C.L. (2011). Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Diseases of Aquatic Organisms* **94**:235–238.

Chatfield, M.W.H. and Richards-Zawacki, C.L. (2011). Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Diseases of aquatic organisms* **94**:235–238.

Chauvenet, A.L.M. *et al.* (2012). Does supplemental feeding affect the viability of translocated populations? The example of the hihi. *Animal Conservation* **15**:337–350.

Cheng, T.L. *et al.* (2011). Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences* **108**:9502–9507.

Choquet, R. *et al.* (2009). U-CARE: Utilities for performing goodness of fit tests and manipulating CApture–REcapture data. *Ecography* **32**:1071–1074.

Choquet, R., Rouan, L. and Pradel, R. (2009). Program E-Surge: A Software Application for Fitting Multievent Models. In: Thomson, D. L., Cooch, E. G. and Conroy, M. J. eds. *Modeling Demographic Processes In Marked Populations*. Springer US, pp. 845–865.

Ciofi, C. and Bruford, M.W. (1999). Genetic structure and gene flow among Komodo dragon populations inferred by microsatellite loci analysis. *Molecular Ecology* **8**:S17-30.

Clare, F. *et al.* (2016). Assessing the ability of swab data to determine the true burden of infection for the amphibian pathogen *Batrachochytrium dendrobatidis*. *EcoHealth*:1–8.

Coddington, E.J. and Cree, A. (1995). Effect of acute captivity stress on plasma concentrations of corticosterone and sex steroids in female whistling frogs, *Litoria ewingi*. *General and Comparative Endocrinology* **100**:33–38.

Collins, J.P. (2010). Amphibian decline and extinction: what we know and what we need to learn. *Diseases of Aquatic Organisms* **92**:93–99.

Collins, J.P. and Storfer, A. (2003). Global amphibian declines: sorting the hypotheses. *Diversity and Distributions* **9**:89–98.

Corlett, R.T. (2015). The Anthropocene concept in ecology and conservation. *Trends in Ecology & Evolution* **30**:36–41.

Cunningham, A.A. (2005). A walk on the wild side—emerging wildlife diseases. *BMJ* **331**:1214–1215.

Cunningham, A.A. (1996). Disease risks of wildlife translocations. *Conservation Biology* **10**:349–353.

Cunningham, A.A. *et al.* (2015). Emerging disease in UK amphibians. *Veterinary Record* **176**:468–468.

Cunningham, A., Danzak, P. and Rodriguez, J. (2003). Pathogen pollution: defining a parasitological threat to biodiversity conservation. *Journal of Parasitology* **89 (suppl.)**:S78-83.

Czechura, G.V. and Ingram, G. (1990). *Taudactylus diurnis* and the case of the disappearing frogs. *Memoirs of The Queensland Museum* **29**:361–365.

Dail, D. and Madsen, L. (2011). Models for estimating abundance from repeated counts of an open metapopulation. *Biometrics* **67**:577–587.

Daltry, J.C. (2002). *Mountain Chicken Monitoring Manual*. Fauna & Flora International, Cambridge, and the Forestry and Wildlife Division, Dominica.

Daltry, J.C. and Gray, G. (1999). Effects of volcanic activity on the endangered mountain chicken frog (*Leptodactylus fallax*). *Froglog* **32**:1–2.

Daskin, J.H. *et al.* (2014). Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians – implications for disease management and patterns of decline. *PLoS ONE* **9**:e100378.

Daskin, J.H., Alford, R.A. and Puschendorf, R. (2011). Short-term exposure to warm microhabitats could explain amphibian persistence with *Batrachochytrium dendrobatidis*. *PLoS ONE* **6**:e26215.

Daszak, P. *et al.* (1999). Emerging infectious diseases and amphibian population declines. *Emerging infectious diseases* **5**:735–748.

Daszak, P., Cunningham, A.A. and Hyatt, A.D. (2000). Emerging infectious diseases of wildlife: threats to biodiversity and human health. *Science* **287**:443–449.

Daszak, P., Cunningham, A.A. and Hyatt, A.D. (2003). Infectious disease and amphibian population declines. *Diversity and Distributions* **9**:141–150.

Dawson, J. *et al.* (2016). Assessing the global zoo response to the amphibian crisis through 20-year trends in captive collections. *Conservation Biology* **30**:82–91.

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

De Vos, J.M. *et al.* (2015). Estimating the normal background rate of species extinction. *Conservation Biology* **29**:452–462.

Dhondt, A.A. *et al.* (2007). Experimental evidence for transmission of *Mycoplasma gallisepticum* in house finches by fomites. *Avian Pathology* **36**:205–208.

Diamond, J., M, (1984). 'Normal' exintction of isolated populations. In: Nitecki, M. H. ed. *Extinctions.* Chicago: Chicago University Press, p. 191–246.

Diamond, J.M. (1989). Overview of Recent Extinctions. In: Western, D. and Pearl, M. eds. *Conservation for the 21st Century.* New York: Oxford University Press, pp. 37–41.

Di Rienzo, A. *et al.* (1994). Mutational processes of simple-sequence repeat loci in human populations. *Proceedings of the National Academy of Sciences* **91**:3166–3170.

Dieringer, D. and Schlötterer, C. (2003). MICROSATELLITE ANALYSER (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes* **3**:167–169.

DiGiacomo, R.F. and Koepsell, T.D. (1986). Sampling for detection of infection or disease in animal populations. *Journal of the American Veterinary Medical Association* **189**:22–23.

Dixo, M. *et al.* (2009). Habitat fragmentation reduces genetic diversity and connectivity among toad populations in the Brazilian Atlantic Coastal Forest. *Biological Conservation* **142**:1560–1569.

Doan, T.M. (2003). Which methods are most effective for surveying rain forest herpetofauna? *Journal of Herpetology* **37**:72–81.

Dodd, C.K.J. and Siegel, R.A. (1991). Relocation, repatriation, and translocation of amphibians and reptiles: are they conservation strategies that work? *Biological Conservation* **47**:336–350.

Doddington, B.J. *et al.* (2013). Context-dependent amphibian host population response to an invading pathogen. *Ecology* **94**:1795–1804.

Durrant, C.J. *et al.* (2014). *Using the Genie® II for Testing the Chytrid Fungus* Batrachytrium Dendrobatids. United Kingdom: Optigene Limited.

Enfield, D.B. and Alfaro, E.J. (1999). The dependence of Caribbean rainfall on the interaction of the Tropical Atlantic and Pacific Oceans. *Journal of Climate* **12**:2093–2103.

Ewen, J.G., Soorae, P.S. and Canessa, S. (2014). Reintroduction objectives, decisions and outcomes: global perspectives from the herpetofauna. *Animal Conservation* **17**:74–81.

Ewing, S.R. *et al.* (2008). Inbreeding and loss of genetic variation in a reintroduced population of Mauritius kestrel. *Conservation Biology* **22**:395–404.

Fa, J. *et al.* (2010). *Leptodactylus fallax*. The IUCN Red List of Threatened Species. Version 2014.3. [Online]. Available at: www.iucnredlist.org [Accessed: 15 October 2015].

Farrer, R.A. *et al.* (2011). Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences* **108**:18732–18736.

Fayer, R. (2004). Cryptosporidium: a water-borne zoonotic parasite. *Veterinary Parasitology* **126**:37–56.

Fellers, G.M. *et al.* (2007). Demise of repatriated populations of Mountain yellow-legged frogs (*Rana muscosa)* in the Sierra Nevada of California. *Herpetological Conservation and Biology* **2**:5–21.

Fentzloff, C. (1984). Breeding, artificial incubation and release of white-tailed sea eagles. *International Zoo Yearbook* **23**:18–35.

Fischer, J. and Lindenmayer, D.B. (2000). An asssessment of the published results of animal relocations. *Biological Conservation* **96**:1–11.

Fisher, M.C. *et al.* (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* **484**:186–194.

Fisher, M.C. *et al.* (2009). Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. *Molecular ecology* **18**:415–429.

Fisher, M.C., Garner, T.W.J. and Walker, S.F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual review of microbiology* **63**:291–310.

Fites, J.S. *et al.* (2014). Inhibition of local immune responses by the frog-killing fungus *Batrachochytrium dendrobatidis*. *Infection and Immunity* **82**:4698–4706.

Fites, J.S. *et al.* (2013). The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. *Science* **342**:366–369.

Florance, D. *et al.* (2011). Excluding access to invasion hubs can contain the spread of an invasive vertebrate. *Proceedings of the Royal Society of London B: Biological Sciences*:rspb20110032.

Fong, G.A., Viña Dávila, N. and López-Iborra, G.M. (2015). Amphibian hotspots and conservation priorities in Eastern Cuba identified by species distribution modeling. *Biotropica* **47**:119–127.

Fong, J.J. *et al.* (2015). Early 1900s detection of *Batrachochytrium dendrobatidis* in Korean amphibians. *PLOS ONE* **10**:e0115656.

Forrest, M.J. and Schlaepfer, M.A. (2011). Nothing a hot bath won't cure: infection rates of amphibian chytrid fungus correlate negatively with water temperature under natural field settings. *PLoS ONE* **6**:e28444.

Forzán, M.J., Gunn, H. and Scott, P. (2008). Chytridiomycosis in an aquarium collection of frogs: diagnosis, treatment, and control. *Journal of Zoo and Wildlife Medicine* **39**:406–411.

Frankham, R. (2008). Genetic adaptation to captivity in species conservation programs. *Molecular Ecology* **17**:325–333.

Frankham, R. (2005). Genetics and extinction. *Biological Conservation* **126**:131–140.

Gallant, A.L. *et al.* (2007). Global rates of habitat loss and implications for amphibian conservation. *Copeia*:967–979.

Garcia, G. *et al.* (2007). Mountain chickens *Leptodactylus fallax* and sympatric amphibians appear to be disease free on Montserrat. *Oryx* **41**:398–401.

Garland, S. *et al.* (2010). BSA reduces inhibition in a TaqMan assay for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **92**:113–116.

Garner, T.W.J. *et al.* (2009). Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Diseases of aquatic organisms* **83**:257–260.

Gascon, C. *et al.* (2007). *Amphibian Conservation Action Plan Proceedings of the IUCN/SSC Amphibian Conservation Summit 2005*. Gland, Switzerland: IUCN-the World Conservation Union.

Gaston, K.J. and Fuller, R.A. (2008). Commonness, population depletion and conservation biology. *Trends in Ecology & Evolution* **23**:14–19.

Geiger, C.C. *et al.* (2011). Elevated temperature clears chytrid fungus infections from tadpoles of the midwife toad, *Alytes obstetricans*. *Amphibia-Reptilia* **32**:276–280.

Geiger, F. *et al.* (2010). Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic and Applied Ecology* **11**:97–105.

Georoff, T.A. *et al.* (2013). Efficacy of treatment and long-term follow-up of *Batrachochytrium dendrobatidis* PCR-positive anurans following itraconazole bath treatment. *Journal of Zoo and Wildlife Medicine* **44**:395–403.

Gervasi, S. *et al.* (2013). Host identity matters in the amphibian-*Batrachochytrium dendrobatidis* system: fine-scale patterns of variation in responses to a multi-host pathogen. *PLoS ONE* **8**:e54490.

Gibson, R.C. and Buley, K.R. (2004). Maternal care and obligatory oophagy in *Leptodactylus fallax*: a new reproductive mode in frogs. *Copeia* **2004**:128–135.

Gillespie, G. and Hollis, G. (1996). Distribution and habitat of the spotted tree-frog, *Litoria spenceri Dubois* (Anura : Hylidae), and an assessment of potential causes of population declines. *Wildlife Research* **23**:49.

Gonzalez, E.G. *et al.* (2014). Island survivors: population genetic structure and demography of the critically endangered giant lizard of La Gomera, *Gallotia bravoana*. *BMC Genetics* **15**:1–17.

Goudet, J. (2003). Fstat (ver. 2.9.4): a program to estimate and test population genetics parameters. [Online]. Available at: http://www.unil.ch/izea/softwares/fstat.html/.

Greenspan, S.E., Longcore, J.E. and Calhoun, A.J.K. (2012). Host invasion by *Batrachochytrium dendrobatidis*: fungal and epidermal ultrastructure in model anurans. *Diseases of Aquatic Organisms* **100**:201–210.

Griffith, B. *et al.* (1989). Translocation as a species conservation tool: status and strategy. *Science* **245**:477–480.

Griffiths, R.A. and Dos Santos, M. (2012). Trends in conservation biology: progress or procrastination in a new millennium? *Biological Conservation* **153**:153–158.

Griffiths, R.A. and Pavajeau, L. (2008). Captive breeding, reintroduction, and the conservation of amphibians. *Conservation Biology* **22**:852–861.

Grogan, L.F. *et al.* (2016). Endemicity of chytridiomycosis features pathogen overdispersion. *Journal of Animal Ecology*:online early.

Harding, G., Griffiths, R.A. and Pavajeau, L. (2015). Developments in amphibian captive breeding and reintroduction programs. *Conservation Biology* **30**:340–349.

Hardman, B. and Moro, D. (2006). Optimising reintroduction success by delayed dispersal: is the release protocol important for hare-wallabies? *Biological Conservation* **128**:403–411.

Hardy, B.M. *et al.* (2015). Itraconazole treatment reduces *Batrachochytrium dendrobatidis* prevalence and increases overwinter field survival in juvenile Cascades frogs. *Diseases of Aquatic Organisms* **112**:243–250.

Harris, R.N. *et al.* (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal* **3**:818–824.

Harris, S. *et al.* (1990). Home-range analysis using radio-tracking data–a review of problems and techniques particularly as applied to the study of mammals. *Mammal Review* **20**:97–123.

Hartup, B.K., Olsen, G.H. and Czekala, N.M. (2005). Fecal corticoid monitoring in whooping cranes (*Grus americana*) undergoing reintroduction. *Zoo Biology* **24**:15–28.

Harvell, C.D. *et al.* (1999). Emerging marine diseases: climate links and anthropogenic factors. *Science* **285**:1505–1510.

Hayes, T.B. *et al.* (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environmental Health Perspectives* **114**:40–50.

Heard, G.W. *et al.* (2015). Refugia and connectivity sustain amphibian metapopulations afflicted by disease. *Ecology Letters* **18**:853–863.

Heard, G.W. *et al.* (2013). Wetland characteristics influence disease risk for a threatened amphibian. *Ecological Applications* **24**:650–662.

Hedges, S.B. and Heinicke, M.P. (2007). Molecular phylogeny and biogeography of West Indian frogs of the genus *Leptodactylus* (Anura, Leptodactylidae). *Molecular Phylogenetics and Evolution* **44**:308–314.

Hess, G. (1996). Disease in metapopulation models: implications for conservation. *Ecology* **77**:1617–1632.

Hess, G.R. *et al.* (2001). Spatial aspects of disease dynamics. In: Hudson, P. J. et al. eds. *The Ecology of Wildlife Diseases.* Oxford, U.K.: Oxford University Press., p. 102–118.

Hoban, S. *et al.* (2014). Comparative evaluation of potential indicators and temporal sampling protocols for monitoring genetic erosion. *Evolutionary Applications* **7**:984–998.

Hofmeyr, M. *et al.* (2004). A second outbreak of rabies in African wild dogs (*Lycaon pictus*) in Madikwe Game Reserve, South Africa, demonstrating the efficacy of vaccination against natural rabies challenge. *Animal Conservation* **7**:193–198.

Holden, W.M. *et al.* (2015). Development of antimicrobial peptide defenses of southern leopard frogs, *Rana sphenocephala*, against the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*. *Developmental and Comparative Immunology* **48**:65–75.

Holden, W.M. *et al.* (2014). Evaluation of amphotericin B and chloramphenicol as alternative drugs for treatment of chytridiomycosis and their impacts on innate skin defenses. *Applied and Environmental Microbiology* **80**:4034–4041.

Holdich, D.M. *et al.* (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowledge and Management of Aquatic Ecosystems*:11.

Holmes, I., McLaren, K. and Wilson, B. (2014). Precipitation constrains amphibian chytrid fungus infection rates in a terrestrial frog assemblage in Jamaica, West Indies. *Biotropica* **46**:219–228.

Homan, R.N., Reed, J.M. and Romero, L.M. (2003). Corticosterone concentrations in free-living spotted salamanders (*Ambystoma maculatum*). *General and Comparative Endocrinology* **130**:165–171.

Horton, D. (2005). *Caribbean Chytrid: The Threat Posed by Chytridiomycosis to the Mountain Chicken (Leptodactylus fallax) and Other Amphibians Endemic to the Lesser Antilles*. MSc thesis, Royal Veterinary College.

Hossack, B.R. *et al.* (2013). Disease in a dynamic landscape: host behavior and wildfire reduce amphibian chytrid infection. *Biological Conservation* **157**:293–299.

Hudson, M.A. *et al.* (2016). In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis epidemic. *Biological Conservation* **195**:37–45.

Hunter, D.A. *et al.* (2010). Presence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in threatened corroboree frog populations in the Australian Alps. *Diseases of Aquatic Organisms* **92**:209–216.

Hyatt, A. *et al.* (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms* **73**:175–192.

IUCN (1998). *Guidelines for Re-Introductions.* Gland, Switzerland: Prepared by the IUCN/SSC Re-introduction Specialist Group. IUCN.

IUCN (2001). *IUCN Red List Categories and Criteria Version 3.1.* [Online]. Available at: http://www.iucnredlist.org/technical-documents/categories-and-criteria/2001-categories-criteria [Accessed: 9 December 2015].

IUCN (2015). *IUCN Red List of Threatened Species. Version 2015-3.* [Online]. Available at: http://www.iucnredlist.org. [Accessed: 4 December 2015].

IUCN/SSC (2013). *Guidelines for Reintroductions and Other Conservation Translocations. Version 1.0.* Gland, Switzerland: IUCN Species Survival Commission.

IUDZG/CBSG (IUCN/SSC) (1993). *The World Zoo Conservation Strategy; The Role of the Zoos and Aquaria of the World in Global Conservation*. Illinois, USA: Chicago Zoological Society.

Iyengar, A. *et al.* (2007). Remnants of ancient genetic diversity preserved within captive groups of scimitar-horned oryx (*Oryx dammah*). *Molecular Ecology* **16**:2436–2449.

Jablonski, D. and Chaloner, W.G. (1994). Extinctions in the fossil record [and discussion]. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **344**:11–17.

James, T.Y. *et al.* (2009). Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. *PLoS Pathog* **5**:e1000458.

Jennelle, C.S. *et al.* (2007). State-specific detection probabilities and disease prevalence. *Ecological Applications* **17**:154–167.

Johnson, M.L. *et al.* (2003). Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of aquatic organisms* **57**:255–260.

Johnson, M.L. and Speare, R. (2005). Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Diseases of aquatic organisms* **65**:181–186.

Johnson, M.L. and Speare, R. (2003). Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. *Emerging Infectious Diseases* **9**:922–925.

Jones, C.G. and Merton, D.V. (2012). A tale of two islands: the rescue and recovery of endemic birds in New Zealand and Mauritius. In: Ewen, J. G. et al. eds. *Reintroduction Biology: Integrating Science and Management.* Oxford, U.K.: Blackwell Publishing.

Jones, E.Y. *et al.* (2006). MHC class II proteins and disease: a structural perspective. *Nature Reviews. Immunology* **6**:271–282.

Jones, H.P. *et al.* (2016). Invasive mammal eradication on islands results in substantial conservation gains. *Proceedings of the National Academy of Sciences* **113**:4033–4038.

Jones, K.E. *et al.* (2008). Global trends in emerging infectious diseases. *Nature* **451**:990–993.

Jones, M.E.B. *et al.* (2012). Treatment of chytridiomycosis with reduced-dose itraconazole. *Diseases of Aquatic Organisms* **99**:243–249.

Joseph, M.B. *et al.* (2013). Taming wildlife disease: bridging the gap between science and management. *Journal of Applied Ecology* **50**:702–712.

Kaiser, H. (1997). Origins and introductions of the Caribbean frog, *Eleutherodactylus johnstonei* (Leptodactylidae): management and conservation concerns. *Biodiversity & Conservation* **6**:1391–1407.

Kaiser, H. (1992). The trade-mediated introduction of *Eleutherodactylus martinicensis* (Anura: Leptodactylidae) on St. Barthélémy, French Antilles, and its implications for Lesser Antillean biogeography. *Journal of Herpetology* **26**:264–273.

Kaiser, H. and Hardy, Jr., J.D. (1994). *Eleutherodactylus martinicensis*. *Catalogue of American Amphibians and Reptiles* **582**:1–4.

Kanafani, Z.A. and Perfect, J.R. (2008). Resistance to antifungal agents: mechanisms and cinical impact. *Clinical Infectious Diseases* **46**:120–128.

Karnauskas, K.B., Donnelly, J.P. and Anchukaitis, K.J. (2016). Future freshwater stress for island populations. *Nature Climate Change* advance online publication:doi:10.1038/nclimate2987.

Kats, L.B. and Ferrer, R.P. (2003). Alien predators and amphibian declines: review of two decades of science and the transition to conservation. *Diversity and Distributions* **9**:99–110.

Keesing, F. *et al.* (2010). Impacts of biodiversity on the emergence and transmission of infectious diseases. *Nature* **468**:647–652.

Kielgast, J. *et al.* (2010). Widespread occurrence of the amphibian chytrid fungus in Kenya. *Animal Conservation* **13**:36–43.

Kilpatrick, A.M., Briggs, C.J. and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in ecology & evolution* **25**:109–118.

Kowalski, T. (2006). *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology* **36**:264–270.

Kriger, K.M. (2009). Lack of evidence for the drought-linked chytridiomycosis hypothesis. *Journal of Wildlife Diseases* **45**:537–541.

Kriger, K.M. and Hero, J.-M. (2006). Survivorship in wild frogs infected with chytridiomycosis. *EcoHealth* **3**:171–177.

Laake, J.L. (2013). *RMark: An R Interface for Analysis of Capture-Recapture Data with MARK. AFSC Processed Rep. 2013-01, 25 P. Alaska Fish. Sci. Cent., NOAA, Natl. Mar. Fish. Serv., 7600 Sand Point Way NE, Seattle WA 98115.*

La Marca, E. *et al.* (2005). Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica* **37**:190–201.

Lampo, M. *et al.* (2006). A chytridiomycosis epidemic and a severe dry season precede the disappearance of *Atelopus* species from the Venezuelan Andes. *The Herpetological Journal* **16**:395–402.

Langhammer, P.F. *et al.* (2013). A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, attenuates in pathogenicity with in vitro passages. *PLOS ONE* **8**:e77630.

Laurance, W.F., McDonald, K.R. and Speare, R. (1996). Epidemic disease and the catastrophic decline of Australian rain forest frogs. *Conservation Biology* **10**:406–413.

Le Comber, S.C. *et al.* (2011). Geographic profiling as a novel spatial tool for targeting infectious disease control. *International Journal of Health Geographics* **10**:35.

Lebreton, J. *et al.* (2009). Modeling individual animal histories with multistate capture–recapture models. In: Caswell, H. ed. *Advances in Ecological Research*. Burlington Academic Press, pp. 87–173.

Lebreton, J.-D. *et al.* (1992). Modeling survival and testing biological hypotheses using marked animals: a unified approach with case studies. *Ecological Monographs* **62**:67–118.

Lederberg, J., Shope, R.E. and Oaks, Jr., S.C. eds. (1992). *Emerging Infections: Microbial Threats to Health in the United States*. [Online]. Washington, D.C.: National Academies Press. Available at: http://www.nap.edu/catalog/2008 [Accessed: 4 January 2016].

Lescure, J. (1979). Étude taxonomique et éco-éthologique d'un amphibien des petites Antilles *Leptodactylus fallax* Muller, 1926 (Leptodactylidae). *Bulletin du Muséum national d'histoire naturelle.* **1**:757–774.

Lever, C. (2001). *The Cane Toad: The History and Ecology of a Successful Colonist*. Otley: Westbury Academic & Scientific Pub.

Levine, A.F.Z. and McPhaden, M.J. (2016). How the July 2014 easterly wind burst gave the 2015-6 El Niño a head start. *Geophysical Research Letters*:2016GL069204.

Lips, K.R. *et al.* (2004). Amphibian population declines in montane southern Mexico: resurveys of historical localities. *Biological Conservation* **119**:555–564.

Lips, K.R. (1998). Decline of a tropical montane amphibian fauna. *Conservation Biology* **12**:106–117.

Lips, K.R. *et al.* (2006a). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America* **103**:3165–3170.

Lips, K.R. *et al.* (2006b). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America* **103**:3165–3170.

Lips, K.R. (1999). Mass mortality and population declines of anurans at an upland site in Western Panama. *Conservation Biology* **13**:117–125.

Longcore, J.E., Pessier, A.P. and Nichols, D.K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* **91**:219–227.

Longcore, J.R. *et al.* (2007). Chytridiomycosis widespread in anurans of northeastern United States. *The Journal of Wildlife Management* **71**:435–444.

Longo, A.V. *et al.* (2013). Lability in host defenses: terrestrial frogs die from chytridiomycosis under enzootic conditions. *Journal of Wildlife Diseases* **49**:197–199.

Longo, A.V. *et al.* (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *Royal Society Open Science* **2**:140377.

Longo, A.V. and Burrowes, P.A. (2010). Persistence with chytridiomycosis does not assure survival of direct-developing frogs. *EcoHealth* **7**:185–195.

Longo, A.V., Burrowes, P.A. and Joglar, R.L. (2010). Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Diseases of aquatic organisms* **92**:253–260.

Lorch, J.M. *et al.* (2011). Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. *Nature* **480**:376–378.

Lorch, J.M. *et al.* (2015). Experimental infection of snakes with *Ophidiomyces ophiodiicola* causes pathological changes that typify snake fungal disease. *mBio* **6**:e01534-15.

Lukacs, P.M. *et al.* (2007). Concerns regarding a call for pluralism of information theory and hypothesis testing. *Journal of Applied Ecology* **44**:456–460.

MacKenzie, D.I. (2009). Getting the biggest bang for our conservation buck. *Trends in Ecology & Evolution* **24**:175–177.

Magin, C. (2003). Dominica's frogs are croaking. *Oryx* **37**:406.

Marano, N., Arguin, P.M. and Pappaioanou, M. (2007). Impact of globalization and animal trade on infectious disease ecology. *Emerging Infectious Diseases* **13**:1807–1809.

Marantelli, G. *et al.* (2004). Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conservation Biology* **10**:173–179.

Marcum, R.D. *et al.* (2010). Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus* (*Bufo*) *boreas boreas*. *Diseases of Aquatic Organisms* **91**:17–21.

Martel, A. *et al.* (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences* **110**:15325–15329.

Martel, A. *et al.* (2011). Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. *Medical mycology* **49**:143–149.

Martel, A. *et al.* (2014). Recent introduction of a chytrid fungus endangers western Palearctic salamanders. *Science* **346**:630–631.

Martin, L. *et al.* (2007). *A Species Action Plan for the Montserrat Mountain Chicken Leptodacylus fallax*. Montserrat: Department of Environment.

Matthews, K.R. (2003). Response of mountain yellow-legged frogs, *Rana muscosa*, to short distance translocation. *Journal of Herpetology* **37**:621–626.

May, R.M., Lawton, J.H. and Stork, N.E. (1995). Assessing extinction rates. In: Lawton, J. H. and May, R. M. eds. *Extinction Rates*. Oxford: Oxford University Press, pp. 1–24.

May, S., Zeisset, I. and Beebee, T.J.C. (2011). Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (*Bufo calamita*) populations. *Conservation Genetics* **12**:805–811.

McCallum, H. (2012). Disease and the dynamics of extinction. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **367**:2828–2839.

McCallum, H. and Dobson, A. (1995). Detecting disease and parasite threats to endangered species and ecosystems. *Trends in Ecology & Evolution* **10**:190–194.

McDonald, K.R. *et al.* (2005). Decline in the prevalence of chytridiomycosis in frog populations in North Queensland, Australia. *Pacific Conservation Biology* **11**:114–120.

McDonald, K.R. (1990). *Rheobatrachus* Liem and *Taudactylus* Straughan and Lee (Anura: Leptodactylidae) in Eungella National Park, Queensland: distribution and decline. *Transactions of the Royal Society of South Australia.* **114**:187–194.

McIntyre, S. (2003). *The Current Status of the Mountain Chicken (Leptodactylus fallax) on Dominica, Eastern Caribbean: An Amphibian in Decline*. MSc thesis, University of East Anglia.

McMahon, T.A. *et al.* (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature* **511**:224–227.

McMahon, T.A. *et al.* (2013). Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. *Proceedings of the National Academy of Sciences* **110**:210–215.

McMahon, T.A. and Rohr, J.R. (2014). Trypan blue dye is an effective and inexpensive way to determine the viability of *Batrachochytrium dendrobatidis* zoospores. *EcoHealth* **11**:164–167.

Mendelson, J.R. *et al.* (2006). Confronting amphibian declines and extinctions. *Science* **313**:48–48.

Meyerson, L.A. and Mooney, H.A. (2007). Invasive alien species in an era of globalization. *Frontiers in Ecology and the Environment* **5**:199–208.

Milner-Gulland *et al.* (2001). Dramatic declines in saiga antelope populations. *Oryx* **35**:340–345.

Mitchell, K.M. *et al.* (2008). Persistence of the emerging pathogen *Batrachochytrium dendrobatidis* outside the amphibian host greatly increases the probability of host extinction. *Proceedings of the Royal Society B: Biological Sciences* **275**:329–334.

Mokany, A. and Shine, R. (2003). Competition between tadpoles and mosquito larvae. *Oecologia* **135**:615–620.

Morell, V. (2008). Into the wild: reintroduced animals face daunting odds. *Science* **320**:742–743.

Morens, D.M. and Fauci, A.S. (2013). Emerging infectious diseases: threats to human health and global stability. *PLOS Pathogens* **9**:e1003467.

Moss, A.S. *et al.* (2008). Chemotaxis of the amphibian pathogen *Batrachochytrium dendrobatidis* and its response to a variety of attractants. *Mycologia* **100**:1–5.

Mountain Chicken Recovery Programme (2014). *Long-Term Recovery Strategy for the Critically Endangered Mountain Chicken 2014-2034.*

Muletz, C.R. *et al.* (2012). Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. *Biological Conservation* **152**:119–126.

Murray, K.A. *et al.* (2009). Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. *Conservation Biology* **23**:1242–1252.

Muths, E. (2003). A radio transmitter belt for small ranid frogs. *Herpetological Review* **34**:345–348.

Myers, N. *et al.* (2000). Biodiversity hotspots for conservation priorities. *Nature* **403**:853–858.

Newell, D.A., Goldingay, R.L. and Brooks, L.O. (2013). Population recovery following decline in an endangered stream-breeding frog (*Mixophyes fleayi*) from subtropical Australia. *PLOS ONE* **8**:e58559.

Nichols, D.K. and Lamirande, E.W. (2001). Treatment of cutaneous chytridiomycosis in blue and yellow poison dart frogs (*Dendrobates tinctorius*). In: Speare, R. ed. *Proceedings: Getting the Jump of Amphibain Disease*. Cairns: James Cook University, p. 51.

NOAH (2015). *National Office of Animal Health Compendium of Animal Medicines: Itrafungol 10 Mg/ml Oral Solution: Contra-Indications, Warnings e.t.c.* [Online]. Available at: www.noahcompendium.co.uk [Accessed: 20 June 2015].

Norris, K. (2004). Managing threatened species: the ecological toolbox, evolutionary theory and declining-population paradigm. *Journal of Applied Ecology* **41**:413–426.

Ogutu-Ohwayo, R. (1990). The decline of the native fishes of lakes Victoria and Kyoga (East Africa) and the impact of introduced species, especially the Nile perch, *Lates niloticus*, and the Nile tilapia, *Oreochromis niloticus*. *Environmental Biology of Fishes* **27**:81–96.

Ohta, T. and Kimura, M. (1973). A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. *Genetical Research* **22**:201–204.

O'Leary, M. (2009). The mathematics of geographic profiling. *Journal of Investigative Psychology and Offender Profiling* **6**:253–265.

Olson, D.H. *et al.* (2013). Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLOS ONE* **8**:e56802.

van Oosterhout, C. *et al.* (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**:535–538.

Pask, J.D., Woodhams, D.C. and Rollins-Smith, L.A. (2012). The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. *Global Change Biology* **18**:1231–1238.

Pearl, C.A. *et al.* (2009). Widespread occurrence of the chytrid fungus *Batrachochytrium dendrobatidis* on Oregon spotted frogs (*Rana pretiosa*). *EcoHealth* **6**:209–218.

Peel, A., Hartley, M. and Cunningham, A. (2012). Qualitative risk analysis of introducing *Batrachochytrium dendrobatidis* to the UK through the importation of live amphibians. *Diseases of Aquatic Organisms* **98**:95–112.

Peery, M.Z. *et al.* (2012). Reliability of genetic bottleneck tests for detecting recent population declines. *Molecular Ecology* **21**:3403–3418.

Peig, J. and Green, A.J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos* **118**:1883–1891.

Peig, J. and Green, A.J. (2010). The paradigm of body condition: a critical reappraisal of current methods based on mass and length. *Functional Ecology* **24**:1323–1332.

Pessier, A.P. *et al.* (1999). Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation* **11**:194–199.

Pessier, A.P. and Mendelson, J.R. eds. (2010). *A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs.* Apple Valley, MN.: IUCN/SSC Conservation Breeding Specialist Group.

Phillips, B.L. *et al.* (2007). Rapid expansion of the cane toad (*Bufo marinus*) invasion front in tropical Australia. *Austral Ecology* **32**:169–176.

Phillips, M.K. and Scheck, J. (1991). Parasitism in captive and reintroduced red wolves. *Journal of Wildlife Diseases* **27**:498–501.

Phillott, A.D. *et al.* (2013). Chytridiomycosis and seasonal mortality of tropical stream-associated frogs 15 years after introduction of *Batrachochytrium dendrobatidis*. *Conservation Biology* **27**:1058–1068.

Pidancier, N., Miquel, C. and Miaud, C. (2003). Buccal swabs as a non-destructive tissue sampling method for DNA analysis in amphibians. *Herpetological Journal* **13**:175–178.

Pilliod, D.S. *et al.* (2010). Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. *Conservation Biology: The Journal of the Society for Conservation Biology* **24**:1259–1267.

Piotrowski, J.S., Annis, S.L. and Longcore, J.E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* **96**:9–15.

Piry, S., Luikart, G. and Cornuet, J.-M. (1999). Computer note. BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. *Journal of Heredity* **90**:502–503.

Pitt, W. and Doratt, R.E. (2006). *Screening for the Evaluation of Selected Chemicals and Pesticides to Control Eleutherodactylus Frogs in Hawaii.* Hilo, Hawaii: USDA, APHIS, WS, NWRC.

Plowright, W. (1982). The effects of rinderpest and rinderpest control on wildlife in Africa. *Symp. Zool. Soc. Lond.* **50**:1–28.

Pollock, J.F. (2006). Detecting population declines over large areas with presence-absence, time-to-encounter, and count survey methods. *Conservation Biology* **20**:882–892.

Pollock, K.H. *et al.* (1990). Statistical inference for capture-recapture experiments. *Wildlife Monographs* **107**:3–97.

Poorten, T.J. *et al.* (2016). Mountain yellow-legged frogs (*Rana muscosa*) did not produce detectable antibodies in immunization experiments with *Batrachochytrium dendrobatidis*. *Journal of Wildlife Diseases* **52**:154–158.

Pounds, A.J. *et al.* (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* **439**:161–167.

Pounds, J.A. *et al.* (1997). Tests of null models for amphibian declines on a tropical mountain. *Conservation Biology* **11**:1307–1322.

Pounds, J.A., Fogden, M.P.L. and Campbell, J.H. (1999). Biological response to climate change on a tropical mountain. *Nature* **398**:611–615.

Prakash, V. *et al.* (2007). Recent changes in populations of resident *Gyps* vultures in India. *Journal of the Bombay Natural History Society* **104**:129–135.

Puschendorf, R. *et al.* (2011). Environmental refuge from disease-driven amphibian extinction. *Conservation Biology* **25**:956–964.

Rachowicz, L.J. *et al.* (2006). Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* **87**:1671–1683.

Rachowicz, L.J. *et al.* (2005). The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology* **19**:1441–1448.

Rachowicz, L.J. and Vredenburg, V.T. (2004). Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Diseases of Aquatic Organisms* **61**:75–83.

R core team (2015). *R: A Language and Environment for Statistical Computing*. [Online]. Vienna, Austria: R Foundation for Statistical Computing. Available at: http://www.R-project.org/.

Raffel, T.R. *et al.* (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Functional Ecology* **20**:819–828.

Ramirez, O. *et al.* (2006). Genetic assessment of the Iberian wolf *Canis lupus signatus* captive breeding program. *Conservation Genetics* **7**:861–878.

Ramsey, J.P. *et al.* (2010). Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infection and Immunity* **78**:3981–3992.

Ranvestel, A.W. *et al.* (2004). Neotropical tadpoles influence stream benthos: evidence for the ecological consequences of decline in amphibian populations. *Freshwater Biology* **49**:274–285.

Raymond, M. and Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**:248–249.

Reed, K.D. *et al.* (2003). Birds, migration and emerging zoonoses: West Nile virus, lyme disease, influenza A and enteropathogens. *Clinical Medicine & Research* **1**:5–12.

Reeder, N.M.M., Pessier, A.P. and Vredenburg, V.T. (2012). A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. *PLOS ONE* **7**:e33567.

Retallick, R.W.R., McCallum, H. and Speare, R. (2004). Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology* **2**:e351.

Ribeiro-Júnior, M.A., Gardner, T.A. and Ávila-Pires, T.C.S. (2008). Evaluating the effectiveness of herpetofaunal sampling techniques across a gradient of habitat change in a tropical forest landscape. *Journal of Herpetology* **42**:733–749.

Richards, S.A. (2008). Dealing with overdispersed count data in applied ecology: overdispersed count data. *Journal of Applied Ecology* **45**:218–227.

Richards, S.A., Whittingham, M.J. and Stephens, P.A. (2011). Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework. *Behavioral Ecology and Sociobiology* **65**:77–89.

Richards, S.J., McDonald, K.R. and Alford, R.A. (1993). Declines in populations of Australia's endemic tropical rainforest frogs. *Pacific Conservation Biology* **1**:66.

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

Rickard, A. (2006). Assessment of translocation of Blanchard's cricket frogs (*Acris crepitans blanchardi*) in south east Michigan. *Endangered Species Update* **23**:3–12.

van Riper, C. *et al.* (1986). The epizootiology and ecological significance of malaria in Hawaiian land birds. *Ecological Monographs* **56**:327–344.

Roche, B. *et al.* (2009). Water-borne transmission drives avian influenza dynamics in wild birds: The case of the 2005–2006 epidemics in the Camargue area. *Infection, Genetics and Evolution* **9**:800–805.

Rodriguez, D. *et al.* (2014). Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. *Molecular Ecology* **23**:774–787.

Rollins-Smith, L.A. *et al.* (2011). Amphibian immune defenses against chytridiomycosis: impacts of changing Environments. *Integrative and Comparative Biology* **51**:552–562.

Rollins-Smith, L.A. (2009). The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1788**:1593–1599.

Ron, S.R. *et al.* (2003). Population decline of the jambato toad *Atelopus ignescens* (Anura: Bufonidae) in the Andes of Ecuador. *Journal of Herpetology* **37**:116–126.

Rosa, G.M. *et al.* (2012). Two remarkable prey items for a chicken: *Leptodactylus fallax* Müller, 1926 predation upon the theraphosid spider *Cyrtopholis femoralis* Pocock, 1903 and the colubrid snake *Liophis juliae* (Cope, 1879). *Tropical Zoology* **25**:135–140.

Rosenblum, E.B. *et al.* (2013). Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *Proceedings of the National Academy of Sciences* **110**:9385–9390.

Rosenblum, E.B. *et al.* (2009). Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. *PLOS ONE* **4**:e6494.

Rossmo, D.K. (2000). *Geographic Profiling*. 1st edition. New York: CRC Press.

Roussel, J.-M., Haro, A. and Cunjak, R.A. (2000). Field test of a new method for tracking small fishes in shallow rivers using passive integrated transponder (PIT) technology. *Canadian Journal of Fisheries and Aquatic Sciences* **57**:1326–1329.

Rousset, F. (2008). GENEPOP'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* **8**:103–106.

Row, J.R. and Blouin-Demers, G. (2006). Kernels are not accurate estimators of home-range size for herpetofauna. *Copeia* **2006**:797–802.

Rowley, J.J.L. and Alford, R.A. (2013). Hot bodies protect amphibians against chytrid infection in nature. *Scientific Reports* **3**:1515 doi:10.1038/srep01515.

Roy, B.A. and Kirchner, J.W. (2000). Evolutionary dynamics of pathogen resistance and tolerance. *Evolution; International Journal of Organic Evolution* **54**:51–63.

Roznik, E.A. and Alford, R.A. (2015). Seasonal ecology and behavior of an endangered rainforest frog (*Litoria rheocola*) threatened by disease. *PloS One* **10**:e0127851.

Rózsa, L., Reiczigel, J. and Majoros, G. (2000). Quantifying parasites in samples of hosts. *The Journal of Parasitology* **86**:228–232.

Ruggeri, J. *et al.* (2015). Seasonal variation in population abundance and chytrid infection in stream-dwelling frogs of the Brazilian Atlantic forest. *PLoS ONE* **10**:e0130554.

Ruiz, A. and Rueda-Almonacid, J.V. (2008). *Batrachochytrium dendrobatidis* and chytridiomycosis in anuran amphibians of Colombia. *EcoHealth* **5**:27–33.

Russell, C.A. *et al.* (2005). Predictive spatial dynamics and strategic planning for raccoon rabies emergence in Ohio. *PLOS Biol* **3**:e88.

Russello, M.A. and Amato, G. (2004). Ex situ population management in the absence of pedigree information. *Molecular Ecology* **13**:2829–2840.

Rödel, M.O. and Ernst, R. (2004). Measuring and monitoring amphibian diversity in tropical forests. I. An evaluation of methods with recommendations for standardization. *Ecotropica* **10**:1–14.

Sabino-Pinto, J. *et al.* (2015). First detection of the emerging fungal pathogen *Batrachochytrium salamandrivorans*in Germany. *Amphibia-Reptilia* **36**:411–416.

Sainsbury, A.W. *et al.* (2000). Grey squirrels have high seroprevalence to a parapoxvirus associated with deaths in red squirrels. *Animal Conservation* **3**:229–233.

Savage, A.E. and Zamudio, K.R. (2016). Adaptive tolerance to a pathogenic fungus drives major histocompatibility complex evolution in natural amphibian populations. *Proc. R. Soc. B* **283**:20153115.

Savage, A.E. and Zamudio, K.R. (2011). MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings of the National Academy of Sciences of the United States of America* **108**:16705–16710.

Scheele, B.C., Guarino, F., *et al.* (2014). Decline and re-expansion of an amphibian with high prevalence of chytrid fungus. *Biological Conservation* **170**:86–91.

Scheele, B.C., Hunter, D.A., *et al.* (2014). Interventions for reducing extinction risk in chytridiomycosis-threatened amphibians. *Conservation Biology* **28**:1195–1205.

Schlaepfer, M.A., Hoover, C. and Dodd, C.K. (2005). Challenges in evaluating the impact of the trade in amphibians and reptiles on wild populations. *BioScience* **55**:256–264.

Schloegel, L.M. *et al.* (2012). Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology* **21**:5162–5177.

Schloegel, L.M. *et al.* (2010). Two amphibian diseases, chytridiomycosis and ranaviral disease, are now globally notifiable to the World Organization for Animal Health (OIE): an assessment. *Diseases of Aquatic Organisms* **92**:101–108.

Schmeller, D.S. *et al.* (2014). Microscopic aquatic predators strongly affect infection dynamics of a globally emerged pathogen. *Current biology: CB* **24**:176–180.

Schwartz, A. and Henderson, R.W. (1991). *Amphibians and Reptiles of the West Indies: Descriptions, Distributions, and Natural History*. Gainesville, Florida: University of Florida Press.

Scott, J.M. *et al.* (2010). Conservation-reliant species and the future of conservation. *Conservation Letters* **3**:91–97.

Scott, M.E. (1988). The impact of infection and disease on animal populations: implications for conservation biology. *Conservation biology* **2**:40–56.

Searle, C.L. *et al.* (2013). Daphnia predation on the amphibian chytrid fungus and its impacts on disease risk in tadpoles. *Ecology and Evolution* **3**:4129–4138.

Searle, C.L. *et al.* (2011). Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. *Conservation Biology* **25**:965–974.

Seddon, P.J., Armstrong, D.P. and Maloney, R.F. (2007). Developing the science of reintroduction biology. *Conservation Biology* **21**:303–312.

Shoo, L.P. *et al.* (2011). Engineering a future for amphibians under climate change. *Journal of Applied Ecology* **48**:487–492.

Skerratt, L.F. *et al.* (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* **4**:125–134.

van der Sluijs, A.M. *et al.* (2013). Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. *Amphibia-Reptilia* **34**:233–239.

Smith, K.F., Acevedo-Whitehouse, K. and Pedersen, A.B. (2009). The role of infectious diseases in biological conservation. *Animal Conservation* **12**:1–12.

Snow, J. (1855). *On the Mode of Communication of Cholera*. London: John Churchill.

Snyder, N.F.R. *et al.* (1996). Limitations of captive breeding in endangered species recovery. *Conservation Biology* **10**:338–348.

Sodhi, N.S. *et al.* (2008). Measuring the meltdown: drivers of global amphibian extinction and decline. *PLoS ONE* **3**:e1636.

Soorae, P.S. ed. (2010). *Global Re-Introduction Perspectives: 2010. Additional Case-Studies from around the Globe.* Abu Dhabi, UAE: IUCN/SSC Re-introduction Specialist Group.

Soorae, P.S. ed. (2011). *Global Re-Introduction Perspectives: 2011. More Case Studies from around the Globe.* Gland, Switzerland: IUCN/SSC Re- introduction Specialist Group and Abu Dhabi, UAE: Environment Agency - Abu Dhabi.

Soorae, P.S. ed. (2013). *Global Re-Introduction Perspectives: 2013. Further Case Studies from around the Globe.* Gland, Switzerland: IUCN/SSC Re- introduction Specialist Group and Abu Dhabi, UAE: Environment Agency - Abu Dhabi.

Soorae, P.S. ed. (2016). *Global Re-Introduction Perspectives: 2016. Case-Studies from around the Globe.* Gland, Switzerland: IUCN/SSC Re- introduction Specialist Group and Abu Dhabi, UAE: Environment Agency - Abu Dhabi.

Soorae, P.S. ed. (2008). *Global Re-Introduction Perspectives: Re-Introduction Case-Studies from around the Globe.* Abu Dhabi, UAE: IUCN/SSC Re-introduction Specialist Group.

Spencer, C.C., Neigel, J.E. and Leberg, P.L. (2000). Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. *Molecular Ecology* **9**:1517–1528.

Spielman, D., Brook, B.W. and Frankham, R. (2004). Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America* **101**:15261–15264.

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

Stockwell, M. *et al.* (2008). The impact of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* on a green and golden bell frog *Litoria aurea* reintroduction program at the Hunter Wetlands Centre Australia in the Hunter Region of NSW. *Australian Zoologist* **34**:379–386.

Stockwell, M.P. *et al.* (2015). Effects of pond salinization on survival rate of amphibian hosts infected with the chytrid fungus. *Conservation Biology: The Journal of the Society for Conservation Biology* **29**:391–399.

Stockwell, M.P., Clulow, J. and Mahony, M.J. (2010). Host species determines whether infection load increases beyond disease-causing thresholds following exposure to the amphibian chytrid fungus. *Animal Conservation* **13**:62–71.

Strayer, D.L. (1999). Effects of alien species on freshwater mollusks in North America. *Journal of the North American Benthological Society* **18**:74–98.

Stuart, S.N. *et al.* (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* **306**:1783–1786.

Stuart, S.N. *et al.* eds. (2008). *Threatened Amphibians of the World.* Lynx Edicions, Barcelona, Spain; IUCN, GLand, Switzerland; and Conservation International, Arlington, Virginia, U.S.A.

Tamukai, K. *et al.* (2011). Treatment of spontaneous chytridiomycosis in captive amphibians using itraconazole. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science* **73**:155–159.

Tapley, B. *et al.* (2015). Amphibians and conservation breeding programmes: do all threatened amphibians belong on the ark? *Biodiversity and Conservation* **24**:2625–2646.

Teixeira, C.P. *et al.* (2007). Revisiting translocation and reintroduction programmes: the importance of considering stress. *Animal Behaviour* **73**:1–13.

Tennessen, J.A. *et al.* (2009). Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana pipiens*) populations suggest intraspecies differences in resistance to pathogens. *Developmental and Comparative Immunology* **33**:1247–1257.

Terrell, V.C.K. *et al.* (2014). Drought reduces chytrid fungus (*Batrachochytrium dendrobatidis*) infection intensity and mortality but not prevalence in adult crawfish frogs (*Lithobates areolatus*). *Journal of Wildlife Diseases* **50**:56–62.

Thorne, E.T. and Williams, E.S. (1988). Disease and endangered species: the black-footed ferret as a recent example. *Conservation Biology* **2**:66–74.

Timmerman, A. *et al.* (1999). Increase El Niño frequency in a climate model forced by future greenhouse warming. *Nature* **398**:394–398.

Tittensor, D.P. *et al.* (2014). A mid-term analysis of progress toward international biodiversity targets. *Science* **346**:241–244.

Tobler, U. and Schmidt, B.R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE* **5**:e10927.

Tuttle, N.C., Beard, K.H. and Al-Chokhachy, R. (2008). Aerially applied citric acid reduces the density of an invasive frog in Hawaii, USA. *Wildlife Research* **35**:676–683.

U.S. Fish and Wildlife Service (2013). *Recovery Plan for the Black-Footed Ferret (Mustela Nigripes)*. Denver, Colorado: U.S. Fish and Wildlife Service.

Venesky, M.D. *et al.* (2012). Selecting for tolerance against pathogens and herbivores to enhance success of reintroduction and translocation. *Conservation Biology* **26**:586–592.

Verity, R. *et al.* (2014). Spatial targeting of infectious disease control: identifying multiple, unknown sources. *Methods in Ecology and Evolution* **5**:647–655.

Viggers, K., Lindenmayer, D. and Spratt, D. (1993). The importance of disease in reintroduction programmes. *Wildlife Research* **20**:687–698.

Voyles, J. *et al.* (2007). Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms* **77**:113–118.

Voyles, J. *et al.* (2009). Pathogenesis of chytridiomycosis, a cause ofcatastrophic amphibian declines. *Science* **326**:582–585.

Vredenburg, V.T. *et al.* (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences* **107**:9689–9694.

Vredenburg, V.T. (2004). Reversing introduced species effects: experimental removal of introduced fish leads to rapid recovery of a declining frog. *Proceedings of the National Academy of Sciences of the United States of America* **101**:7646–7650.

Wake, D.B. and Vredenburg, V.T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences* **105**:11466–11473.

Walker, S.F. *et al.* (2007). Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms* **77**:105–112.

Walker, S.F. *et al.* (2008). Invasive pathogens threaten species recovery programs. *Current Biology* **18**:R853–R854.

Walsh, P.D. *et al.* (2007). Potential for Ebola transmission between gorilla and chimpanzee social groups. *The American Naturalist* **169**:684–689.

Wang, S. *et al.* (2014). Population size and time since island isolation determine genetic diversity loss in insular frog populations. *Molecular Ecology* **23**:637–648.

Warkentin, I.G. *et al.* (2009). Eating frogs to extinction. *Conservation Biology* **23**:1056–1059.

Weldon, C. *et al.* (2004). Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases* **10**:2100–2105.

Wells, K.D. (2010). *The Ecology and Behavior of Amphibians*. Chicago: University of Chicago Press. pp. ****.

Whiles, M.R. *et al.* (2006). The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. *Frontiers in Ecology and the Environment* **4**:27–34.

White, A.W. and Pyke, G.H. (2008). Frogs on the hop: translocations of green and golden bell frogs *Litoria aurea* in Greater Sydney. *Australian Zoologist* **34**:249–260.

White, G.C. and Burnham, K.P. (1999). Program MARK: Survival Estimation from Populations of Marked Animals. *Bird Study* **46 Supplement**:120–138.

Whitfield, S.M. *et al.* (2012). Temporal variation in infection prevalence by the amphibian chytrid fungus in three species of frogs at La Selva, Costa Rica. *Biotropica* **44**:779–784.

Wiles, G.J. *et al.* (2003). Impacts of the brown tree snake: patterns of decline and species persistence in Guam's avifauna. *Conservation Biology* **17**:1350–1360.

Wilson, G.J., Carter, S.P. and Delahay, R.J. (2011). Advances and prospects for management of TB transmission between badgers and cattle. *Veterinary Microbiology* **151**:43–50.

Wimsatt, J. *et al.* (2006). The quest for a safe and effective canine distemper virus vaccine for black-footed ferrets. In: *Proceedings of the Symposium on the Status of the Black-Footed Ferret and Its Habitat*. Fort Collins, CO, USA: Scientific Investigations Report 2005-5293. U.S. Geological Survey, pp. 248–266.

Wingate, D.B. (2010). The successful elimination of cane toads, *Bufo marinus*, from an island with breeding habitat off Bermuda. *Biological Invasions* **13**:1487–1492.

Wobeser, G. (2002). Disease management strategies for wildlife. *Revue Scientifique Et Technique (International Office of Epizootics)* **21**:159–178.

Wobeser, G.A. (2007). *Disease in Wild Animals*. Berlin, Heidelberg: Springer-Verlag.

Woodhams, D.C. *et al.* (2008). Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. *EcoHealth* **5**:268–274.

Woodhams, D.C. *et al.* (2011). Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology* **8**:8.

Woodhams, D.C. *et al.* (2007). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation* **10**:409–417.

Woodhams, D.C. *et al.* (2012). Treatment of amphibians infected with chytrid fungus: learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. *Diseases of Aquatic Organisms* **98**:11–25.

Woodhams, D.C., Alford, R.A. and Marantelli, G. (2003). Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms* **55**:65–67.

Woodworth, B.L. *et al.* (2005). Host population persistence in the face of introduced vector-borne diseases: Hawaii amakihi and avian malaria. *Proceedings of the National Academy of Sciences of the United States of America* **102**:1531–1536.

Woolaver, L.G. *et al.* (2015). *Species Action Plan for the Conservation of the Madagascar Pochard 2014-2014.* Durrell Wildlife Conservation Trust & IUCN/SSC.

Young, H.S. *et al.* (2014). Declines in large wildlife increase landscape-level prevalence of rodent-borne disease in Africa. *Proceedings of the National Academy of Sciences* **111**:7036–7041.

Young, R.P. ed. (2008). A Biodiversity Assessment of the Centre Hills, Montserrat*. Durrell Conservation Monograph No. 1*. Jersey, Channel Islands: Durrell Wildlife Conservation Trust.

Young, S., Berger, L. and Speare, R. (2007). Amphibian chytridiomycosis: strategies for captive management and conservation. *International Zoo Yearbook* **41**:85–95.

Zhang, L. *et al.* (2016). Reintroduction and post-release survival of a living fossil: the Chinese giant salamander. *PLOS ONE* **11**:e0156715.

Zippel, K. *et al.* (2011). The Amphibian Ark: a global community for ex situ conservation of amphibians. *Herpetological Conservation and Biology* **6**:340–352.

Appendices

Appendix A. Microsatellite primer development

Microsatellite primers for the mountain chicken were isolated commercially by Ecogenics (Switzerland). Eight polymorphic markers (Appendix B) were then standardised using a Qiagen Multiplex PCR Kit using the following reaction mix: 5 µl of Multiplex mix, 0.2ul of 0.2 pmol/µl of forward primer, 0.2µl of 0.2pmol/µl of reverse primer, 1µl of DNA and 3.6µl of nuclease free ddH_2O. PCR conditions were as follows: initial denaturation at 95^oC for 15min, followed by 35 cycles of: 30s at 94^oC, 90s at specific annealing temperature (Appendix B), extension for 60s at 72^oC; and a final extension of 30min at 72^oC. All genotyping was performed at DNA Sequencing and Services (Dundee) and microsatellite scoring was performed using GeneMapper (v4).

The microsatellite genotypes will be submitted to Dryad upon acceptance of this manuscript for publication, and the corresponding accession code will be provided here.

Appendix B. Polymorphic microsatellites identified for Leptodactylus fallax

For each marker the annealing temperature (Tm ^{o}C), number of observed alleles (NOA), repeat motif (mot), PCR products allele size range in base pairs (range), combinations of primers for multiplexing (M) are shown.

Primer Code	Tm (°C)	Primer sequence (5'-3')	NOA	Mot	Range	M
Lepfal_010673	62	AGCAATTCTTGTTGCCTCCC	5	(TAGA)	217-241	3
		AGCCTAAGTTCTTGCAGGGC				
Lepfal_015759A	64	AAGATCAGCCAGGGACAGAC	10	(TTTC)	189-234	3
		CACTGTGATATTTAGGGGTGC				
Lepfal_000867	64	CGTGAGAAAGACTAGGGCAC	10	(TAGA)	200-244	1
		AAAAGGGAGCACTCCACAGG				
Lepfal_002969	60	AGCATCACAGGGAACCAGTC	5	(AC)	169-191	2
		GCTCCTGAAGTACAAACGCC				
Lepfal_003035	62	ACATACAGAACTGCTTACATGTCC	5	(TG)	126-134	1
		GCTTTGTCACTGGCTCCAAG				
Lepfal_011628	60	ATGATTGGCCCCAGTGTATG	4	(CA)	207-234	2
		GATCGCAGAACCTGGACCTC				
Lepfal_013956	58	AGCGTTCGATTAGTAGCTGTG	5	(AC)	134-162	2
		AGTTCACCCCAACGTAGGAC				
Lepfal_017957	58	TGTATGATGTGGGCCTTCCC	6	(TG)	189-242	1
		CACCACTGAAATAACCTATCATTTGTC				

Appendix C. Mountain chickens found dead or with severe signs of chytridiomycosis on Dominica between 2003-2004

Date	Site	Number dead	Number with signs of chytridiomycosis
13/12/2002	Galion	2	0
13/01/2003	Bagatelle	0	1
24/01/2003	Galion	0	2
30/01/2003	Elmshall	1	0
01/02/2003	La Haut	1	0
17/02/2003	Bois Cotlette	1	0
19/02/2003	Petit Coulibri	1	1
25/02/2003	Petit Coulibri	0	1
25/02/2003	Soufriere	1	1
06/03/2003	Dublanc Valley	1	0
19/03/2003	Bagatelle	0	1
26/03/2003	Bois Cotlette	0	1
28/03/2003	Dublanc Valley	3	1
01/04/2003	La Haut	4	0
26/12/2003	Milton Estate	1	0
17/01/2004	Elmshall	1	0
12/02/2004	Upper Kings Hill	1	0
14/04/2004	Coulibistre	1	0
15/06/2003	Fond St. Jean	2	4
26/06/2003	Fond St. Jean	1	5

Appendix D. Summary statistics of all loci in all populations

NA = Number of alleles, H_O = Observed heterozygosity, H_E = Expected heterozygosity. Loci significantly (p=<0.05) deviating from HWE indicated in shaded cell, red = heterozygote deficiency, green = heterozygote excess. Significant (p= <0.05) F_{IS} values are shown in bold. Allelic richness only presented for groups.

	Locus	NA	H_O	H_E	F_{IS}
Founders	0673_p1	3	0.545	0.450	-0.224
	759A_p1	6	0.818	0.688	-0.200
	0867_p1	3	0.545	0.437	-0.263
	2969_p1	3	0.727	0.662	-0.103
	3035_p1	2	0.364	0.485	0.259
	1628_p1	3	0.000	0.606	1.000
	3956_p1	3	0.182	0.589	0.701
	7957_p1	5	0.909	0.684	-0.351
Wild	0673_p1	4	0.473	0.441	-0.072
	759A_p1	6	0.838	0.724	-0.158
	0867_p1	6	0.581	0.528	-0.101
	2969_p1	3	0.622	0.650	0.044
	3035_p1	3	0.500	0.529	0.055
	1628_p1	3	0.041	0.588	0.931
	3956_p1	4	0.081	0.516	0.844
	7957_p1	6	0.740	0.741	0.001
Post Dom	0673_p1	3	0.529	0.558	0.053
	759A_p1	6	0.294	0.627	0.539
	0867_p1	5	0.412	0.592	0.311
	2969_p1	3	0.353	0.426	0.176
	3035_p1	3	0.235	0.314	0.256
	1628_p1	2	0.353	0.471	0.256
	3956_p1	2	0.000	0.148	1.000
	7957_p1	4	0.647	0.770	0.164
Mont	0673_p1	4	0.550	0.514	-0.071
	759A_p1	8	0.825	0.745	-0.108
	0867_p1	6	0.533	0.501	-0.064
	2969_p1	3	0.622	0.656	0.052
	3035_p1	4	0.492	0.506	0.029
	1628_p1	3	0.025	0.571	0.956
	3956_p1	4	0.100	0.477	0.791
	7957_p1	6	0.765	0.722	-0.059
Pre Dom	0673_p1	7	0.621	0.743	0.168
	759A_p1	8	0.759	0.736	-0.031
	0867_p1	10	0.759	0.814	0.069
	2969_p1	5	0.370	0.387	0.044
	3035_p1	4	0.357	0.611	0.420
	1628_p1	4	0.138	0.356	0.616
	3956_p1	5	0.148	0.546	0.733
	7957_p1	4	0.793	0.702	-0.132
Dom	0673_p1	7	0.596154	0.677931	0.122
	759A_p1	8	0.615385	0.710232	0.135
	0867_p1	10	0.634615	0.744586	0.149
	2969_p1	5	0.36	0.391919	0.082
	3035_p1	5	0.313725	0.558144	0.44
	1628_p1	4	0.230769	0.434653	0.472
	3956_p1	5	0.088889	0.453433	0.806

Appendix E. Description of DNA extraction method for skin swabs

Briefly, we removed the tip of the swab using a sterile blade and placed in a sterile Eppendorf. We then added 60 µl of PrepMan Ultra (Applied Biosystems) with 30 to 40 mg of 0.5 mm zirconium/silica beads (Biospec Products). We homogenised the sample for 45 seconds in a TissueLyser 2 (Qiagen, Ltd.). After briefly centrifuging (2 min at 4000 rpm in a benchtop centrifuge) to settle all material to the bottom of the tube, we repeated the homogenisation and centrifugation steps. We then placed the homogenised sample in a 100 °C water bath for 10 min, cooled for 2 min, then centrifuged at 4000 rpm for 3 min. We recovered as much supernatant as possible and stored it at –20 °C until ready to be analysed.

Appendix F. Bd infection intensity treatment group comparison using linear mixed models.

During treatment comparison (weeks 1-15)

Bd infection intensities in the IT and control groups were compared using a linear mixed model constructed with package {lme4} (Bates et al. 2015) in R. Treatment group and time were included as fixed effects and frog ID was included as a random effect. Models are ranked using AIC corrected for small sample size (AICc). Standard errors for variable estimates were produced using 10000 simulations of the model in the {arm} package (Gelman and Su 2015) in R.

Table F1: Model selection table for linear mixed effects model of Bd infection intensity (Genomic equivalents) comparison between the IT and control groups during treatment (weeks 1-15). Model selection was carried out using AICc.

Fixed effects	Random effects	K	AICc	Delta AICc	AICc Weight	Log likelihood
Group * Time	Frog ID	6	2147.125	0.0000	0.9997	-1067.496
Group + Time	Frog ID	5	2163.159	16.0340	0.0003	-1076.532
Group	Frog ID	4	2171.464	24.3394	0.0000	-1081.700
Time	Frog ID	4	2182.987	35.8620	0.0000	-1087.462
.	Frog ID	3	2196.183	49.0577	0.0000	-1095.072

During the treatment period, there is clear support for the top model over the other models (AICc weight = 0.9997). We therefore use only parameter estimates from this model in the graph overleaf.

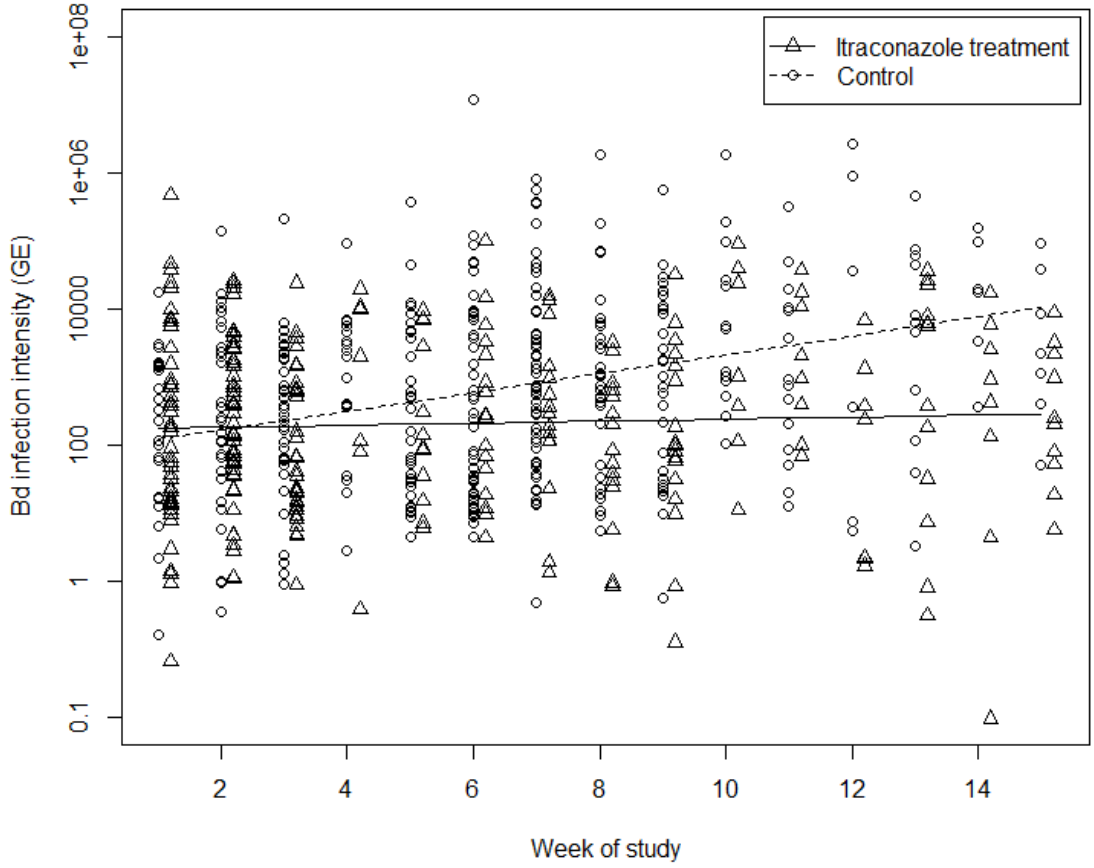


Figure F2. Bd infection intensity comparison (Genomic equivalents) between the IT and control groups during the treatment period. The y-axis is logged in order to display data which varies over many orders of magnitude. Linear mixed model prediction for top model (treatment group*time) is plotted. IT group data are plotted with an x-offset of +0.1 for display purposes.

Post treatment comparison (weeks 16-24):

Table F2: Model selection table for linear mixed effects model of Bd infection intensity (Genomic equivalents) comparison between the IT and control groups after treatment (weeks 16-24). Model selection was carried out using AICc.

Fixed effects	Random effects	K	AICc	Delta AICc	AICc Weight	Log lik.
.	Frog ID	3	685.4048	0.0000	0.5772	-339.6334
Group	Frog ID	4	686.2000	0.7953	0.3878	-338.9844
Time	Frog ID	4	692.5821	7.1773	0.0160	-342.2211
Group + Time	Frog ID	5	693.1020	7.6972	0.0123	-341.4810
Group * Time	Frog ID	6	694.3212	8.9164	0.0067	-340.9150

As no model received overwhelming support (AICc weight of top model = 0.5772), we considered all models with a delta AIC <7 for inference (Burnham & Anderson, 2002). The top model had no variation in group or time, and the only model with a delta AIC <7 included group dependency. There was, however, very weak evidence for a group difference in Bd infection intensity in the post treatment period (summed Akaike weight=0.401; evidence ratio=0.7). The model averaged estimate for the difference in Bd infection intensity between the control and IT groups was 1.45 GE (Unconditional SE=1.82), suggesting that any difference was not ecologically important and after treatment ended, there was no prolonged benefit of treatment with itraconazole.

References for Appendix F

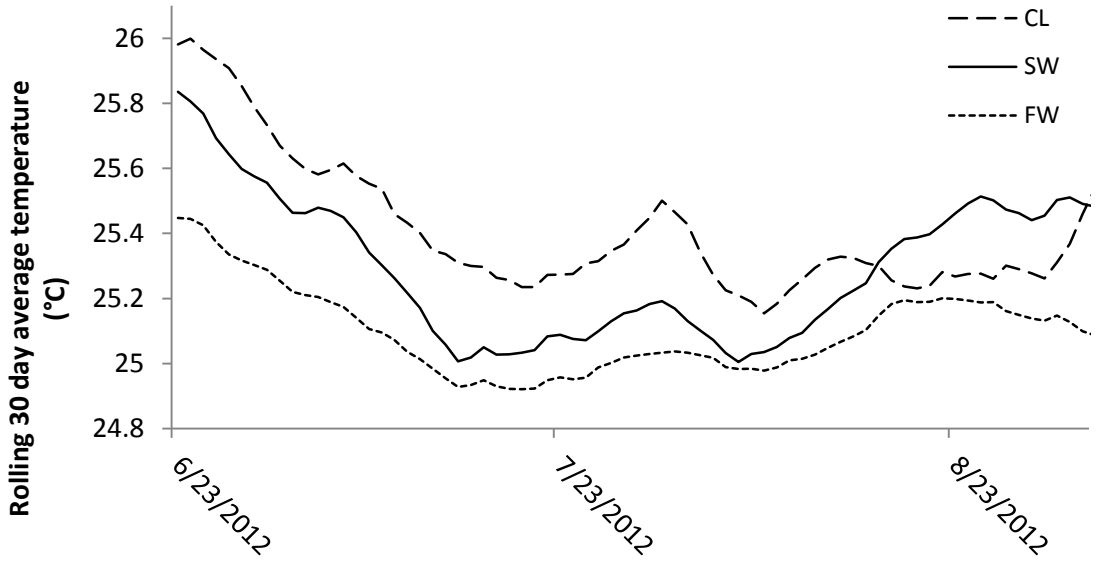
Bates, D., Machler, M., Bolker, B., and Walker, S. 2015. Fitting linear mixed-effects models using {lme4}. Journal of Statistical Software **67**: 1-48. doi:10.18637/jss.v067.i01

Burnham, K.P., and Anderson, D.R. 2002. Model selection and multimodel inference: a practical information-theoretic approach. Springer-Verlag, New York.

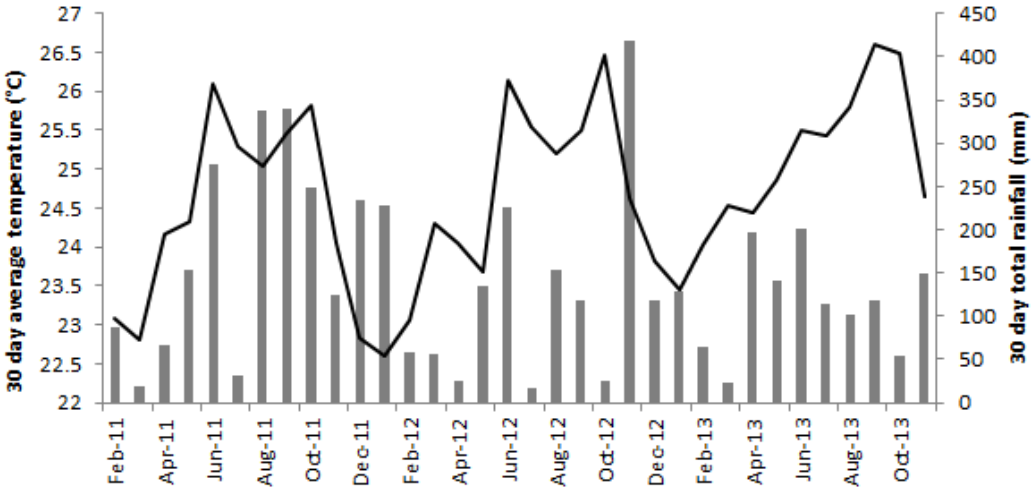
Gelman, A., and Su, Y-S. 2015. arm: data analysis using regression and multilevel/heirachical models. R package version 1.8-6. http://CRAN-R-project.org/package=arm

Appendix G. Comparison of temperate data from each study site on Montserrat in 2012.

There are only limited differences meaning SW temperature data was used to represent every site the analyses.



Appendix H. Monthly estimates of 30 day mean temperature and 30 day accumulated rainfall averages for Montserrat. Black line represented temperature, and grey bars represent rainfall. These variables were significantly correlated meaning they could not be used in the same multivariate analyses.



Appendix J. qPCR results from first Dominican population of mountain chickens discovered for seven years in 2011. The Bd infection loads recorded in these animals were sufficiently high they likely resulted in the extirpation of the majority of this population shortly after these surveys.

Date	Sex/Age	PCR result	Bd load (zsp. equiv.)
10/08/11	Female	NEG	0
10/08/11	Male	NEG	0
03/09/11	Male	NEG	0
04/10/11	Female	NEG	0
04/10/11	Female	NEG	0
08/12/11	Female	POS	4
08/12/11	Juvenile	POS	32475
09/12/11	Juvenile	POS	7055
05/01/12	Female	POS	10021
06/01/12	Female	POS	4146
19/01/12	Juvenile	POS	10455

Appendix K. Description of procedure used to implant mountain chickens destined for release with intracoelomic transmitter

Mountain chickens were fitted with radiotransmitters intracoloemically (Werner, 1991) as external radio-transmitters resulted in skin abrasions (B. Tapley, pers. comm.).

The transmitters (BD2-HX and PD2-HX) had external helix antennae which were modified for use internally. The antennae were coiled around a 10 mm piece of Tygon tubing, which extended beyond the end of the transmitter. Externally this transmitter is expected to have 100 - 200 m range in normal conditions.

In order to insert the transmitter, an incision of approximately 10 mm was made on the left side of the frog, approximately 1.5 cm lateral to mid line, through the skin and muscle (Fig. K1). Bleeding was minimal in all cases. The transmitter was disinfected by immersion in F10 1:500 for 5 - 10 minutes, then rinsed with saline and inserted. The muscle was closed with absorbable suture (vicryl 4/0) with a simple continuous pattern. The skin was closed with non-absorbable, 4/0 monocryl in a continuous, everting pattern. Knots were secured with tissue glue. Ceftazidime was given intra-muscularly every three days for the next nine days.

Wounds were checked (no problems were observed) and stitches removed before transport.

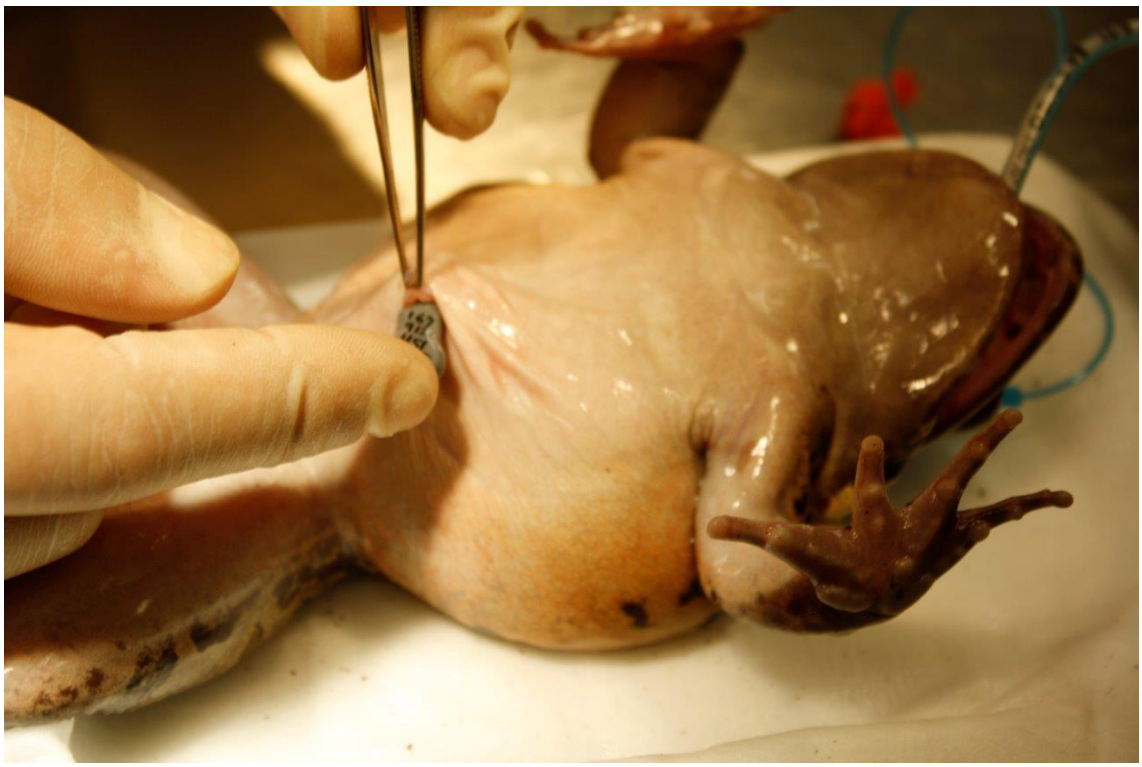


Figure K1. Photo taken during the procedure to implant a radiotransmitter into a captive bred mountain chicken prior to release. Photo credit: Gerardo Garcia / Durrell.

References for Appendix K

Werner, J.K. (1991). A radio-telemetry implant technique for use with *Bufo americanus*. *Herpetological Review* **22**:94-95.

Appendix L. Creation of scaled mass index for mountain chickens

Scaled mass indices for the mountain chicken were created as described in (Peig and Green 2010; 2009). Pairs of SVL and body mass from 266 unique mountain chickens caught during 2005, prior to the chytridiomycosis epidemic were utilised to estimate the ln (SVL) - ln (mass) relationship. Individuals were excluded from the creation of the index if they were below 100 mm (the minimum size of a mountain chicken in the release cohort) in order to prevent contaminating the dataset with juveniles which would likely have a different SVL-mass relationship. Separate scaled mass indices were created for each sex as there is a sex difference in the SVL - mass relationship in many amphibian species (confirmed in mountain chickens through preliminary analysis in these data).

A standardised major axis regression was used to fit the relationship between the natural log of the mass (g) and the natural log of the SVL (mm) (Peig and Green 2009) / Fig. B-1). The slope of this regression was used to scale body masses of the reintroduced animals to their mass if they had a standardised SVL.

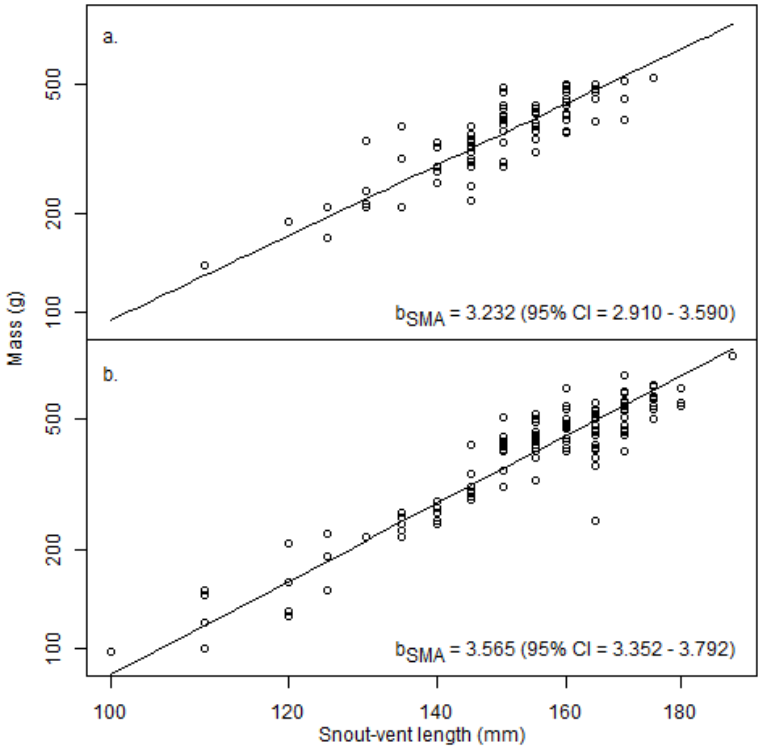


Figure B-1. Log SVL vs. log mass for 266 wild a. male and b. female mountain chickens captured during 2005. A standardised major axis regression is used to estimate the relationship between the two in order to generate scaled masses for reintroduced mountain chickens.

Appendix M. Final captures and deaths of reintroduced mountain chickens recovered, clinical signs of chytridiomycosis and maximum Bd-infection load recorded prior to disappearance or death. Where this value is not in the week prior to disappearance or death, the number of weeks prior is specified. Signs of chytridiomycosis: SS = skin sloughing, WL = weight loss, RV = red ventral skin, L = lethargy, MT = muscle tremors, LC = lost coordination, WE = white line under eye.

REL	Date last seen	Date dead	ID	Sex	Max Bd load (zsp. equiv.)	Signs of chytridiomycosis	Other notes
1	18/05/2011		1	M	63322	RV	
1	03/04/2011		2	F	11623	SS	
1	28/04/2011		3	F	16210	L	
1	08/06/2011		4	M	40486	SS, WE	
1	15/04/2011		5	F	143	None	
1	29/06/2011		6	M	2081	None	
1	16/11/2011		7	F	33722	SS, RV	
1	14/09/2011		8	M	1714	None	
1	25/05/2011		9	F	67860	SS, MT	Cut on left hind leg, healed later
1	06/04/2011		10	F	30	None	
1	06/04/2011		11	F	10	None	Cut on toe, healed later
1	08/06/2011		12	M	6313	L	
1	25/05/2011		13	M	50107	RV, MT	
1		25/05/2011	14	F	48734	RV	Euthanised: severe signs of chytridiomycosis
1		08/06/2011	15	F	5321 (4 weeks prior)	SS	
1	01/06/2011		16	F	19886	SS, RV	Scratches on leg, healed later
1	21/04/2011		17	M	Negative	None	
1	01/06/2011		18	M	5459	SS, RV	
1	04/05/2011		19	F	32098	RV, SS, MT	
1	26/02/2011		20	M	Negative	None	
1	22/06/2011		21	F	4204	None	Broken toe, healed later
1	04/05/2011		22	M	19923	MT	Wound on lower back, healed later
1	29/06/2011		23	F	7359	RV	
1	24/03/2011		24	F	2	None	Appeared to be predating crayfish
1		25/05/2011	25	M	18090	RV	
1	13/04/2011		26	M	Negative	None	
1		03/03/2011	27	F	Negative	None	

REL	Date last seen	Date dead	ID	Sex	Max Bd load (zsp. equiv.)	Signs of chytridiomycosis	Other notes
1	18/05/2011		28	M	53499	RV	
1		07/04/2011	29	M	5379	SS, MT	
1	08/06/2011		30	M	81085	RV	
1	04/05/2011		31	M	20270	SS, RV, MT	Seen eating gecko
1	07/02/2011		32	M	Negative	None	
1	02/03/2011		33	M	2	None	
1	01/03/2011		34	M	Negative	None	
1	08/06/2011		35	M	2	None	
1	18/05/2011		37	M	4353	RV	
1		20/04/2011	38	M	8901	SS, RV	
1	18/05/2011		39	M	77	WE	
1	24/03/2011		40	M	5460	SS, RV	
1	08/06/2011		41	M	127898	SS, RV, WE	
1	18/05/2011		42	M	662.358 (6 weeks prior)	None	
1	At release		43	F	Not swabbed	Lost too early	
1		01/06/2011	44	F	79 (6 weeks prior)	SS, RV	
1	At release		45	M	Not swabbed	Lost too early	
1	At release		46	F	Not swabbed	Lost too early	
1	At release		47	F	Not swabbed	Lost too early	
1	08/06/2011		48	M	37514	RV	
1	04/05/2011		49	F	5446	SS	
1		24/03/2011	50	F	6372	SS, LC	
1	03/03/2011		51	M	1	None	
1	15/06/2011		52	F	17014	L	
1	At release		53	M	Not swabbed	Lost too early	
1		24/08/2011	54	F	30623	SS	
1	04/05/2011		55	F	150	MT	
1	06/04/2011		56	F	Negative	None	
1	25/05/2011		57	F	124533	RV, MT	
1	01/06/2011		58	F	177120	RV, MT	Broken leg and wound, healed later
1	15/06/2011		59	M	68075	None	Scar from healed cut on leg

REL	Date last seen	Date dead	ID	Sex	Max Bd load (zsp. equiv.)	Signs of chytridiomycosis	Other notes
1	28/04/2011		60	F	41443	SS, RV, WE	
1	25/05/2011		61	F	6757	WL	Underweight after first infection with Bd
1	20/07/2011		62	M	2918	MT	
1	30/03/2011		63	M	Negative	None	Underweight
1	21/03/2011		64	M	Negative	None	
2		19/04/2012	65	M	168498	SS, WL, RV	
2		10/03/2012	66	M	43984	SS, L	Dehydrated
2		30/03/2012	67	M	1996	L	Dehydrated
2	08/05/2012		68	M	1126	None	
2	30/04/2012		69	F	11210 (5 weeks before)	RV	
2	18/04/2012		70	F	65930	None	
2	05/04/2012		71	M	13	None	Often basking in sun
2	01/04/2012		72	M	3	None	
2		26/03/2012	73	F	10637	RV	Euthanised: wound & air in body cavity
2		14/03/2012	74	F	355	None	Crushed by rock
2	30/04/2012		75	M	44161	RV	
2		18/03/2012	76	F	34338	SS, MT, RV	Euthanised: severe signs of chytridiomycosis
2		22/03/2012	77	M	94738	SS, RV	
2	30/04/2012		78	F	3769	SS	Healed wound
2	18/04/2012		79	F	2234	RV	
2		26/04/2012	80	M	63107	SS, RV	
2	19/04/2012		81	F	100226	SS, RV, MT	Dehydrated later
2	28/04/2012		82	M	1266	SS	
2	08/05/2012		83	F	382663	SS, RV	
2	19/04/2012		84	M	988	SS	Seen basking in sun, healed wound
2	30/04/2012		85	M	4221	LC	Seen basking in sun
2		28/04/2012	86	F	15491	SS, WE	
2		06/04/2012	87	F	12044	SS, RV	
2	07/06/2012		88	M	7693	SS	
2	21/04/2012		89	F	74	None	

REL	Date last seen	Date dead	ID	Sex	Max Bd load (zsp. equiv.)	Signs of chytridiomycosis	Other notes
2		24/04/2012	90	F	110068	SS, WL	
2	30/04/2012		91	F	2000	None	
2		07/03/2012	92	M	42 (3 weeks prior)	-	Too decomposed to examine
2		06/03/2012	93	F	1	No	Euthanised: severe emphysema dyspena signs
2	07/06/2012		94	M	15957	None	
2	06/04/2012		95	F	483	WL	
2	30/04/2012		96	F	47818	SS, RV, MT	Healing wound
2	02/03/2012		97	M	1548	None	
3	28/11/2012		99	F	Negative	None	
3	17/03/2013		102	F	621	None	
3		30/01/2013	104	M	198941	SS	
3	01/02/2013		105	M	6065	SS	Dehydrated, seen eating tarantula
3	24/04/2013		107	F	394800	RV	
3		18/02/2013	108	F	159488	RV	
3		03/02/2013	109	M	99015	SS, RV, L, MT	
3		17/03/2013	111	F	12961	SS, RV, L, MT, WE	
3	04/09/2013		112	M	Negative	None	
3	21/03/2013		114	M	10124	SS, RV	Healed wound from wrestling?
3	15/11/2012		115	F	Not swabbed	Lost too early	
3	At release		116	M	Not swabbed	Lost too early	
3	24/11/2012		117	M	Negative	None	
3	01/05/2013		118	M	6480	None	
3		07/02/2013	119	F	12170	RV, LS	
3		03/02/2013	120	F	3748	SS, RV	
3	14/03/2013		121	F	2810	None	
3	08/03/2013		122	F	58	None	Healed wound on leg
3	At release		123	F	Not swabbed	Lost too early	
3	25/03/2013		124	F	47	None	Some muscle loss
3		09/01/2013	125	F	163005	SS, RV, MT, LC	
3	24/11/2012		127	F	Negative	None	

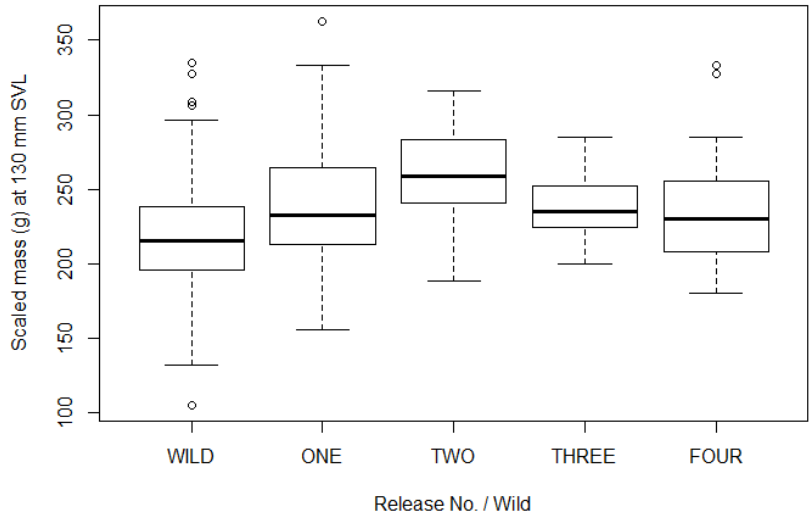
REL	Date last seen	Date dead	ID	Sex	Max Bd load (zsp. equiv.)	Signs of chytridiomycosis	Other notes
3	26/11/2012		128	M	Negative	None	
3	24/12/2012		129	F	Negative	None	
4	20/10/2014		1	F	17	None	Healed toe fracture
4	27/11/2014		2	F	176	None	
4	10/04/2015		3	M	Negative	None	
4	03/12/2014		4	M	1092	None	
4	14/08/2014		5	F	Negative	None	Eggs visible
4	08/08/2014		6	F	Negative	None	
4	11/07/2014		7	M	Negative	None	
4	24/09/2014		8	F	9	None	Eggs visible
4	24/09/2014		9	M	Negative	None	Healed wound
4	22/08/2014		10	F	Negative	None	Produced foam nest
4	16/09/2014		11	F	Negative	None	
4	01/12/2014		12	F	9	None	
4	20/10/2014		13	M	Negative	None	
4	15/10/2014		14	M	Negative	None	
4	14/09/2014		15	F	Negative	None	Eggs visible, healed wound
4	24/07/2014		16	M	Negative	None	
4	At release		17	M	Not swabbed	Lost too early	
4	27/11/2014		18	M	Negative	None	
4	24/09/2014		19	M	Negative	None	
4	13/08/2014		20	F	Negative	None	Eggs visible, healed wound
4	30/08/2014		21	F	Negative	None	
4	10/09/2014		22	F	Negative	None	
4	13/07/2014		23	M	Negative	None	
4	03/12/2014		24	M	574	None	
4	01/10/2014		25	F	Negative	None	Eggs visible
4	20/08/2014		26	M	Negative	None	
4	19/11/2014		27	M	3	None	
4	21/09/2014		28	M	Negative	None	Healed wound on knee

REL	Date last seen	Date dead	ID	Sex	Max Bd load (zsp. equiv.)	Signs of chytridiomycosis	Other notes
4	13/07/2014		29	F	Negative	None	
4	25/09/2014		30	M	Negative	None	
4	05/11/2014		31	M	Negative	L, MT	Underweight
4	12/09/2014		32	F	Negative	None	Eggs visible, healed wound
4	At release		33	M	Not swabbed	Lost too early	
4	27/11/2014		34	M	Negative	None	
4	01/10/2014		35	M	147	None	
4	24/09/2014		36	F	Negative	None	
4	At release		37	M	Not swabbed	Lost too early	
4	14/08/2014		38	F	Negative	None	
4	03/07/2014		39	F	Not swabbed	Lost too early	
4	24/09/2014		40	M	Negative	None	
4	27/10/2014		41	M	1	None	Large wound healed
4	06/10/2014		42	M	Negative	None	
4	13/07/2014		43	F	Negative	None	
4	12/11/2014		44	M	Negative	None	
4	02/07/2014		45	F	Not swabbed	Lost too early	
4	28/09/2014		46	M	Negative	None	
4	03/07/2014		47	M	Not swabbed	Lost too early	
4	24/09/2014		48	M	Negative	None	
4	At release		49	F	Not swabbed	Lost too early	
4	10/07/2014		50	F	Negative	None	
4	01/12/2014		51	F	Negative	None	
4	19/11/2014		52	M	13	None	
4		15/08/2014	NA	NA	Negative (carcass)	NA	Heavily decomposed in pond, no ID

Appendix N. *Eleutherodactylus johnstonei* Bd infection prevalence in surveys undertaken during release four (wet season).

Date	Bd positive	Prevalence (%)	Prev. 95% CI
03/07/14	0/60	0.00	0.00 - 6.25
13/08/14	1/60	1.67	0.09 - 8.88
08/10/14	0/60	0.00	0.00 - 6.25
09/12/16	10/60	16.6	8.89 - 28.2

Appendix O. Comparison of scaled mass of mountain chickens in each release cohort prior to release and of wild individuals caught during 2005. (At 130 mm snout-ventral length)



Appendix P. Choice of method to determine home range estimates for the reintroduced mountain chickens.

Kernel density estimation is regarded as the gold standard for home range estimation for most taxa (Worton 1987). Automated optimisation routines such as least-squares cross validation (LSCV) and reference selection (h_{ref}) exist to select bandwidths for KDE utilisation distributions, the most important step in KDE home range estimation (Gitzen and Millspaugh 2003; Worton 1989; Silverman 1986). Preliminary analysis suggested LSCV and h_{ref} bandwidth selection procedures generated under- and over- estimates for home range size respectively for the data in the current study (as reported in Kie (2013)). This is common in home range estimation for herpetofauna as these animals often use 'favourite' sites repeatedly rendering the data highly auto-correlated (Row and Blouin-Demers 2006). This results in extremely variable bandwidth estimates using LSCV to which home range is very sensitive (Row and Blouin-Demers 2006; Worton 1995). Although subsampling has been recommended to reduce autocorrelation (e.g. Swihart and Slade 1985), it has been shown to reduce the biological relevance of home range estimates (de Solla, Bonduriansky and Brooks 1999) and so no sub-sampling was performed.

References for Appendix P

de Solla, S.R., Bonduriansky, R. and Brooks, R.J. (1999). Eliminating autocorrelation reduces biological relevanece of home range estimates. *Journal of Animal Ecology* **68**:221–234.

Gitzen, R.A. and Millspaugh, J.J. (2003). Comparison of least-squares cross-validation bandwidth options for kernel home-range estimation. *Wildlife Society Bulletin* **31**:823–831.

Kie, J.G. (2013). A rule-based ad hoc method for selecting a bandwidth in kernel home-range analyses. *Animal Biotelemetry* **1**:13.

Row, J.R. and Blouin-Demers, G. (2006). Kernels are not accurate estimators of home-range size for herpetofauna. *Copeia* **2006**:797–802.

Silverman, B.W. (1986). *Density Estimation for Statistics and Data Analysis*. CRC Press.

Swihart, R.K. and Slade, N.A. (1985). Testing for independence of observations in animal movements. *Ecology* **66**:1176–1184.

Worton, B.J. (1987). A review of models of home range for animal movement. *Ecological Modelling* **38**:277–298.

Worton, B.J. (1989). Kernel methods for estimating the utilization distribution in home-range studies. *Ecology* **70**:164–168.

Worton, B.J. (1995). Modelling radio-tracking data. *Environmental and Ecological Statistics* **2**:15–23.