DOCTOR OF PHILOSOPHY

Conservation of an endangered amphibian

The case of the Natterjack toad (Epidalea calamita) in Ireland

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Conservation of an endangered amphibian:

the case of the Natterjack toad (*Epidalea calamita*)

in Ireland

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“One of the basic steps in saving a threatened species is to learn more about it: its diet, its mating and reproductive processes, its range patterns, its social behaviour”

Dian Fossey, National Geographic 1970

“Natterjack toad”. Illustration by Kate Diamond katediamond.org

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Summary

Amphibians have been declining globally since at least the 1970s and are the most endangered class of vertebrates with over 40% of species threatened with extinction. The Natterjack toad (Epidalea calamita) is the rarest amphibian in Ireland, regionally Red-listed as Endangered. The species is subject to substantial Government conservation efforts, including regular monitoring and surveillance, a Pond Creation Scheme and an ongoing Head-start and Translocation Programme to facilitate new pond colonisation.

This thesis aimed to update the Natterjack toad's conservation status in Ireland, establish temporal trends assessing threats and pressures, describe its genetic integrity and population structure and evaluate the efficacy of conservation measures. The new information generated spans habitat selection, spatial ecology, population biology, metapopulation dynamics, genetic diversity, biogeography and effects of climate change.

Natterjack toad annual egg string counts suggested a -23% decline in the number of egg stringsbetween 2004 and 2018 with local extirpation at one site. Assessment of perceived threats and pressures suggests that declines are likely driven by poor habitat quality. Conservation programmes failed to significantly arrest decreases in the number of egg strings offsetting further declines by only 4%. Nevertheless, the conservation value of artificially created ponds should not be underestimated as they had 43% higher aquatic macroinvertebrate species richness and 33% higher macroinvertebrate abundance than natural ponds. Mark-recapture using photo ID and genetic fingerprinting suggested that extrapolation of total population estimates from egg strings alone may underestimate the census size by up to 83% due to substantial sex ratio deviation from 1:1 with up to 7 males per female at breeding ponds. Genetic studies indicated high genetic diversity with no evidence of genetic bottlenecks or inbreeding depression despite considerable declines in the number of egg strings. The Natterjack toad population in Ireland displayed significant genetic spatial structuring, best explained by barriers to dispersal and gene flow inhibited by coniferous forestry plantations, bog, marsh, moor and heath, scrub, anthropogenic presence and rivers, and facilitated by sand dunes and coastal grasslands. Suitable bioclimatic-habitat niche space for the species is likely to expand northward and to higher elevations under projected global climate change with models predicting increase in the number of egg strings and earlier spawning by the end of the 21 century. However, limited dispersal capability and ongoing threats and pressures mean potential benefits of climatic change are unlikely to be realised.

Continued population monitoring and surveillance is recommended while it is suggested that future research should include: estimation of sex ratio variation between metapopulations, use of acoustic monitoring to assess the male population at breeding sites, use of environmental or eDNA in assessing species presence including colonisation of new ponds and calibration of water DNA densities using population abundance derived from egg strings, a greater focus on disease and pathogens, and investigation of terrestrial habitat use and hibernacula availability.

Species conservation strategies should focus on working with landowners and farmers to improve habitat quality, water quality and the availability of breeding ponds to maximise connectivity between breeding sites facilitating dispersal. Recommendations are made to conservation practitioners with respect to genetic structuring and identified genetic entities.

A major challenge lies in breaching the boundaries between academic research, Government and conservation management decision making and practical on-the-ground conservation action by various stakeholders (principally landowners and farmers) to make conservation programmes more effective and efficient.

Chapter 1

General Introduction

Biodiversity loss is the most critical global challenge humanity is currently facing, threatening both ecosystem service delivery and human health and well-being (Ceballos *et al.* 2010; Dirzo & Raven 2003; Mace *et al*. 2005; Ehrlich & Ehrlich 2013; Ceballos *et al.* 2015). A growing body of evidence suggests that current species extinction rates are higher than pre-human background rates by two to three orders of magnitude (Pimm *et al*. 2006; Steadman 2006), comparable to the previous five mass extinction events of Earth's history (Barnosky *et al.* 2011). Even common and widespread species that are not currently endangered, have experienced declines and local extirpations at the population-level, directly threatening ecosystem functioning (Ceballos & Ehrlich 2009). The effect of the current 6th mass extinction event extends across all taxonomic groups, but some taxonomic groups, for example amphibians, are more sensitive to anthropogenic pressure than others and have been disproportionately affected (Isaac *et al.* 2012).

1.1 Amphibian extinction crisis

Since the beginning of 1980s herpetologists and conservation biologist have described amphibian declines and extinctions (Collins & Storfer 2003). At the First World Congress of Herpetology scientists expressed concerns about global trends in amphibian populations (Wake 1991, 1998; Bishop *et al.* 2012). At that time, over 500 species were under threat, suggesting that declines began as early as 1970s (Sherman & Morton 1993; Drost & Fellers 1996). Even though some scientists expressed doubts about the magnitude of the amphibian decline (Pechmann & Wilbur 1994, Pechmann *et al.* 1991), extensive research efforts since have shown that such declines are

widespread, extend beyond normal demographic fluctuations and are a result of anthropogenic activities (Pounds *et al.* 1997; Houlahan *et al.* 2000; Collins & Storfer 2003).

Amphibians are now accepted as the group with the highest proportion of threatened species (Beebee & Griffiths 2005). According to the IUCN Red List, 41% of the world's amphibian species are threatened with extinction (Figure 1.1; IUCN 2020), declining more rapidly than any other vertebrate group (Regan *et al.* 2001; Stuart *et al.* 2004). This is likely to be an underestimation of the proportion of threatened amphibians if Data Deficient and undescribed species (estimated at 3,500 species) are considered (Zippel & Mendelson 2008; Giam *et al.* 2012). Addressing the amphibian extinction crisis represents "the greatest species conservation challenge in the history of humanity" (Zippel & Mendelson 2008).

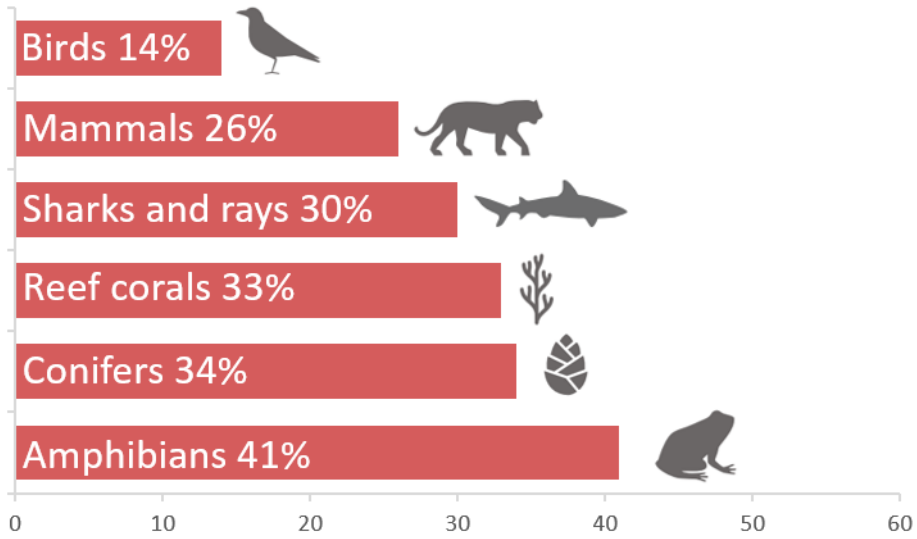

Figure 1.1 Proportion of threatened species across different taxonomic groups (IUCN 2020).

1.2 Threats and pressures

The amphibian extinction crisis has been attributed to a range of threats and pressures, often interacting synergistically (Sodhi *et al.* 2008). The principal cause of amphibian decline is habitat destruction and fragmentation (Gallant *et al.* 2007; Gardner *et al*. 2007). Amphibians are ectotherms and have permeable skin, hence all aspects of their physiology, behaviour and life history are strongly dependent on external environmental conditions (Duellman & Trueb 1986; Zug *et al.* 2001; Pough *et al.* 2004). In addition, most species have complex terrestrial-freshwater life cycles which require two distinct environments further narrowing their habitat tolerances (Houlahan & Findlay 2003). Therefore, amphibians have complex multiple habitat requirements making them disproportionately sensitive to any habitat modifications.

Pollution has been proposed as a likely cause of global amphibian declines, impacting 25% (570) of threatened species (IUCN 2020). Their sensitivity to chemical contaminants in the environment is often attributed to their permeable skin, anamniotic eggs and reliance on freshwater ecosystems where toxins accumulate (Bishop *et al.* 2012). Increased use of agrochemicals like fertilisers (Rouse *et al.* 1999), insecticides (Rohr & Crumrine 2005) and herbicides (Jones *et al*. 2010) over the past century have been a major contributor to amphibian declines. For instance, historical use of cholinesterase-inhibiting pesticides have been related to population declines of four California frog species (*Rana aurora draytonii*, *R. boylii*, *R. casadea* and *R. muscosa*) (Davidson 2004). However, evaluating the impact of toxins in laboratory and field experiments has its challenges like focusing on a single or only few pesticides, mainly calculating lethal levels and extrapolating experimental results on

a small group of animals to population-level effects, hence the impact of chemical pollution remains poorly understood.

Amphibian declines have been observed even in pristine and well protected natural habitats, providing evidences for the significant role disease play in the amphibian extinction crisis (Drost & Fellers 1996; Daszak *et al.* 2003; Martel *et al.* 2013). In particular, emerging infectious diseases caused by ranavirus and chytrid fungus have been associated with mass mortalities, directly linked to recent extinctions and population declines of hundreds of species (Laurance *et al.* 1996; Vredenburg *et al.* 2010; Teacher *et al.* 2010; Kik *et al.* 2011). Chytridiomycosis caused by *Batrachochytrium dendrobatidis* (*Bd*, Berger *et al.* 1998) and the recently discovered *B. salamandrivorans* (*Bsal*, Martel *et al.* 2014) has played a role in the decline of over 500 amphibian species, including 90 species currently presumed extinct (Scheele *et al.* 2019). The effect of chytridiomycosis on amphibian biodiversity is described as the greatest documented loss of biodiversity attributed to a disease (Skerratt *et al.* 2007; Scheele *et al.* 2019).

Other challenges facing amphibian conservation are spread of invasive species such as predatory introduced fish (e.g. rainbow trout *Oncorhynchus mykiss*, Zamora *et al.* 2018; mosquitofish *Gambusia affinis,* Komak & Crossland 2000; Pyke & White 2000; exotic ornamental fish like *Carassius auratus* and *Gambusia affinis,* Denoël *et al.* 2005), over-exploitation for food and traditional medicine and the unregulated international pet trade (Carpenter *et al.* 2007; Natusch & Lyons 2012), causing direct mortalities and increased disease risk to wild populations (Rowley *et al.* 2010; Fisher & Garner 2007). Recent studies suggest that climate change can pose an additional serious threat to amphibian populations (e.g. Cohen *et al.* 2019; Bosch *et al.* 2018)

given their high dependence on temperature and humidity in combination with low ability to disperse and track range shifts (Blaustein *et al.* 2001; Carey & Alexander 2003). However, evidence that climate change is directly causing amphibian declines and extinction is controversial (Carey & Alexander 2003; McCallum 2005; Rohr *et al.* 2008; Yiming *et al.* 2013). Synergisms between multiple factors i.e. disease and climate change (Bosch *et al*. 2007; Pounds *et al.* 2006; Laurance 2008) may accelerate the rate of amphibian decline in the future (Sodhi *et al.* 2008; Hof *et al*. 2011).

1.3 Ecological importance of amphibians

Amphibians provide vital ecosystem services such as ecosystem engineering, nutrient cycling, and energy transfer between aquatic and terrestrial systems (Hocking & Babbitt 2014). Amphibians contribute to biological pest control and prevention of pathogen outbreaks by controlling disease-vectors like mosquitos (DuRant & Hopkins 2008; Valencia-Aguilar *et al.* 2013). Some amphibian species like the tree frog *Xenohyla truncate* play important role in seed dispersal (Silva *et al.* 1989; Silva & Britto-Pereira 2006). Tadpoles are known to significantly impact algal and perithyton community structure in freshwater communities altering primary production and organic matter dynamics (Whiles *et al.* 2006; Altig *et al.* 2007). Amphibians are an important food source for other species, thus their declines may cause significant food web shifts. Zipkin *et al.* (2020) documented the collapse of Neotropical (amphibian eating) snake communities in areas with mass amphibian mortality. Trophic cascades have profound effects on other taxonomic groups and whole ecosystems through top-down and bottom-up processes. Hence, the amphibian

extinction crisis demands urgent action to prevent, arrest and reverse population declines to secure the persistence of amphibian species and their associated ecosystem service delivery in the future.

1.4 The Natterjack toad

The Natterjack toad *(Epidalea calamita)* is widely distributed throughout Europe, ranging from the Iberian Peninsula in the west and as far east as the Baltic coast with several isolated populations in Great Britain and Ireland (Figure 1.2; Sillero *et al.* 2014). The species inhabitats a wide range of conditions throughout its European range but it is most often associated with open habitats on sandy substrate, dry heath or grassland where shallow pools form. The Natterjack toad is among the few amphibians that can tolerant brackish water. The lethal threshold for salinity is considered to be at 10-11ppt (Gomez-Mestre *et al.* 2003), even though research in the UK suggests a lower tolerance of 4.5ppt (Beebee 1985). During the day, toads stay borrowed in moist sandy soil or under debris and stones. They are nocturnal being most active at night when they predate invertebrates and emerge *en mass* to breed. Adult toads do not have many natural predators due to poisonous glands on their skin which produce pugnacious compounds. Tadpoles are also toxic to avoid fish predation (Boomsma & Arntzen 1985; Denton 1991).

The Natterjack toad has a prolonged breeding season compared to other Anurans. At dusk male toads gather at breeding pools and call to attract females. The operational sex ratio during the breeding season is male-biased resulting in intense competition among male toads (Arak 1983). While all males are sexually mature,

some (often small individuals with weak calls) will adopt a silent satellite behaviour and try to intercept females attracted to calling males (Arak 1988). Reproductive males are found at breeding sites throughout the whole breeding season while females come to breeding sites exclusively for spawning and leave immediately afterwards (Sinsch 1988). After arriving at the pond, half of the females initiate amplexus with the first encountered male toad, while the other half moves between several calling males. Females prefer loud, rapidly repeated calls (large males) and try to reject non-calling males, probably as a strategy to avoid mating with males of other species, like the common toad *Bufo bufo* (Arak 1988). Polygyny is considered to be a common anuran breeding strategy where large males dominate breeding sites and available females resulting in only a few males successfully breeding. Multiple paternity of egg clutches as a result of polyandrous mating (several males mate simultaneously with a female) and clutch piracy (pirate males search for recently laid egg clutches, clasp them and release their sperm) have been reported in several amphibian species (e.g. Vieites *et al.* 2004; Knopp & Merilä 2009; Byrne & Roberts 2012). However, multiple paternity has not yet been recorded in Natterjack toads and it might be unlikely due to their segregated-pair breeding behaviour (May *et al.* 2011). Spawning occurs between April and July in mainly ephemeral pools. The number of breeding female toads varies greatly depending on the weather conditions each year (warmth and rainfall), the height of the water table and the number of pools which form (Smith & Skelcher 2019). Therefore, the species' reproduction can be highly successful in some years while completely failing in others (Beebee & Griffiths 2000; Baker 2011). Each female usually lays a single egg string during the breeding season. Fertility varies between populations from over 7,000 eggs per string

in the south of Spain to approximately 2,000 eggs per string in Ireland where the species is at the north-western limit of its distribution (Aubry *et al.* 2010). Eggs usually hatch within 10 days if favourable environmental conditions present i.e. warm temperatures. Tadpoles feed on algae and detritus. Metamorphosis occurs approximately six weeks after hatching. Survival rates of egg strings and tadpoles are typically very low (overall premetamorphic survival betweem 0% and 6%; Aubry *et al*. 2010). Predation by invertebrates e.g. predatory diving beetles, early pond desiccation (drying up) and fungal infections are among the main threats (Becart *et al*. 2007).

Figure 1.2 Natterjack toad (*Epidalea calamita*) distribution across Europe (IUCN 2020).

Despite its widespread distribution in Europe, the Natterjack toad population is declining (Beja *et al.* 2016). There have been recent concerns about its current status, with its range having contracted by >50% during the latter half of the 20th century. Recent surveys indicate that the species' range may have contracted even further, with very poor and irregular breeding activity recorded in the most westerly parts of its current range (Bécart *et al.* 2007). The species is listed on Annex IV of the EU Habitats and Species Directive (92/43/EEC) with EU member states (including the Republic of Ireland) required to report regularly to the European Commission on the species' conservation status [under Article 17]. The last two Article 17 reports assessed the species' conservation status throughout Europe as 'unfavourable' except for the Mediterranean region (European Topic Centre 2012).

1.5 The Natterjack toad in Ireland

In Ireland, the Natterjack toad is the most range restricted and rarest amphibian, regionally Red-listed as 'Endangered' (King *et al*. 2011; Figure 1.3). Prior to the initiation of the Pond Creation Scheme in 2008 by the National Parks and Wildlife Service (NPWS), toads were restricted to 12 discrete sites in County Kerry; all designated as Special Areas of Conservation or SACs (Bécart *et al*. 2007). Natterjack toads are protected under the Irish Wildlife Act 1976 amended in 2000. The last monitoring project for the species (Sweeney *et al.* 2013) evaluated the population trend as declining.

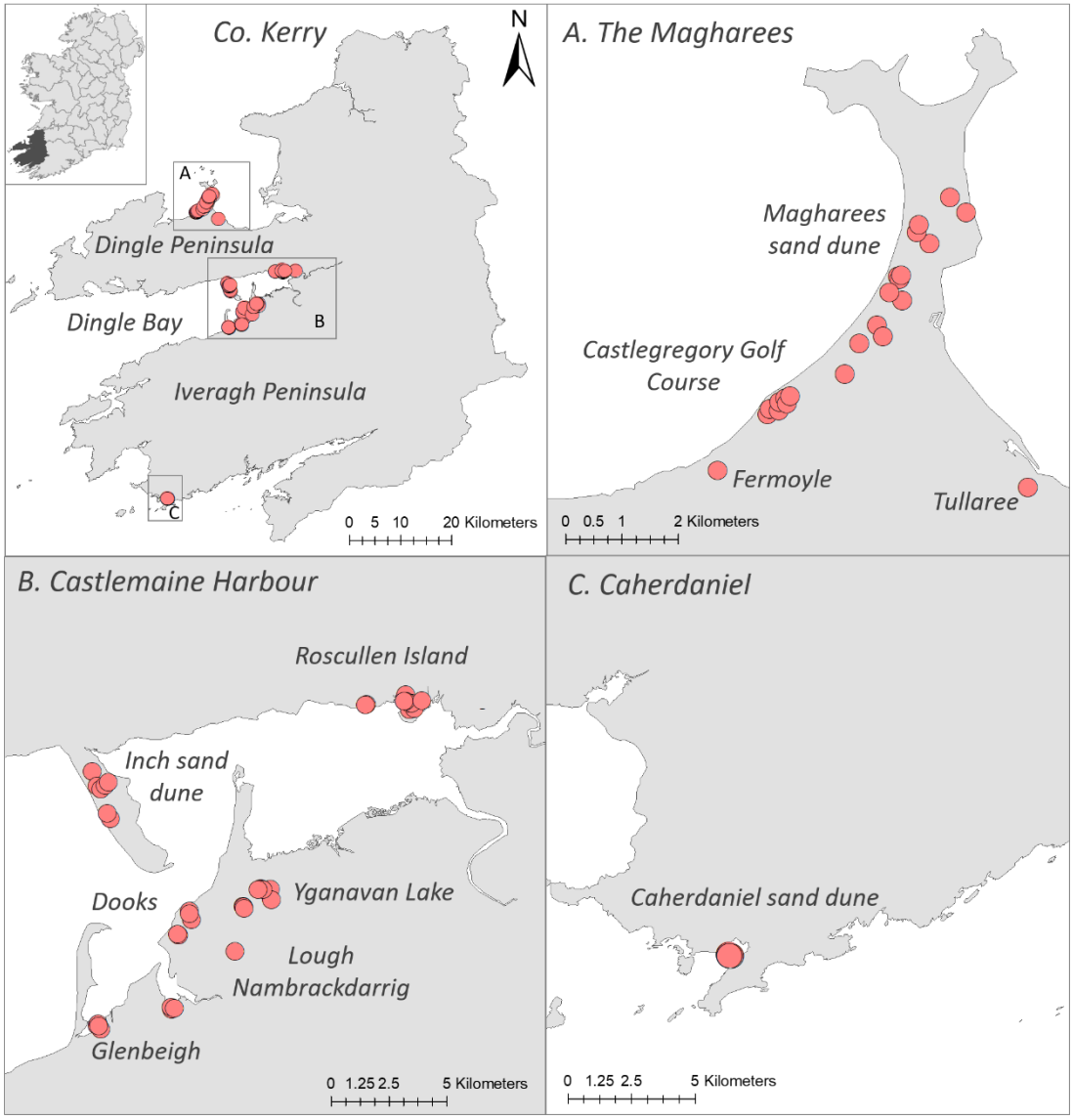

Figure 1.3 Natterjack toad breeding sites in Co. Kerry, Ireland.

Ireland lost more than half of its farmland ponds between the late 1880s and the early 21st century (Reid *et al.* 2014) with pond loss identified as the single most important driver of Natterjack toad population declines (Beebee 2002). One hundred new artificially created ponds were made as part of the National Parks and Wildlife Service (NPWS) Pond Creation Scheme initiated in 2008. The programme's goal was to increase the number of suitable breeding sites around Castlemaine Harbour and Fermoyle (Figure 1.3) and to restore the toad to its historical range. Most natural

breeding sites occur on sand dune habitats or within associated scrub. Artificially created ponds are exclusively on links golf courses (i.e. amenity grasslands created on sand dunes) or, as part of the Pond Creation Scheme in improved or semi-improved agricultural grasslands within agricultural farmland. Initial indications suggest that toads have started to colonise some of the new ponds with breeding activity in 16% of constructed ponds during 2011-12 (Sweeney *et al.* 2013). In 2014, a Head-start and Translocation Programme was launched to enhance pond colonization rates where egg strings and tadpoles were collected from the wild, grown through to metamorphosis in captivity and then returned to the wild. There are no data available yet on the efficacy and success of these translocations. We identified seven Natterjack toad populations on Co Kerry (Magharees, Inch, Roscullen, Dooks, Yganavan, Glenbeigh and Caherdaniel) that are spatially disjunct from one another by distance and poor habitat. Each of the seven populations consisted of one or more breeding sites (in total twelve) characterised as a group of breeding ponds in close proximity (<500m) and lack of visiable barriers to species movement (Figure 1.3).

1.6 Aims and objectives

The main aim of the current research was to generate new knowledge on the Natterjack toad ecology and conservation status in Ireland by applying multidisciplinary approaches to inform management guidelines to prevent future population declines and aid species recovery. Each chapter addresses specific objectives outlined briefly below:

Chapter 2 used 14 years of intensive monitoring and surveillance data to assess the current conservation status of the Natterjack toad in Ireland. This chapter aimed to quantify temporal trends in the number of egg strings and recruitment, evaluate the success of habitat restoration measures on toad's productivity, analyse breeding site preferences and identify perceived threats and pressures.

Chapter 3 aimed to test the efficacy of artificially created Agri-Environment Scheme ponds in replicating the conditions of naturally occurring ponds in the same landscape, now largely restricted to natural, non-agricultural habitats. The specific objectives were to compare environmental parameters, aquatic and terrestrial invertebrate taxa richness, abundance and community structure and to establish indicator taxa that differentiated natural and artificial communities.

Chapter 4 aimed to compare population size estimates derived from unadjusted spawn counts and Capture-Mark-Recapture (CMR) using passive photo-identification to recognise individuals from their ventral markings verified by molecular genetic fingerprinting using microsatellite markers on DNA recovered from skin swabs. One of the main objectives was to provide recommendations on best practises for monitoring a pond-breeding amphibian species.

Chapter 5 investigated the population genetic structure of the Natterjack toad in Ireland in order to inform conservation management. The specific objectives were to provide genetic characterisation of each extant population, reconstruct parentage of offspring samples, estimate the effective and census population size, evaluate the impact of pond characteristics on effective population size, detect any genetic

bottleneck effect(s) due to historical or recent population decline, quantify genetic differentiation between populations and assign genetic clusters.

Chapter 6 investigated the impact of habitat fragmentation and loss on spatial genetic structure and gene flow between twelve metapopulations of the Natterjack toad in Ireland. The specific objectives were to use microsatellite markers to estimate genetic structure and quantify pairwise genetic distances between remaining breeding sites, quantify climatic and habitat landscape variability, relate genetic distance to geographic distance and landscape dispersal resistance explicitly testing isolation-by-distance (IBD) and isolation-by-resistance (IBR) models.

Chapter 7 evaluated the impact of climate change on Natterjack toad's distribution range and breeding behaviour. This chapter aimed to characterize the bioclimatic-habitat niche space of the Natterjack toad throughout its distribution in Europe and to assess the impact of climate on the toad's environmental suitability, number of laid egg strings and breeding time in Ireland.

Chapter 8 integrates results from chapters 2-7 on Natterjack toad biology, ecology and genetics in Ireland to provide new insights to inform species-specific conservation strategies for this endangered amphibian to help secure its future at its western most range edge margin.

Chapter 2

Conservation efforts fail to halt the decline of the regionally endangered Natterjack toad (*Epidalea calamita*) in Ireland

This chapter was published as a Government Report:

Reyne M, Aubry A, Martin Y, Helyar S, Emmerson M, Reid N (2019) *Natterjack Toad (Epidalea calamita) Monitoring and Conservation Status 2016-2018* Irish Wildlife Manuals, No. 107. National Parks and Wildlife Service, Department of Culture, Heritage and the Gaeltacht, Ireland.

A manuscript based on this chapter is *in print* as:

Reyne M, Aubry A, Emmerson M, Marnell F, Reid N (*in print*) Conservation efforts fail to halt the decline of the regionally endangered Natterjack toad (*Epidalea calamita*) in Ireland. *Biological Conservation.*

2.1 Abstract

Amphibian declines are of major conservation concern worldwide. The Natterjack toad (*Epidalea calamita*) is the rarest amphibian in Ireland, regionally Red-listed as Endangered. The species is at the north-western edge of its European range in Ireland and is subject to substantial conservation efforts, including regular monitoring and surveillance, a pond creation agri-environment scheme, and a head-start and translocation programme to facilitate pond colonisation. We used large-scale intensive monitoring and surveillance data from 2004 to 2018 to quantify temporal trends in egg string production analysing breeding site preferences and occurrence of perceived threats and pressures. Despite substantial conservation efforts, egg string production declined by 23% in Ireland over a 14-year period (-1.6%/year). Twenty-two of 100 artificial ponds had been colonised by 2018, but artificial sites accounted for <10% of eggs laid and had prevented further declines by only 4%. Natterjack toad spawning was associated with ponds with a large surface area, situated in sand dune habitat, with high water conductivity, and a high percentage cover of aquatic vegetation at the substrate with short terrestrial vegetation in the surrounding vicinity. Threats and pressures are related to poor water quality at breeding sites and abandonment of surrounding agricultural land leading to unsuitable terrestrial vegetation. Given the Natterjack toad's population trend in Ireland, continued monitoring and surveillance is vital, while we advocate protection of occupied sand dunes, active engagement with farmers and landowners to ensure compliance with habitat maintenance recommendations and improved habitat connectivity to facilitate colonisation of artificial ponds.

2.2 Introduction

Declines in amphibian populations have been reported worldwide over the past few decades even in common and widespread species (Young *et al.* 2001; Stuart *et al.* 2004; Nyström *et al*. 2007; Adams *et al.* 2013; Grant *et al.* 2016; Petrovan & Schmidt 2016). Nowadays, amphibians are widely recognised as the vertebrate group with the highest proportion of species threatened with extinction assessed by IUCN (IUCN 2020). While there is little evidence to support one single global cause, local stressors include habitat destruction and fragmentation (Cushman 2006), contamination (Mann *et al.* 2009; Brühl *et al.* 2013), spread of pathogens (Berger *et al.* 1998; Lips 1999; Martel *et al.* 2013), invasive species (Johnson *et al.* 2011), illegal harvest and trade (Schlaepfer *et al.* 2005). Global factors such as climate change may pose an additional serious threat to amphibian populations worldwide and contribute to the ongoing amphibian crisis (e.g. Carey & Alexander 2003; Pounds *et al.* 2006; Griffiths *et al.* 2010). Responses to these stressors are context-dependent varying between populations and species (Blaustein & Kiesecker 2002), thus continued monitoring of declining populations is essential to assess extinction risk (O'Grady *et al*. 2004) and implement effective management to arrest declines, and where possible, restore populations.

Amphibians may experience high amplitude fluctuations in population size (Alford & Richards 1999; Marsh 2001; Newman & Squire 2001), hence assessing long-term population trends can be challenging (Williams *et al*. 2002; Green 2003). Anuran species breeding in highly variable environments like temporal ponds, are particularly difficult to monitor as they exhibit greater variation in population size than those

breeding in permanent water bodies or terrestrial habitats (Green 2003; Loman & Andersson 2007; Raithel *et al.* 2011). For many amphibian species surveys usually are conducted during the breeding season using counts of egg strings or spawn clumps (for females) or call vocalisations (for males). Egg-mass counts is a common method for monitoring pond-breeding amphibians in Europe (e.g. Grant *et al.* 2005; Paton & Harris 2009; Meek 2018). While the relationship between egg-mass counts and number of adult females and population size may vary over years (Richter *et al.* 2003; Greenberg & Tanner 2005), egg-mass count has been shown to be an easy solution for long-term monitoring of the annual reproductive effort and population health for some amphibians including the Natterjack toad (e.g. Loman & Andersson 2007; Raithel *et al.* 2011).

The Natterjack toad (*Epidalea calamita*) is widely distributed throughout Europe, ranging from the Iberian Peninsula in the west and as far east as the Baltic coast with several isolated populations in Ireland and Great Britain (Gasc *et al.* 1997). Despite its widespread distribution, the Natterjack toad population is generally declining with its range having contracted by >50% during the latter half of the 20th century (Beja *et al*. 2016). Population declines of common and widespread amphibian species are of particular concern as often those species are overlooked when setting conservation strategies (Sterrett *et al.* 2019). Recent surveys indicate that the species' range may have contracted even further, with very poor and irregular breeding activity recorded in the most westerly parts of its current range (Bécart *et al.* 2007). The species is listed on Annex IV of the EU Habitats and Species Directive with EU member states required to report regularly to the European Commission on

the species' conservation status under Article 17 [92/43/EEC]. The last two Article 17 reports assessed the species' conservation status throughout Europe as 'unfavourable' except for in the Mediterranean region (European Topic Centre 2012). In Ireland, the Natterjack toad is regionally Red-listed as Endangered (King *et al*. 2011) and is subject to considerable conservation efforts over the past decade aiming to increase its distribution range and population size. Long-term monitoring data and intensive management of the species provide an excellent opportunity to evaluate the impact of conservation efforts on a declining amphibian species.

Ireland has lost more than half of its farmland ponds since that late 19th century (Reid *et al*., 2014) with pond loss being identified as the single most important driver of Natterjack toad population declines (Beebee 2002; Rannap *et al.* 2007). One hundred new ponds were created on wet agricultural grasslands as part of the National Parks and Wildlife Service (NPWS) Pond Creation Scheme initiated in 2008. Under this scheme farmers are paid to dig ponds and maintain the surrounding land for toads. The programme's goal was to increase the number of suitable breeding sites within the species core range and to restore the toad's historical range. Breeding activity was recorded in 16% of constructed ponds during 2011-12 (Sweeney *et al*. 2013). In 2016, a head-start and translocation program was launched by NPWS in conjunction with Fota Wildlife Park, Co. Cork and Dingle Oceanworld, Co. Kerry. Egg strings and tadpoles were collected from the wild, grown through to metamorphosis in captivity and then returned to the wild in late summer supplementing existing populations and as part of an assisted colonisation effort to sites in the Pond Creation Scheme. Currently, there is no data available by which to assess the success of the

program, as breeding will occur 4 to 5 years post release when toads have reached sexual maturity and return to the ponds for breeding (Beebee 1979). Other methods like eDNA (Reyne *et al.* 2021) could be used to monitor artificial pond colonisation rate by detecting species presence before field signs of colonisation (breeding) are observed.

In this study, we assessed the population breeding performance and temporal trend of the most range restricted and rarest amphibian species in Ireland, the Natterjack toad. We used data from 14 years of intensive monitoring and surveillance to quantify temporal trends of the Natterjack toad's egg string production and recruitment in Ireland. We also evaluated the success of the habitat restoration measurements on the toad's productivity, analysed breeding site preferences and identified perceived threats and pressures. We discuss the importance of our results in the context of the global amphibian crisis and make recommendations for species conservation strategies.

2.3 Methods

2.3.1 Study area

The Natterjack toad is highly range restricted in Ireland, found naturally only in County Kerry, Ireland (Figure 2.1). All areas where Natterjack toads were recorded in the past were monitored. Additionally, all 100 newly constructed ponds in the NPWS Natterjack toad Pond Creation Scheme were regularly (see below) surveyed in order to provide assessment of the success of the habitat restoration program. Natterjack toad breeding ponds were shallow (<1m) but varied in origin, hydroperiod and size. Natural ponds that were traditional breeding sites included large ephemeral pools that formed in sand dune slacks (Magharees, Inch and Caherdaniel), permanent ponds on links golf courses (Dooks Golf Course and Castlegregory Golf Course) and shallow bay areas along lake shores (Lough Gill, Lough Yganavan and Lough Nambrackdarrig). All artificial ponds in the NPWS Pond Creation Scheme were created on wet agricultural grassland within farm fields. Often grazing livestock were present to maintain a short sward in compliance with the habitat management recommendations of the Scheme.

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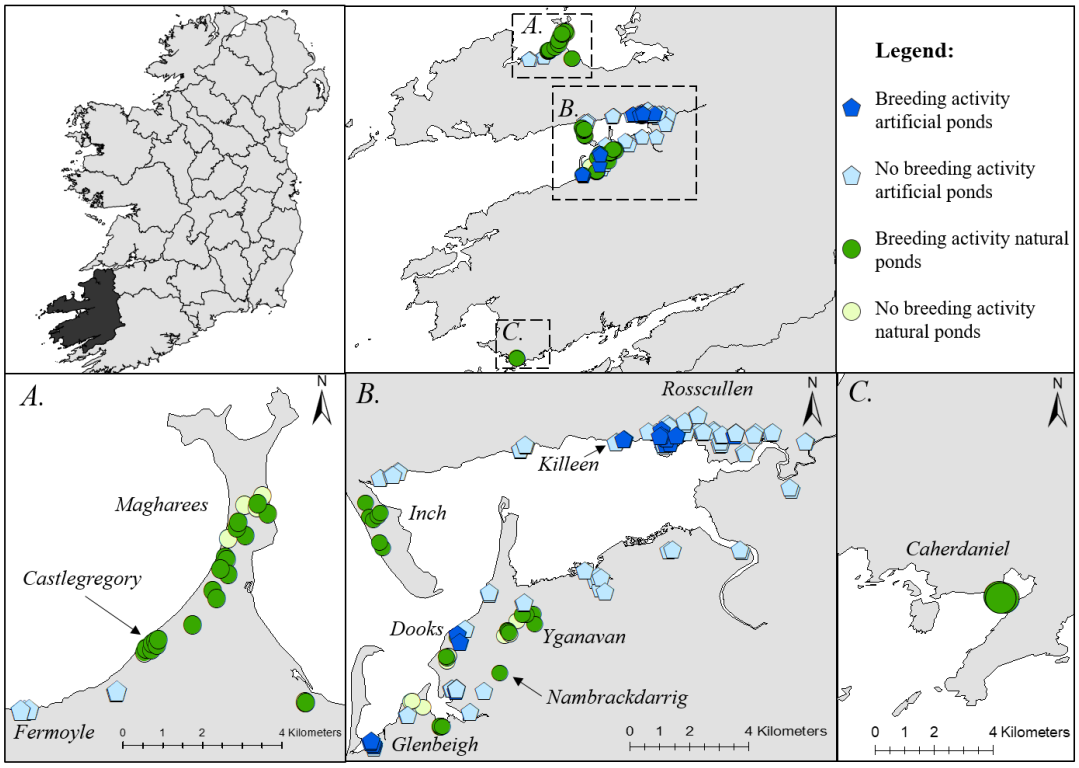

Figure 2.1 Map of the study area showing locations of the surveyed ponds.

2.3.2 Population monitoring

We used two measurements to monitor Natterjack toad reproduction. Egg string count was used as a proxy of the female breeding population size and toadlet abundance as an indicator of breeding success. Even though egg string counts cannot be used to directly calculate population size (Schmidt 2004, 2005), they were used to monitor relative change in the number of females spawning over time assuming constant detection probability.

Ponds were visited every seven to ten days through the duration of the breeding season (April – July) in 2016-18. This visit interval was chosen to ensure that egg strings could not be deposited and hatch between visits resulting in missed egg strings. We recorded the total number of egg strings in each pond by walking the

perimeter searching shallow water and aquatic vegetation. Surveys of deeper water away from the perimeter were made using a zigzag transect method. For each pond, we mapped egg string locations and stages of development based on Gosner stages (Gosner, 1960) in order to avoid double counting during consecutive visits. The earliest stage (Gosner stages 2-6) consisted of two lines of recently laid eggs; the second stage (Gosner stages 7-14) had a single line of eggs; and the third stage (Gosner stage 16) consisted of well-developed embryos with defined tails. It was assumed that each breeding female deposited one egg string (Buckley & Beebee 2004), thus, egg string counts were taken as a proxy of the number of breeding females.

Only sites where egg strings occurred were surveyed for toadlets. Quadrats ($0.25m^2$) were placed regularly every 10m along the water's edge up to a distance of 5m from the shore. Toadlet numbers in each quadrat were recorded. Toadlet abundance was calculated by multiplying mean toadlet density by the area surveyed i.e. density in toadlets/m^2 multiplied by the area surveyed around each pond based on pond circumference. The exact toadlet counts were used to estimate toadlet abundance for each breeding site and survey year. We investigated the relationship between toadlet abundance and number of egg strings using a Spearman Rank correlation.

2.3.3 Temporal trends

Linear trends in egg string counts were fitted to values of the current surveys 2016-18 and those from previous surveys i.e. 2004-06 (Becart *et al*. 2006) and 2011-12

(Sweeney *et al.* 2013). Average percentage change over time in egg string counts were calculated as a difference between the start and end of the fitted trend line and not the difference between the first and last values of egg string counts (to avoid comparing potential extremes). A temporal trend was described for each of twelve breeding sites and overall, for Ireland. We also calculated a temporal trend for Ireland excluding egg strings recorded at artificially created ponds, in order to evaluate the impact of the NPWS Pond Creation Scheme on species productivity.

2.3.4 Breeding site preferences

We selected 17 variables that best characterised Natterjack toad habitat to investigate the effects of aquatic and terrestrial parameters on breeding activity (Table 2.1). Differences in the environmental parameters were tested between ponds with Natterjack toad presence and absence using Mann Whitney-U tests. Presence was defined as ponds where breeding activity was observed at least once during the 2016-18 surveys, while absence was defined as ponds where Natterjack toad breeding activity was not detected during the same surveys. Each environmental variable was fitted as an independent explanatory variable in a Generalized Linear Mix Model (GLMM) where pond ID was the random factor and the number of egg strings was the dependant variable with multimodel selection used to choose the single best model from all subset regressions using the Akaike Information Criterion corrected for sample size (AICc), number of parameters and AIC weights (Burnham & Anderson 2002). GLMMs were run using IBM SPSS Statistics v24 (dependant variable had negative binomial distribution). All predictor variables were tested for

multicollinearity with one of a pair of highly correlated variables (correlation coefficient >0.7) excluded from analysis (the one with the lowest correlation coefficient with egg string numbers). Variables were standardized to have $\bar{x}$ =0 and σ =1 prior to the analysis.

2.3.5 Threats and pressures

Threats and pressures perceived as present at each breeding site were categorised according to criteria listed in the official European Union guidelines for the Natura 2000 Standard Data Form and recorded under EU Habitat Directive codes (Table 2.2) (European Topic Centre 2018). The count of each threat and pressure was also expressed as a percentage of all ponds and those with Natterjack toads present.

Table 2.1 Description of 17 environmental parameters collected as explanatory variables in the study.

Environmental parameter	Description
Pond type	Natural (0) and artificial (1).
Habitat	Surrounding terrestrial habitat type (within 100m radius of the pond) defined by Fossitt (2000); 1) agricultural grassland (GA1), 2) wet grassland (GS4), 3) fixed dunes (CD2) and 4) amenity grassland i.e. links golf courses converted from fixed sand dunes (GA2), 5) Scrub (WS1).
Activity	Three site management practises were recognized at the study area: farming, golf course management and discontinued use (abandonment).
pH	pH ± 0.05 measured with a Hanna Combo tester HI98129.
Conductivity	Conductivity (µS/cm) ± 2% measured with a Hanna Combo tester HI98129 ranging from 0 to 3,999 µS/cm.
Salinity	Salinity was measured with Extech RF20 portable refractometer in parts per thousands.
Dissolved Oxygen (DO)	Dissolved oxygen (mg/L) measured using a YSI 550A meter ranging from 0 to 50 mg/L.
Temperature	Water temperature (C°) ± 2% measured with a Hanna Combo tester HI98129
% aquatic plants $_{surface}$	Percentage of pond surface covered by floating aquatic plants.
% aquatic plants $_{substrate}$	Percentage of pond substrate covered by aquatic plants.
% plant litter	Percentage of pond substrate obscured by plant litter such as dead leaves.
% filamentous algae $_{substrate}$	Percentage of pond substrate covered by filamentous algae.
% emergent vegetation	Percentage of pond surface with emergent vegetation i.e. plants rooted in the substrate but projecting above the surface e.g. reeds.
% bare substrate	Percentage of pond substrate that was unvegetated e.g. bare sand.
Pond age	Artificial ponds varied in age from 9 years old (created in 2009) to 3 years old (created in 2015).
Surface area (m^2)	Pond length (*a*) and width (*b*) were measured by an Insight 1000 LH Laser Rangefinder. The two dimensions were used to estimate the surface area (*A*) by using the formula for an ellips*e*.
Area dried up (%)	The surface area that dried was estimated as the difference between the largest and smallest surface area that were recorded for each pond as measured every 2 weeks throughout the field survey in 2016. Results were converted into percentage of the largest measurement.
Predator abundance	Predator pressure was estimated as a total number of predatory macroinvertebrates (water beetles and their larvae, dragonfly and damselfly larvae, water bugs and leeches) in sweep and bottle trap samples per pond. For details see Reyne *et al.* (2020).

Table 2.2 List of pressures and threats to the Natterjack toad in Ireland according to the Article 17 report format of the Habitat Directive for the period 2013-2018.

Code	Description
A06	Abandonment of grassland management (e.g. cessation of grazing or of mowing). Sites with 80% of the surrounding sward higher >20cm.
I02	Other invasive alien species. Presence of New Zealand pygmyweed (*Crassula helmsii*) and sea buckthorn (*Hippophae rhamnoides*).
J01	Mixed source pollution to surface and ground waters (eutrophication). Presence of algal blooms, high conductivity as a result of decomposition of organic material, decreased water transparency.
K02	Drainage
L01	Abiotic natural processes (salinization). Ponds with water salinity above 4ppt.
L02	Natural succession resulting in species composition change (other than by direct changes of agricultural or forestry practices). Ponds with 80% of the surface covered with emergent vegetation.
L06	Interspecific faunal and floral relations (competition and predation). Predation was measured as the abundance of predatory invertebrates (for detailed methodology see Reyne et al. 2020). Competition was recorded as presence of Common frogs (*Rana temporaria*) and/or Smooth newts (*Lissotriton vulgaris*).

2.4 Results

A total of 168 ponds (68 natural and 100 artificially created ponds) were monitored from 2016 to 2018. During this period, we conducted 4,704 pond visits. There was substantial variation in the number of egg strings between years with 3,216 egg strings laid in 2016, 1,457 egg strings in 2017 and 2,685 egg strings in 2018. Natural breeding sites accounted for the vast majority (>90%) of egg string production with the single most productive area being the Magharees sand dune system (Appendix A: Table A.1). The Natterjack toad spawned in 22 artificial ponds over the three breeding seasons (not all occupied every year; Figure 2.1) with breeding in 16 ponds during 2016, 10 during 2017 and 13 during 2018.

The highest number of toadlets was recorded in 2018 (Appendix A: Table A.1). Toadlet abundance was highly correlated with egg string production (r_s=0.694, p<0.001, n=33; Appendix A: Figure A.1a) largely driven by the large numbers of egg strings and toadlets at the Magharees. This positive correlation remained statistically significant even after excluding the Magharees from the analysis although the relationship was marginally less strong (r_s=0.619, p<0.001, n=30; Appendix A: Figure A.1b).

Fecundity declined at almost all breeding sites from 2004 to 2018 (Table 2.3, Figure 2.2). The greatest declines over time (>90%) were observed at Roscullen Island and Dooks Golf Courses, with the small population at Fermoyle now believed to be locally extirpated. The only two populations with an increase egg string counts were the Magharees and Inch. However, the first systematic survey of Inch peninsula was only conducted in 2016 and since then new breeding ponds were discovered. Overall,

Natterjack toad egg string production declined by 23% (ranging from -100% to +900%) over a period of 14 years equivalent to -1.6% per year. The egg string productivity would have declined by 27% over the period of 14 years equivalent to -1.9% per year without the NPWS Natterjack toad Pond Creation Scheme (Table 2.3). At Roscullen Island and Killen breeding activity was recorded only at artificial ponds.

Natterjack toad breeding was significantly associated with ponds with a large surface area, neutral pH, high conductivity, high oxygenation, low plant litter, a high coverage of aquatic plants at the substratum and short grassland swards surrounding the pond edges (Table 2.4).

Pond type was strongly associated with habitat type (r_p=0.822, p<0.001) and land management activity (r_p=0.689, p<0.001) i.e. artificial ponds were mostly constructed on marginal grasslands used for agriculture, while conductivity and salinity were highly correlated (r_p=0.721, p<0.001). We, therefore, excluded pond type, land management activity and salinity from further analysis. When environmental variables were fitted simultaneously in a single GLMM, those variables in the single best model, suggested number of egg strings varied significantly between habitat types (Table 2.5) with sand dune ponds being most productive (Appendix A: Figure A.2). Breeding was more likely in ponds with a large surface area, high conductivity with a high coverage of aquatic plants at the substratum, suggesting these are the key combination of conditions most strongly determining productivity. Number of egg strings had a positive trend with the coverage of aquatic plants at the surface and a negative trend with aquatic predator abundance (numbers of predatory aquatic macroinvertebrates); and whilst both variables were included in the single

best model neither was statistically significant at the conventional 95% level yet their inclusion suggests they may contribute to some variation in breeding activity.

The main threats and pressures that the Natterjack toad is perceived to face at breeding sites was poor water quality i.e. toxic, or deoxygenating algal blooms, abandonment of agricultural land and lack of grazing (Figure 2.3). Invasive species including New Zealand pygmyweed (*Crassula helmsii*) and sea buckthorn (*Hippophae rhamnoides*) were present at five breeding sites. High salinity (>4 ppt) as a result of sea water incursion was recorded at four breeding sites making them not suitable for breeding and resulting in dead egg strings. Water drainage was only observed directly once in 2016 at the Magharees that led to a total loss of 485 egg strings. However, it is possible that some of the ponds that dried up might have been impacted by ground water abstraction for agricultural or recreational purposes. The frequency of threats did not differ between all ponds and the subset of ponds used by the Natterjack toad for breeding (p>0.05; Figure 2.3).

Table 2.3 Natterjack toad egg string count for each breeding site and overall, in Ireland (Co Kerry). The slope of linear trends (see Figure S1) are presented as the average change from the start and end of the line of best fit (not the beginning and end value for numbers of observed egg strings) and have been standardised to have a mean of zero and a standard deviation of 1 allowing direct comparisons of coefficients. Similarly, average percentage change values (%) represent the difference in the fitted line not the raw data.

Breeding site	**2004**	**2005**	**2006**	**2011**	**2012**	**2016**	**2017**	**2018**	**β ± SE**	**% change**
Magharees	228	983	1,183	381	224	2,261	386	1,519	0.063 ± 0.068	+114
Castlegregory Golf Course	573	868	992	472	421	354	495	405	-0.132 ± 0.049	-54
Fermoyle	3	0	0	0	0	0	0	0	-0.091 ± 0.062	-100
Tullaree	12	35	51	23	1	1	3	16	-0.109 ± 0.057	-87
Inch	-	-	-	-	-	17	18	392	0.867 ± 0.498	900
Killeen	-	-	-	7	23	12	6	8	-0.142 ± 0.166	-63
Roscullen island	91	532	873	17	220	79	50	64	-0.105 ± 0.059	-96
Dooks Golf Course	45	568	209	10	2	50	54	22	-0.098 ± 0.061	-96
Yganavan	219	269	419	66	101	155	146	23	-0.125 ± 0.052	-77
Nambrackdarrig	8	12	16	0	0	1	9	1	-0.108 ± 0.058	-87
Glenbeigh	52	67	55	11	23	44	59	11	-0.083 ± 0.064	-48
Caherdaniel	98	333	313	102	92	242	231	224	-0.001 ± 0.073	-6
Total Ireland (excluding Pond Creation Scheme)	1,329	3,667	4,111	1,059	923	3,095	1,363	2,608	-0.033 ± 0.070	-27
Total Ireland	**1,329**	**3,667**	**4,111**	**1,089**	**1,107**	**3,216**	**1,457**	**2,685**	**-0.035 ± 0.071**	**-23**

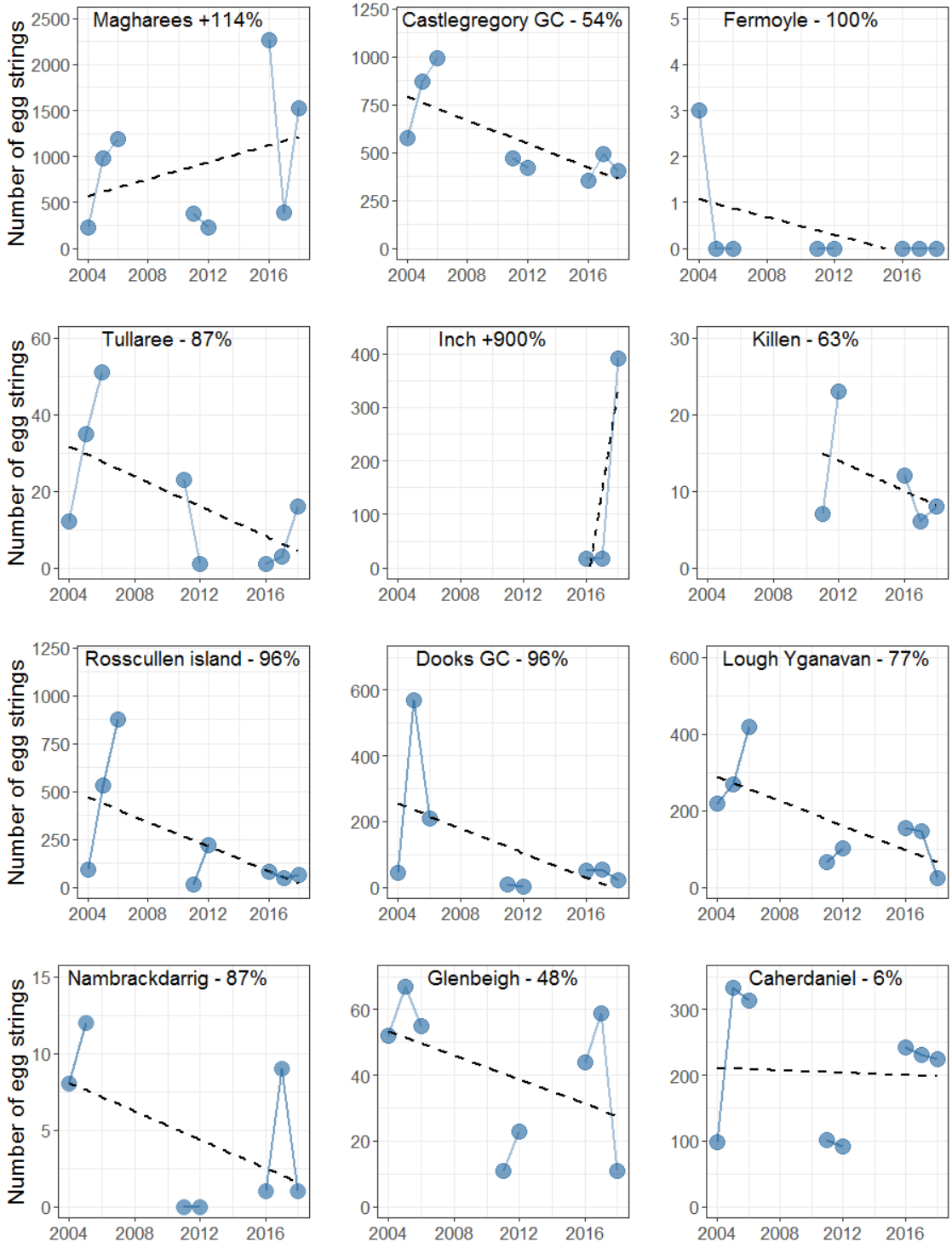

Figure 2.2 Number of egg strings recorded at each breeding site along with the linear trend and percentage change.

Table 2.4 Mean ± 1 standard deviation (SD) values of environmental parameters associated with ponds with and without Natterjack toads and the statistical results for a test of difference. Significant values are marked with an asterisk.

Environmental parameter	Natterjack toad		Mann Whitney		
	Absence	Presence	U	Z	*p*
Size					
Surface area (m^2)	106.4 ± 295.0	1152.5 ± 2405.4	9802.5	-9.052	<0.001*
Area that dried (%)	60.6 ± 27.7	63.4 ± 30.3	21225.0	-1.418	0.156
Water					
Temperature	16.0 ± 2.0	15.6 ± 1.6	23817.0	-0.132	0.895
pH	6.7 ± 0.7	7.4 ± 0.7	11379.0	-9.383	<0.001*
Conductivity (µS/cm)	502.9 ± 917.3	618.4 ± 671.5	15253.5	-6.501	<0.001*
Salinity (ppt)	1.8 ± 2.5	1.6 ± 1.5	22513.5	-1.102	0.270
Oxygen					
mg/l	6.7 ± 2.4	7.2 ± 2.4	16720.5	-5.410	<0.001*
% saturation	65.5 ± 23.1	78.8 ± 39.3	16268.5	-5.746	<0.001*
Predator abundance	62.5 ± 50.4	65.1 ± 60.1	8873.0	-1.057	0.291
Vegetation (%)					
Emerged vegetation	24.6 ± 25.7	28.3 ± 26.7	22489.5	-1.134	0.257
Bare substrate	31.5 ± 31.6	17.5 ± 23.7	21624.0	-1.685	0.092
Aquatic plants $_{Surface}$	15.7 ± 22.5	18.3 ± 24.6	23510.5	-0.386	0.699
Plant litter	44.4 ± 32.9	35.6 ± 29.4	17936.5	-4.489	<0.001*
Aquatic plants $_{substrate}$	24.8 ± 28.2	53.4 ± 36.2	15654.5	-6.317	<0.001*
Filamentous algae $_{substrate}$	6.6 ± 12.7	7.9 ± 17.3	23058.0	-0.845	0.398
Sward height (%)					
<5cm	18.9 ± 23.6	34.5 ± 27.8	17331.5	-5.058	<0.001*
5-20cm	27.8 ± 25.2	21.6 ± 21.9	19983.5	-3.028	0.002*
>20cm	53.1 ± 30.7	43.6 ± 27.6	21336.0	-1.988	0.047*

Table 2.5 GLMMs results for associations between environmental parameters and number of egg strings. Significant values are marked with an asterisk.

Environmental parameters	*F*	*β* ± SE	n.df.	d.df.	*p*
Model	19.983	2.411 ± 0.449	9	441	<0.001*
Habitat type	26.145	Multifactorial	4	441	<0.001*
Conductivity	8.152	0.743 ± 0.260	1	441	0.005*
Aquatic plants surface	0.101	0.044 ± 0.140	1	441	0.751
Aquatic plants substrate	12.988	0.545 ± 0.151	1	441	<0.001*
Surface area	0.572	0.610 ± 0.359	1	441	0.090
Predator abundance	2.886	-0.151 ± 0.199	1	441	0.450

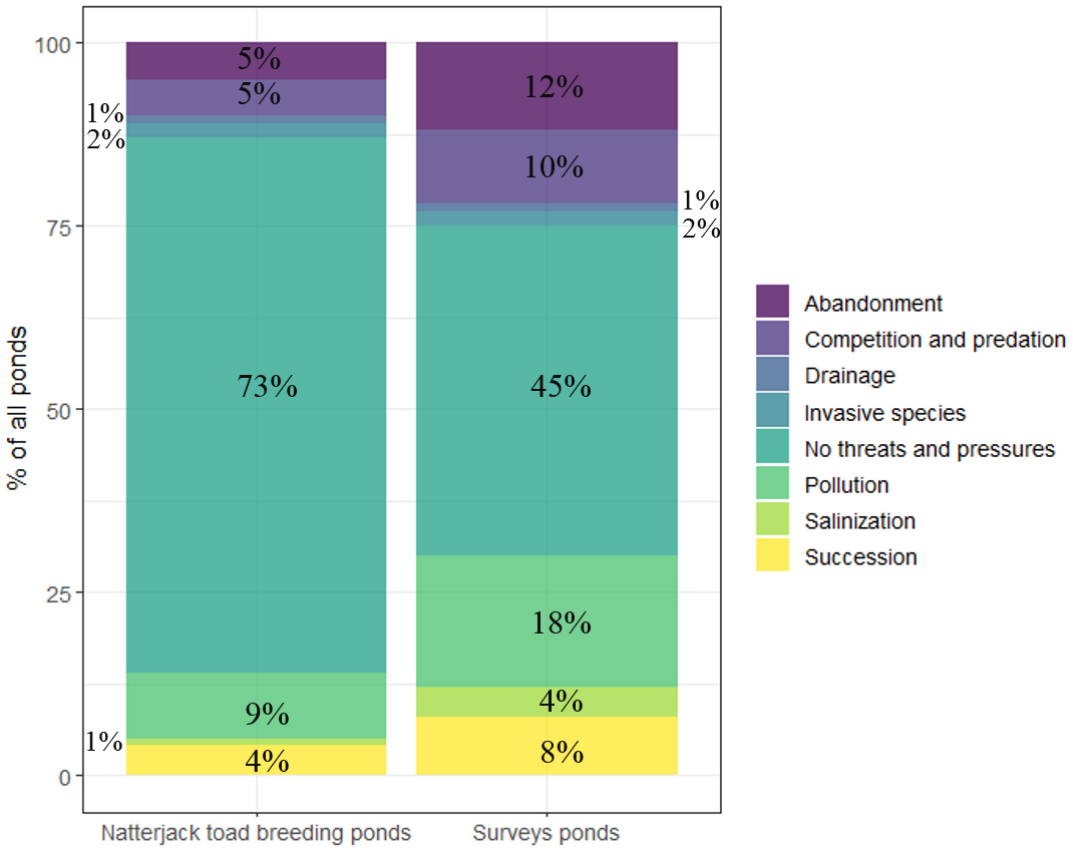

Figure 2.3 Threats and pressures to the Natterjack toad in Ireland according to the Article 17 report format of the Habitats Directive

2.5 Discussion

Egg string production declined by 23% over 14 years (2004 to 2018) throughout Natterjack toad's highly restricted range in Ireland. Toadlet production (and by inference likely recruitment) was highly correlated withthe number of egg strings. The species may be locally extirpated from at least one site where it bred previously. Despite 22% of artificially created ponds being occupied, they accounted for <10% of all eggs laid and had reduced declines by only 4%. It seems likely this may be related to artificial ponds providing a less favoured environment which was shown to be significantly different from the conditions of natural ponds. Natterjack toads were perceived to be at greatest threat from poor water quality and lack of suitable terrestrial habitat maintenance around breeding sites. Without addressing the prevalence and impact of threats and pressures, and ensuring any ponds created in future more closely approximate the conditions of natural ponds, further declines in the reproduction success and population seem likely.

Long-term monitoring and surveillance are necessary to detect changes in population trends, especially for pond breeding amphibians where interannual high fluctuations in breeding activity can be pronounced (Buckley & Beebee 2004). Short-term variations in egg string counts for example as a result of different climatic conditions (Smith & Skelcher 2019), historical hydroperiod, variation in predation pressure or generational lag effect (Di Minin & Griffiths 2011) can be hard to distinguish from the genuine underlying temporal trends if long-term data are not available, leading to erroneous interpretation of change. During the current study, egg string production varied markedly between survey years. Egg string numbers

declined by 55% from 2016 to 2017 before increasing by 85% during 2018. Interannual variability reflects variability in female breeding effort as much as true change in egg string production which broadly appears to be in decline both long-term (2004-2018) and short-term (2016-18). In some populations (i.e. Roscullen Island and Dooks Golf Course) egg string production declined by >90% with no evidence of breeding at one small population (i.e. Fermoyle) since 2004. However, not all populations declined. Number of egg strings at the largest population at Magharees sand dune system increased by 114% but this was insufficient to offset the sum decline among other populations. Creation of artificial breeding pools in sand dunes can provide additional suitable breeding sites for the Natterjack toad and aid successful metamorphosis (Baker *et al.* 2011; Buckley *et al.* 2014; Smith & Skelcher 2019), thus ensuring toadlet recruitment even in dry years when ponds are particularly ephemeral.

Egg string production may not directly reflect population recruitment, hence toadlet abundance was also examined as an additional indicator of Natterjack toad productivity. Toadlet abundance was highly correlated ($r^2=0.90$) with egg string counts and thus was largely redundant as a measure of reproduction given it exhibited much the same variation as egg string counts whilst requiring substantial additional survey effort. Long-term studies on the Natterjack toad in Great Britain found similar positive relationship between egg string counts and toadlet production (Beebee & Buckley 2014; Smith & Skelcher 2019) suggesting that simply monitoring egg string numbers only as indicator of reproductive effort is the most effective use of time and resources.

Presence of Natterjack toad breeding activity was mostly associated with natural ponds (traditional breeding sites). Over 78% of breeding activity was recorded on sand dune systems with Natterjack toads generally being associated with coastal habitats elsewhere within their range utilising sandy soil for burrowing (Beebee 2002). Natterjack toad presence was associated with ponds of neutral (rather than acidic) pH; high oxygenation facilitating respiration and rapid development of eggs and tadpoles; high conductivity indicative of high primary production and high aquatic plant and litter coverage of the substrate providing underwater refuges and a food source for tadpoles (Kopp *et al*. 2006). Natterjack toads were also associated with short terrestrial vegetation in the immediate vicinity of the pond (within 100m) raising the issue of appropriate management which should include regular grazing to ensure swards are well maintained. Protection of sand dune systems is essential for the survival of the Natterjack toad in Ireland. Targeted moderate grazing by cattle can be beneficial for grassland management on sand dunes, however, intensive grazing can be damaging by negatively impacting the hydroperiod of ponds formed in sand dune slacks and the quality (nutrient enrichment) of water; consequently, negatively impacting tadpole survival (Bridson 1978). Maximising the benefits of conservation grazing requires site-specific management measures and these are best arranged by landowner and farmer agreement after stakeholder engagement.

The NPWS Pond Creation Scheme has created over 100 new ponds in agricultural grasslands within the range of the Natterjack toad in Ireland. Of these only 22 have been successfully colonised to date. At Roscullen Island and Killeen breeding was exclusively in artificially created ponds, suggesting that the species might have been locally extinct at those two sites if no conservation measures were

implemented. Amphibians often have a patchy distribution at a landscape level (Marsh & Trenham 2001) and persistence in the landscape depends on dispersal and colonisation of suitable habitats (Semlitsch 2002). Dispersing Natterjack toadlets choose environments with less resistance and show a preference for open areas (e.g. bare sand) actively avoiding agricultural environments (grassy fields) where locomotion is impeded by vegetation density (Stevens *et al*. 2006). Thus, a relatively small proportion of newly created ponds in farmland have been colonised successfully, suggesting not only that a small proportion of the constructing ponds have suitable breeding condition, but most of the ponds might be located too far away from the source population. Indeed those ponds that have been successfully colonised are in close proximity to the likely source population. For instance, the most productive artificially created ponds at Roscullen Island were in close proximity to two natural breeding ponds that previously existed in the area. Pond Creation Schemes aiming to conserve amphibians should take into account presence of vital source populations and suitable pathways for dispersal when creating new habitat or consider assisted migrations in order to overcome problems impeding dispersal and colonisation.

While natural ponds were in reasonable condition (at 73% of natural ponds no threats or pressures were detected), most of the artificial ponds were in a poor state. In species with highly restricted ranges like the Natterjack toad the only opportunity for habitat creation is often in suboptimal habitat (in this case agricultural grasslands and abandoned farmlands) around the fringes of the current range, as the latter is already saturated. Hence, active management of the artificial ponds is essential to mitigate some of the existing threats and pressure and help offset future reductions

in egg string production and recruitment (Di Minin & Griffiths 2011). Land abandonment and poor sward management are known threats (Rannap *et al.* 2007) as is water pollution. These issues need to be addressed through active engagement with farmers and landowners to ensure compliance with habitat maintenance recommendations and to reduce agricultural runoff causing waterbody eutrophication.

Natterjack toad monitoring and conservation in Ireland is a good example of long-term collaboration between researchers, private organisations, conservation practitioners and landowners. Unfortunately, despite substantial conservation efforts the species is still in decline highting the complexity of amphibian conservation. Major challenges still remain regarding development of reliable monitoring methods, accessing amphibian trends with a high degree of confidence at a wider scale and identification of the main drivers of population declines. Pond restoration practises may fail to provide suitable breeding habitats as a result of poor planning, lack of regional scale connectivity and low compliance with management recommendations especially on private land. In the case of the Natterjack toad a combination of continued monitoring of breeding success, management of breeding sites especially regarding maintenance of short swards and good water quality, protection of sand dunes, improved habitat connectivity (including assisted migration) to facilitate colonisation of artificial ponds, active stakeholder engagement with local farmers and landowners and further research on survival and recruitment of toadlets raised in captivity are needed to prevent continued decline and to safeguard the species at the north-western edge of its European range.

Chapter 3

Artificial agri-environment scheme ponds do not replicate natural environments despite higher aquatic and terrestrial invertebrate richness and abundance

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3.1 Abstract

Farmland ponds are a highly threatened freshwater habitat which has undergone dramatic losses during the last 200 years. Agri-environment schemes (AES) incentivise farmers to adopt farming methods to benefit biodiversity, yet there are a paucity of data evaluating the success of artificially created AES ponds as analogues of natural ponds in an attempt to recreate lost environments. We examined variation in environmental parameters and aquatic and terrestrial invertebrate communities between 38 natural ponds and 91 artificial ponds that were created in south-west Ireland. Artificial ponds in agricultural grassland did not replicate natural ponds in adjacent semi-natural habitats differing significantly in size, pH, conductivity, productivity and surrounding vegetation structure i.e. sward height. These differences significantly influenced aquatic and terrestrial invertebrate community structure with a suite of indicator taxa in both natural and artificial ponds. The conservation value of artificial ponds in agricultural grasslands should not be underestimated as they had 43% higher aquatic species richness and 33% higher aquatic species abundance than natural ponds in adjacent semi-natural habitats. We demonstrate that artificial agri-environment scheme ponds created in agricultural grasslands, whilst not direct analogues of natural ponds in adjacent semi-natural habitats, do fulfil a role in preserving high local biodiversity albeit representing a different community of species. Creation of ponds in farmland as well as in adjacent natural habitats could provide a wider range of environmental conditions and richer associated macroinvertebrate communities, increasing landscape connectivity and further enhancing regional biodiversity.

3.2 Introduction

Agricultural intensification raises concerns about biodiversity loss and degradation in associated ecosystem service delivery (Smith *et al.* 2002; Deacon *et al.* 2018). Agri-Environmental Schemes (AES) aim to reverse the negative consequences of converting natural habitats to agriculture by compensating farmers for financial losses associated with modifying agricultural practises to benefit biodiversity (Weibull *et al.* 2003; Whittingham 2011). AES have become key to environmental and agricultural policy and are one of the main mechanisms by which wildlife conservation projects are financed (Batary *et al*. 2015). AES yield mixed outcomes with most lacking adequate monitoring resulting in a paucity of data by which to assess success (Kleijn *et al.* 2003). Nevertheless, in some instances AES are capable of reversing population declines whilst increasing species richness and abundance of common taxa (Kleijn *et al.* 2006; Perkins *et al.* 2011). Most of the successful AES have focused on terrestrial taxonomic groups, for example, birds (Batary *et al*. 2015). While some work has been done on lotic systems (e.g. Jones *et al.* 2017), little is known on the impact of AES on freshwater ponds and associated aquatic biodiversity.

Freshwater ponds are important habitats for aquatic biodiversity. This is particularly the case in agricultural landscapes where ponds act as dynamic sinks and sources of regional biodiversity within an otherwise uniform, monocultural matrix (Davies *et al.* 2008; Sayer *et al*. 2012; Céréghino *et al.* 2014). The number and distribution of ponds throughout Europe have undergone dramatic declines; for example, Ireland lost 54% of its farmland ponds between the late 1800s and the early 2000s because of agricultural intensification and large-scale land drainage schemes

(Reid *et al.* 2004). Pond degradation reduces habitat quality, increases aquatic habitat fragmentation and leads to loss of associated freshwater species (Wood *et al.* 2003). Loss of suitable breeding ponds can negatively affect amphibian population dynamics (Gibbs *et al.* 2005), species abundance (Hartel *et al.* 2010) and richness (Simon *et al.* 2009) with similar results for other taxonomic groups, for example, dragonflies (Kadoya *et al.* 2008).

In an attempt to recreate lost freshwater habitats, artificially created AES ponds are constructed on farmland throughout Europe each year as analogues of natural ponds; now largely restricted to adjacent non-agricultural habitats (Batary *et al.* 2015). Artificial ponds play an important role in metapopulation dynamics, serving as steppingstones for dispersal (Casas *et al.* 2012) and can sustain an important fraction of regional aquatic diversity, making a significant contribution to freshwater biodiversity (Ruggiero *et al.* 2008; Oertli 2018; Williams *et al.* 2020) and benefiting non-aquatic species such as bats by providing foraging habitats (Sirami *et al.* 2013). However, little is known about the degree to which artificial ponds replicate the biotic and abiotic environments of natural ponds and, therefore, their contribution to habitat and landscape restoration.

Ecosystem restoration is the practice of renewing and restoring damaged or destroyed ecosystems by active human intervention. Due to their complexity, it is often impossible to restore a degraded ecosystem to a state of pre-anthropogenic impact, and thus, all ecological restoration projects result in the creation of novel environments that, at best, mimic some natural analogue (Lundholm *et al.* 2010). Natural and restored environments frequently differ in, for example, climatic

conditions, soils, community structure and species interactions (Pickett *et al.* 2001). Identifying the abiotic and biotic differences between natural habitats and their artificial replicas may help inform conservation management practises (Lundholm *et al.* 2010).

This study aimed to test the efficacy of the agri-environmental pond creation scheme for the Natterjack toad nested in agricultural grasslands in replicating the conditions of natural ponds in the same landscape, now largely restricted to adjacent semi-natural habitats. While the pond creation scheme did not prove to be beneficial to the Natterjack toad, it can play an important role in enhancing biodiversity on farmland. The specific objectives were to compare environmental parameters, aquatic and terrestrial invertebrate species richness, abundance and community structure, and to establish indicator species that differentiated natural and artificial communities. Explicitly quantifying artificial pond biodiversity is of value in demonstrating the effectiveness of restoration ecology highlighting both the limitations and successes of creating artificial analogues of natural environments.

3.3 Methods

A total of 129 ponds were surveyed in Co. Kerry, Ireland; 38 natural ponds and 91 artificially constructed ponds created as part of an existing National Parks and Wildlife Service (NPWS) Pond Creation Scheme initiated in 2008 (Appendix C: Figure C.1), funded by the Republic of Ireland Government. Any experiment as a pure scientific investigation of the differences between natural and artificial ponds would necessitate a fully factorial design avoiding any confound between pond type and habitat type. Yet here, natural ponds occurred mainly in sand dune habitat. Ponds within links golf courses (hereafter, amenity grassland) were natural sand dune ponds, thus natural in this instance refers to the origin of the pond and not the surrounding habitat. Artificial ponds were created exclusively in adjacent coastal grasslands. In this real-world scenario, AES ponds were constrained to agricultural grasslands. Our comparison between natural and artificial ponds is, nevertheless, valuable, not despite of, but because ponds occupied different habitats. In the studied landscape, almost 100% of farmland ponds had been historically drained (Reid *et al.* 2014). Artificial ponds in agricultural grassland were typically on sandy soils within <200m of the coastal margin and spatially adjacent to remaining semi-natural habitats containing natural ponds within the same overall landscape. All ponds, whether natural or artificial were shallow (<1m deep). Government funded the current study in order to assess the efficacy of their pond creation scheme and thus, our study quantifying the value of artificial ponds for biodiversity was deemed of applied value.

3.3.1 Invertebrate sampling

The aquatic macroinvertebrate assemblage of each pond was surveyed using an approach combining sweep netting and baited bottle trapping. This combined approach enabled a comprehensive estimate of taxa richness (following e.g. Becerra-Jurado *et al*. 2008). Sampling was conducted during June and July 2016 with both samples pooled within each pond. In some cases, due to low water levels, only sweep netting was used and thus only ponds where both types of sampling were successful were used in analyses lowering our final sample size to 86 ponds. The terrestrial invertebrate assemblage of each pond was surveyed using pitfall traps. Traps were left *in-situ* for a period of four weeks (between late April and late May 2017). At the time of sampling, most of the artificial ponds (78%) were eight or nine years old, with the remaining ponds being less than 8 years old. For a detailed outline of the sampling procedure for aquatic and terrestrial macroinvertebrates see Appendix C.

We recognise that our trapping methods may not have sampled the entire invertebrate community uniformly, but our aim was not a definitive parochial species list per pond but relative comparison with all ponds sampled using the same method during the same season.

3.3.2 Environmental parameters

A total of 17 environmental parameters were collected to describe the aquatic and terrestrial environment associated with each pond (Table 3.1). Continuous measurements were taken once a month for each pond throughout the study period and the mean recorded.

Table 3.1 Description of 15 environmental parameters collected as explanatory variables in the study.

Environmental parameter	Description
Pond type	Natural (0) and artificial (1).
Habitat	Surrounding terrestrial habitat type (within 100m radius of the pond) defined by Fossitt (2000); 1) improved agricultural grassland (GA1), 2) wet grassland (GS4), 3) fixed dunes (CD2) and 4) amenity grassland i.e. links golf courses converted from fixed sand dunes (GA2).
Activity	Three site management practises were recognized at the study area: farming, golf course management and discontinued use (abandonment).
pH	pH ± 0.05 measured with a Hanna Combo tester HI98129.
Conductivity	Conductivity (µS/cm) ± 2% measured with a Hanna Combo tester HI98129 ranging from 0 to 3,999 µS/cm.
Salinity	Salinity was measured with Extech RF20 portable refractometer in parts per thousands.
Dissolved Oxygen (DO)	Dissolved oxygen (mg/L) measured using a YSI 550A meter ranging from 0 to 50 mg/L.
% aquatic plants $_{surface}$	Percentage of pond surface covered by floating aquatic plants.
% aquatic plants $_{substrate}$	Percentage of pond substrate covered by aquatic plants.
% plant litter	Percentage of pond substrate obscured by plant litter such as dead leaves.
% filamentous algae $_{substrate}$	Percentage of pond substrate covered by filamentous algae.
% emergent vegetation	Percentage of pond surface with emergent vegetation i.e. plants rooted in the substrate but projecting above the surface e.g. reeds.
% bare substrate	Percentage of pond substrate that was unvegetated e.g. bare sand.
Pond age	Artificial ponds varied in age from 9 years old (created in 2009) to 3 years old (created in 2015).
Surface area (m^2)	Pond length (*a*) and width (*b*) were measured by an Insight 1000 LH Laser Rangefinder. The two dimensions were used to estimate the surface area (*A*) by using the formula for an ellips*e*.
Area dried up (%)	The surface area that dried was estimated as the difference between the largest and smallest surface area that were recorded for each pond as measured every 2 weeks throughout the field survey in 2016. Results were converted into percentage of the largest measurement.
Presence of vertebrate predators	Vertebrate predator (fish and amphibians) presence/absence data recorded for each pond during invertebrate surveys.

3.3.3 Statistical analysis

Single variable tests of differences in the mean values of environmental parameters were used to describe variation in pond environments i.e. Mann Whitney-U between pond types and Kruskal-Wallis χ2 tests between habitat types. The proportion of ponds that where loosely categorised as permanent (taken here as <50% of the surface dried) and ephemeral (>50% dried) were compared using a χ^2 test of association. The relationship between the surface area of each pond that dried and species richness and abundance was investigated using linear regression.

Sample-based rarefaction curves were generated and compared to account for the uneven sample size between natural and artificial ponds. Violin plots were used to visualise results as they explicitly display data density. These analyses were performed using the package *vegan* for R3.6.3 (Oksanen *et al.* 2019; R Core Team 2019).

Variation in aquatic and terrestrial invertebrate species richness and abundance were examined using Generalized Linear Models (GLMs). Multimodel selection was used to choose the single best approximating model from all subset regressions using the Akaike Information Criterion corrected for sample size (AICc). Only models within two AICc units of the best model were considered. GLMs were run using IBM SPSS Statistics v24.

Multivariate ordination analyses were used to examine variation in aquatic and terrestrial invertebrate community composition using PRIMER6 with PERMANOVA$^+$ software. Permutational multivariate analysis of variance (PERMANOVA) was used to compare community assemblages between different pond and habitats types.

Distance-based Linear Models (DistLMs) were used to explain variation in the macroinvertebrate community based on environmental parameters using resemblance tables and permutations with the best model selected based on the lowest AICc value. Distance-based Redundancy Analysis (dbRDA) biplots created using *ggplot2* (Wickham 2016) were used to visualise patterns in similarity between pond or habitat types. We also created density plots for each dbRDA axis and calculated significance levels to visualise how much variation in the dataset was accounted for by each axis. Full details are provided in Appendix C.

Indicator Species Analysis (ISA) was used to identify taxa responsible for differences in invertebrate assemblages between pond or habitat types. ISA produces an Indicator Value (IV) based on the concept that an ideal indicator species will be found exclusively within a given group (McCune & Grace 2002). Indicator values were tested for statistical significance using a randomised Monte-Carlo test. Analysis were performed in PC-Ord v6.0 (McCune & Grace 2002). The association between the relative abundance of each identified indicator species with the environmental parameters was analysed using all subset regression and GLM as described above.

3.4 Results

In total, 56 aquatic macroinvertebrate species (Appendix C: Figure C.2a) were recorded from 86 ponds of which 19 (22%) were natural and 67 (78%) artificial ponds. In total, 87 spider species (Appendix C: Figure C.2b) were recorded at 126 ponds (pitfall traps at 3 ponds caught no spiders) of which 37 (29%) were natural and 89 (71%) artificial ponds.

The surface area of artificial ponds was typically an order of magnitude smaller than natural ponds (Table 3.2). Artificial ponds had significantly lower pH and conductivity, had less emergent and substratum vegetation including filamentous algae and were surrounded by a lower percentage cover of short sward. Ponds varied significantly in size between habitat types being substantially larger in fixed dunes and amenity grasslands while smallest in improved and wet grasslands (Appendix C: Table C.2). Surrounding sward heights varied significantly between habitat types with wet grasslands having the tallest vegetation.

Table 3.2 Mean values ± 1 standard deviation (SD) for environmental parameters associated with natural and artificial ponds and a test of difference

Environmental parameter	Pond type		Mann Whitney		
	Natural	Artificial	U	Z	*p*
Size					
Surface area (m^2)	452.8 ± 620.7	46.9 ± 22.2	384.5	-4.126	0.028
Area that dried (%)	59.0 ± 28.5	42.0 ± 24.2	420.0	-2254	0.024
Water					
pH	7.4 ± 0.6	6.7 ± 0.7	166.5	-4.555	<0.001
Conductivity (µS/cm)	998.7 ± 1141.6	494.0 ± 808.6	278.0	-3.345	0.001
DO (mg/l)	5.9 ± 2.8	6.0 ± 2.2	562.5	-0.260	0.765
Vegetation (%)					
Emerged vegetation	46.6 ± 31.7	25.2 ± 26.1	405.5	-1.976	0.048
Bare substrate	12.1 ± 22.3	20.2 ± 25.8	443.5	-1.639	0.101
Aquatic plants surface	26.3 ± 35.0	19.9 ± 28.8	540.5	-0.529	0.597
Plant litter	38.9 ± 33.8	52.1 ± 35.4	496.5	-0.987	0.323
Aquatic plants	62.1 ± 44.4	34.3 ± 35.8	334.5	-2.790	0.005
Filamentous algae	6.8 ± 12.0	3.0 ± 9.7	433.0	-2.344	0.019
Sward height (%)					
<5cm	36.5 ± 28.3	21.2 ± 25.0	1098.5	-3.318	0.001
5-20cm	28.0 ± 23.4	10.5 ± 17.1	837.5	-4.690	0.001
>20cm	49.9 ± 30.1	51.0 ± 27.9	1712.0	-0.088	0.930

Aquatic macroinvertebrate species richness and abundance differed significantly between pond types (Table 3.3) even after accounting for unbalanced sampling using rarefaction (Figure 3.1). Artificial ponds communities were 43% more species rich (artificial: 7.2, 95% CI: 6.5-7.9; natural: 4.2, 95% CI: 3.1-5.3) with a 33% higher abundance (artificial: 37.4, 95% CI: 30.2-44.6; natural: 21, 95% CI: 9.6-32.4). Aquatic species richness was positively related to the percentage cover of substratum aquatic plants whilst both richness and abundance showed a negative trend with conductivity (Table 3.3). Aquatic species abundance was negatively related to the percentage of plant litter while positively related to the area of the pond that dried up. Habitat was not retained in the top GLMs of aquatic macroinvertebrate richness and abundance (Table 3.3) yet rarefaction suggested significant variation (p<0.001) after accounting for unbalanced sampling (Figure 3.1a) with highest richness and abundance at ponds in agricultural grasslands. Furthermore, pond age, ephemerality and vertebrate predators had no significant effect on aquatic species richness and abundance (see Appendix C). Pond type was not retained in the top GLM of spider species richness and abundance (Table 3.3) with no significant difference after accounting for unbalanced sampling using rarefaction (Figure 3.1b). Habitat was retained in the top GLM of spider richness and abundance but was not significant (p<0.05) with rarefaction suggesting that most habitats were similar except that spider richness was lowest at ponds in wet grassland (Figure 3.1b). Both spider species richness and abundance were positively related to short swards (Table 3.3).

Table 3.3 GLM results for **a)** species richness and **b)** abundance for aquatic and spider species.

Environment *Model* Independent variable	***F***	***β* ± SE**	**n.df.**	**d.df.**	***p***
Aquatic species					
a) *Species richness*	7.904	8.245 ± 0.874	3	82	<0.001
Pond type	9.601	-2.115 ± 0.683	1	82	0.003
Conductivity	2.967	-0.489 ± 0.284	1	82	0.890
Aquatic plants substrate	7.343	0.773 ± 0.285	1	82	0.008
b) *Species abundance*	3.964	3.012 ± 0.207	4	81	0.005
Pond type	5.859	-0.573 ± 0.237	1	81	0.018
Conductivity	3.483	-0.172 ± 0.092	1	81	0.066
Plant litter	5.863	-0.221 ± 0.091	1	81	0.018
Area dried up	0.536	0.075 ± 0.102	1	81	0.466
Spider species					
a) *Species richness*	3.339	8.358 ± 0.144	4	124	0.012
Habitat type	1.269	Multifactorial	3	124	0.288
Short sward	5.385	0.005 ± 0.002	1	124	0.022
b) *Species abundance*	2.338	28.109 ± 8.358	4	124	0.059
Habitat type	0.120	Multifactorial		124	0.948
Short sward	8.023	0.330 ± 0.116	1	124	0.005

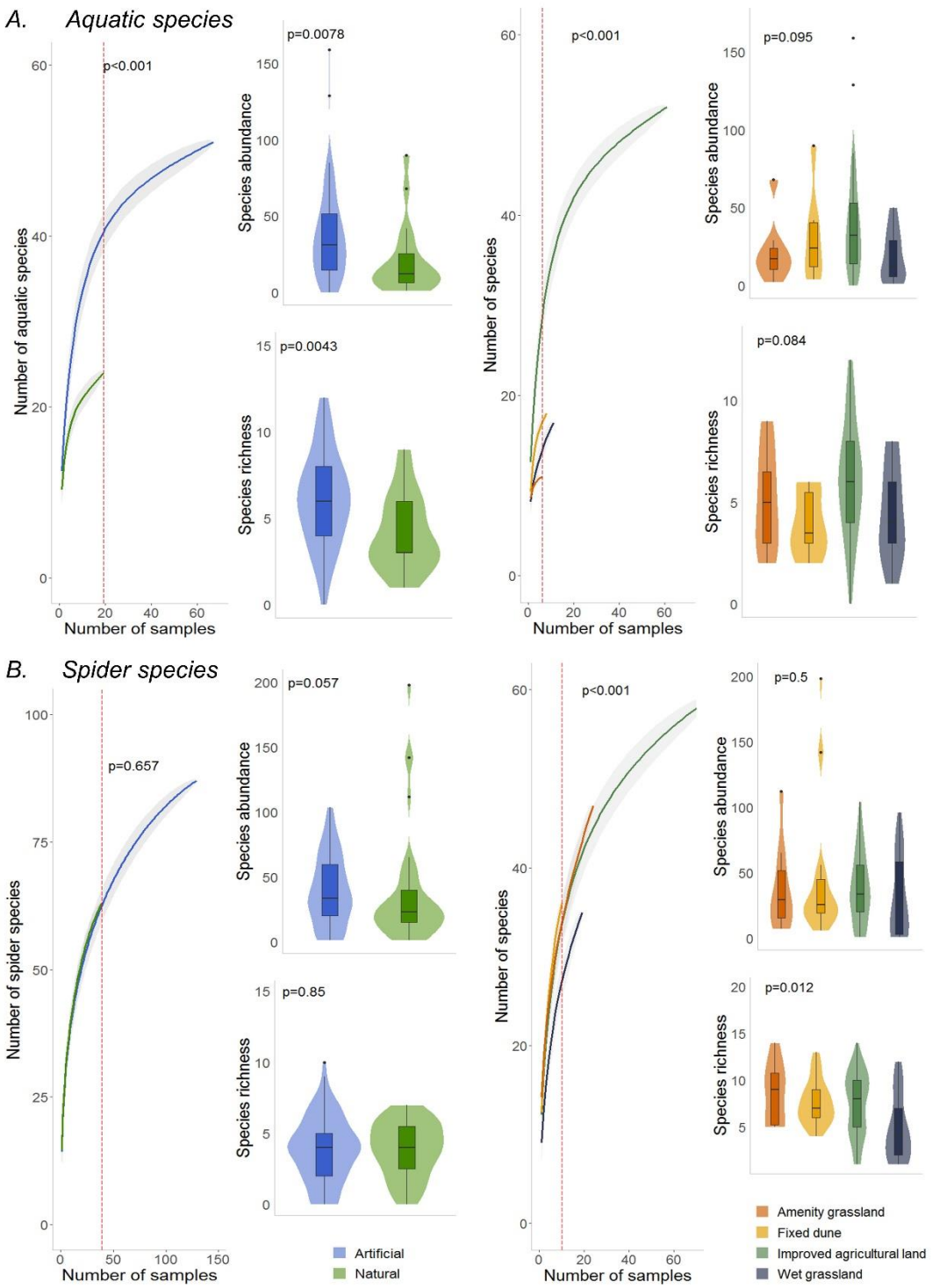

Figure 3.1 Sample-based rarefaction curves and violin plots of taxa richness and abundance across pond and habitat types for **a**) aquatic and **b**) spider species.

Multivariate ordination analyses suggested that aquatic and terrestrial invertebrate communities differed significantly in structure between pond and habitat types (Figure 3.2, Appendix C: Table C.4). Pairwise comparisons indicated that aquatic communities differed significantly between pond types while ponds in amenity grassland differed from those in all other habitat types as well as ponds in fixed dunes differing from those in agricultural land (Appendix C: Table C.4). DistLM suggested that differences in aquatic communities were driven by pH, conductivity and the percentage of the substratum covered by aquatic plants (Figure 3.2; Appendix C: Table C.5). All three environmental parameters differed significantly between pond types (Table 3.2) and habitats (Appendix C: Table C.2). Pairwise comparisons indicated that spider communities differed significantly between pond types and all habitats (Figure 3.2; Appendix C: Table C.4). DistLM suggested those differences were driven by sward height (Appendix C: Table C.5) with all categories of sward heights differing significantly between habitats (Appendix C: Table C.2).

Indicator Species Analysis suggested that artificial ponds were characterised by four aquatic and five spider indicator species while natural ponds were characterised by three aquatic and three spider indicator species (Table 3.4). GLMs suggested that the abundance of each indicator species was driven by species-specific environmental parameters (Table 3.5 and Table 3.6) indicative of varying ecologies, but many factors influencing their abundance differed significantly between natural and artificial ponds i.e. conductivity, cover of substratum aquatic plants and the surrounding coverage of short swards.

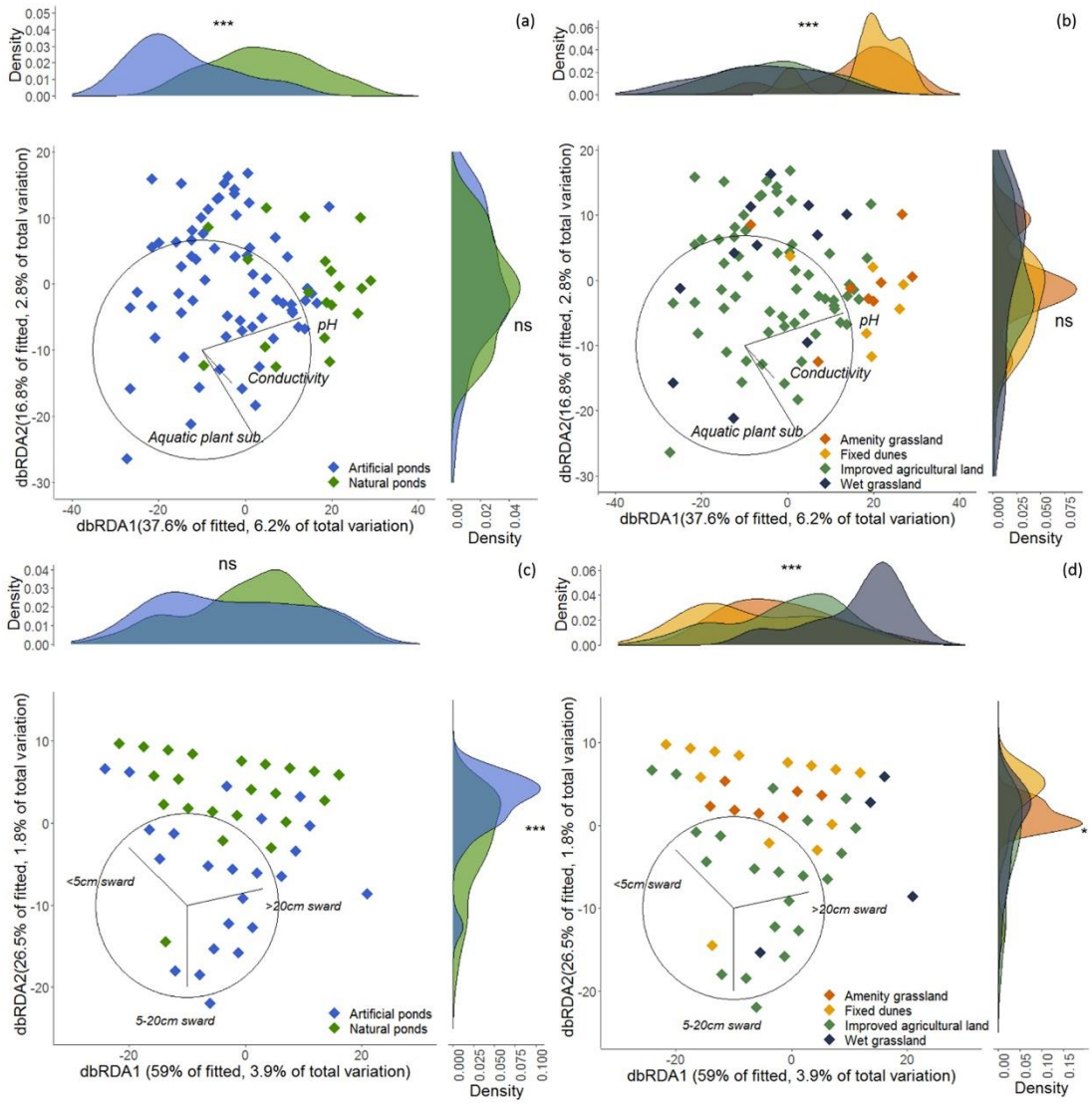

Figure 3.2 Aquatic macroinvertebrate (a-b) and terrestrial spider community (c-d) structure between pond type (left column) and habitat types (right column) as illustrated by Distance-based Redundancy Analysis (dbRDA) plots of Distance-based Linear Models (DistLMs) based on associated environmental parameters. Each symbol represents a single pond. Regular spacing of ponds within the biplot space for the terrestrial communities is the result of percentage cover (estimated to the nearest 10%) within sward height categories. Environmental vectors are proportional to their contribution to the total variation explained and are in direction of increasing values. Marginal density graphs show distribution of data for dbRDA values and significant level (*ns* not significant, * p<0.05, ** p<0.01, *** p<0.001).

Table 3.4 Species Indicator Analysis for **a)** aquatic macroinvertebrates and **b)** terrestrial spiders between pond types showing Indicator Values (IV) for those with significant results.

Taxa Pond type	**Species**	**IV**	***p***
a) Aquatic species			
Artificial	*Notonecta glauca*	0.449	0.001
	Lestes sponsa	0.431	0.003
	Dytiscus marginalis	0.328	0.040
	Agabus bipustulatus	0.288	0.034
Natural	*Agabus nebulosus*	0.352	0.003
	Haemopis sanguisuga	0.293	0.011
	Helophorus fulgidicollis	0.293	0.012
b) Spider species			
Artificial	*Pachygnatha clercki*	0.680	<0.001
	Pardosa pullata	0.382	<0.001
	Bathyphantes gracilis	0.322	<0.001
	Oedothorax fuscus	0.263	0.006
	Ceratinella brevipes	0.226	0.035
Natural	*Alopecosa pulverulenta*	0.501	<0.001
	Pirata piraticus	0.258	0.011
	Tenuiphantes tenuis	0.199	0.037

Table 3.5 GLM results for each significant aquatic macroinvertebrate indicator species with environmental parameters (single best model as selected by AICc values).

Indicator species Environmental parameters	***F***	***β* ± SE**	**n.df.**	**d.df.**	***p***
Aquatic indicator species					
Notonecta glauca	2.216	1.066 ± 0.266	3	82	0.092
Pond type	4.282	-0.705 ± 0.341	1	82	0.042
Conductivity	1.565	-0.344 ± 0.275	1	82	0.215
Bare ground	0.055	0.063 ± 0.267	1	82	0.815
Lestes sponsa	0.511	0.608 ± 0.277	2	83	0.602
Pond type	0.470	-0.237 ± 0.346	1	83	0.495
Conductivity	0.402	-0.140 ± 0.221	1	83	0.528
Dytiscus marginalis	5.545	0.407 ± 0.118	2	83	0.005
Pond type	8.114	-0.339 ± 0.119	1	83	0.006
Conductivity	3.516	-0.223 ± 0.119	1	83	0.064
Agabus bipustulatus	1.063	6.337 ± 1.369	3	82	0.369
Pond type	1.000	-1.559 ± 1.452	1	82	0.286
Plant litter	0.369	-0.854 ± 1.406	1	82	0.545
Agabus nebulosus	3.778	0.977 ± 0.527	4	81	0.007
Pond type	5.903	1.460 ± 0.601	1	81	0.017
Dissolved oxygen	0.595	-0.459 ± 0.595	1	81	0.443
Aquatic plants surface	7.980	1.572 ± 0.556	1	81	0.006
Aquatic plants substrate	1.705	0.710 ± 0.544	1	81	0.195
Haemopis sanguisuga	6.049	1.166 ± 0.255	6	79	<0.001
Pond type	0.431	0.050 ± 0.076	1	79	0.513
Habitat	6.575	Multifactorial	1	79	0.001
Dissolved oxygen	0.716	0.056 ± 0.067	1	79	0.400
Aquatic plants substrate	0.961	0.065 ± 0.066	1	79	0.330
Helophorus fulgidicollis	0.257	0.070 ± 0.049	2	83	0.774
Pond type	0.416	0.033 ± 0.051	1	83	0.521
Filamentous algae	0.194	-0.022 ± 0.051	1	83	0.660

Table 3.6 GLM results for each significant terrestrial spider species with environmental parameters (single best model as selected by AICc values).

Indicator species Environmental parameters	***F***	***β* ± SE**	**n.df.**	**d.df.**	***p***
Spider indicator species					
Alopecosa pulverulenta					
Habitat	10.977	Multifactorial	3	118	<0.001
Bathyphantes gracilis					
Habitat	2.903	Multifactorial	3	118	0.038
Ceratinella brevipes					
Short sward	1.151	-0.062 ± 0.058	1	120	0.286
Oedothorax fuscus	6.289	0.699 ± 3.138	4	117	<0.001
Habitat	4.245	Multifactorial	3	117	0.007
Short sward	9.543	2.302 ± 0.745	1	117	0.003
Pachygnatha clercki	0.980	0.004 ± 0.283	4	117	0.422
Habitat	0.869	Multifactorial	3	117	0.459
Short sward	1.734	-0.088 ± 0.067	1	117	0.191
Pardosa pullata					
Habitat	10.349	Multifactorial	3	118	<0.001
Pirata piraticus	3.838	8.240 ± 5.805	4	117	0.006
Pond type	2.628	-0.460 ± 0.284	1	117	0.108
Habitat	2.505	Multifactorial	3	117	0.063
Tenuiphantes tenuis	1.689	0.497 ± 0.202	4	117	0.157
Short sward	1.535	0.059 ± 0.048	1	117	0.218
Habitat	2.105	Multifactorial	3	117	0.103

3.5 Discussion

Artificially created AES ponds constructed in agricultural grassland, whilst not direct analogues of natural ponds in adjacent non-agricultural habitats within the same landscape, do appear to fulfil an important role in preserving local biodiversity despite differences in community structure. Artificial ponds had significantly higher aquatic macroinvertebrate species richness and abundance than natural ponds but neither pond type differed with respect to surrounding terrestrial invertebrates; specifically, spiders taken as bioindicators. Our results indicate the importance of newly created farmland ponds in maintaining aquatic biodiversity and are in line with other studies that demonstrate a significant contribution of artificial ponds in maintaining high regional freshwater biodiversity (e.g. Biggs *et al.* 2007; Davies *et al.* 2008; Simaika *et al.* 2016).

Overall, artificial ponds in agricultural grassland did not replicate natural ponds in adjacent semi-natural habitats in their abiotic and biotic characteristics and showed considerable environmental variation. In line with predictions derived from island biogeography (McArthur & Wilson 1967), we might expect larger ponds to be more species rich with more complex community structure than small ponds. However, our results indicated that higher aquatic species richness and abundance was observed in artificial ponds which were significantly smaller in size than natural ponds. Oertli *et al.* (2002) suggested that multiple small ponds can maintain higher species richness and have higher conservation value than a single large pond. Indeed, pond density at a landscape level as well as presence of connectivity to nearby sources of biodiversity are major factors contributing to aquatic invertebrate richness

(Gledhill *et al.* 2008; Thiere *et al.* 2009). A large number of highly dispersed artificial ponds embedded in a uniform farmland landscape can create habitat heterogeneity; a key feature in supporting regional or beta-diversity (Froneman *et al.* 2001; Scheffer *et al.* 2006). Other factors that have been identified to impact macroinvertebrate community structure like water permanency, presence of vertebrate predators (amphibians and fish), and pond age had no significant impact on aquatic invertebrate abundance and richness, and are discussed in Appendix C.

Natural and artificial ponds differed in water chemistry. Artificial ponds were characterized by more acidic conditions which typically reduces macroinvertebrate species richness (Nicolet *et al.* 2004). However, artificial ponds were also characterized with lower conductivity which typically supports a wider range of species (Batzer *et al.* 2004; Hinden *et al.* 2005). Natural and artificial ponds differed in their productivity (as indicated by plant coverage) and surrounding sward height. Ponds with complex aquatic vegetation structure support increased richness and abundance of macroinvertebrates through provision of refuges and more abundant prey populations (Deacomn *et al.* 2018; Zelnik *et al.* 2018). Artificial ponds were surrounded by taller vegetation that can act as a buffer for surface run-off and aid improved water quality (Usio *et al.* 2017). High sward in close proximity to the pond can benefit *Odonata* species by providing suitable roosting areas and shelter (Rouquette & Thompson 2007). Consistent with other studies that suggest macroinvertebrates respond to different physical and chemical environmental conditions (e.g. Simaika *et al.* 2016; Deacon *et al.* 2018) it can be expected that the differences described above in the biotic and abiotic characteristics of the natural and

artificial ponds significantly influenced aquatic macroinvertebrate richness, abundance and community structure.

Aquatic macroinvertebrate diversity was 43% more species rich with a 33% higher abundance at artificial than natural ponds. The Intermediate Disturbance Hypothesis (Connell 1978) suggests that higher species richness should be expected at an intermediate level of disturbance as some members of established communities will coexist with species tolerant of disturbance increasing overall diversity. Several studies on macroinvertebrate communities in streams and rivers suggest higher species richness when moderate disturbance was present (e.g. Townsend *et al.* 1997; Mccabe & Gotelli 2000). Indeed, Sayer *et al.* (2012) showed that pond management focused on arresting natural succession and restoring macrophyte-dominated communities can be highly beneficial for aquatic biodiversity by enhancing species richness while no evidence of species loss was observed. Intermediate levels of disturbance could generate some of the macroinvertebrate species richness as artificial ponds were constructed on agricultural land where regular disturbance was present (e.g. grazing by livestock, removing of the emergent vegetation by farmers in line with AES requirements) further delaying ecological succession. Pond communities are dynamic in space and time which can explain some of the variation observed in snap-shot studies (Jeffries 2011; Hassall *et al.* 2012). Natural ponds were older thus representing more stable climax communities while newer (artificial) ponds were in a phase of neutral assembly. Thus, early colonising species such as *Agabus bipustulatus* as well as ubiquitous species such as *Notonecta glauca* and carnivorous diving beetles such as *Dytiscus marginalis*, which depend on established

prey populations, were found in artificial ponds. Moreover, presence of open areas and lower percentage cover of substrate by aquatic plants in artificial ponds may provide a more heterogeneous habitat where refuge as well as open hunting water is present thus elevating the overall species richness and abundance (Bloechl *et al.* 2010). Landscape configuration e.g. distance between ponds, quality of neighbouring ponds and habitat connectivity is likely to play a role in pond colonisation rates (Resetarits & Binckley 2009, 2013). For instance, artificial ponds created for mitigation of developments for great crested newts (*Triturus cristatus*) are often isolated and poorly colonised, therefore of limited conservation value (Lewis *et al*. 2007). While we did not find any significant impact of pond type on spider abundance and richness, they did differ between ponds in different habitats with lowest spider richness in wet grasslands driven by a negative relationship with tall swards. Whilst tall swards may support arboreal spider species, ground hunting species such as wolf spiders (*Lycosidae*) may be adversely affected by the thick rank grass of wet grasslands.

Multivariate analysis suggested that aquatic macroinvertebrate and terrestrial spider communities differed significantly in structure between pond types and habitats and that environmental parameters (pH, conductivity and percentage of substrate covered by aquatic plants for aquatic invertebrates and sward height for spiders) explained part of the compositional variation. Wood *et al.* (2000) found that pond communities were very strongly influenced by the aquatic vegetation cover and that even ponds in very close proximity to each other, but with different percentage cover of aquatic plants, had significantly different community structure. In the

current study, aquatic macroinvertebrate communities of artificial ponds were characterised by four indicator species, all being highly mobile predators that are habitat generalists, indicating that artificial ponds play a key role in maintaining populations of common species (Bloechl *et al.* 2010). Spider communities differed among pond types and habitats and those differences were driven entirely by surrounding sward height**.** Natural ponds were characterised by spider species often found in wetlands (*Pirata piraticus* and *Tenuiphantes tenuis*) or sand dunes (*Alopecosa pulverulenta*), while artificial ponds were characterised by widespread spider species that are grassland specialists (Harvey *et al*. 2002). The total variation in the community structure explained by recorded environmental parameters was low especially for spiders; where the only parameter recorded was sward height. Other processes apart from environmental heterogeneity could drive observed differences.

Our study has significant applied conservation implications. AES ponds whilst not analogues of adjacent natural environments, are nevertheless, important in conserving aquatic macroinvertebrates and have the potential to increase regional aquatic biodiversity. Observed differences between natural and artificial ponds in this study may be unsurprising as they were confounded by habitat variation thus generating different environmental conditions. However, in the current context, AES pond creation aimed to create greater freshwater resources in the landscape to augment natural ponds and replace lost farmland ponds, thus knowing their biodiversity value relative to remaining extant ponds in adjacent semi-natural habitats is of applied value. Farmland ponds support >30% of Irish beetle fauna

proving an important habitat in a transformed agricultural landscape (Gioria *et al.* 2010). Constructed ponds are relatively easy to build, cost effective and are colonised quickly by aquatic macroinvertebrates. Bloechl *et al.* (2010) found that ponds were colonised by 36 *Coleoptera* and *Heteroptera* species within two years of ponds construction. Some studies have highlighted the potential of artificial ponds for conservation of other biota e.g. farmland birds (Lewis-Phillips *et al*. 2019), hence a wider taxonomic assessment may be needed to determine their full role in landscape restoration ecology. It is important to note that in the same way that we now seek to preserve 'farmland birds' that have adapted to a heavily damaged environment, we should be careful when setting as targets habitats that may be heavily impacted by their surrounding landscape. Restoration practises should aim to create farmland ponds reflecting at best, high-quality analogues found in pristine habitats like natural grasslands and meadows rather than natural ponds within agricultural landscapes. Accordingly, management and restoration should focus on promoting macrophyte communities, arresting natural succession (through removal of emergent vegetation and plant litter) and increasing habitat complexity within pond networks providing open and closed water surfaces, complex vegetation structures, variability in pond permanency/ephemerality and buffer strips with both short and tall vegetation providing a wider range of habitats. Creation of ponds in farmland as well as in adjacent natural habitats could provide a wider range of environmental conditions and richer associated macroinvertebrate communities, increasing landscape connectivity and further enhancing regional biodiversity.

Chapter 4

Combining spawn egg counts, individual photo-ID and genetic fingerprinting to estimate the population size and sex ratio of an endangered amphibian

A manuscript based on this chapter was published as:

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4.1 Abstract

For pond breeding amphibians, spawn egg counts are frequently used for population abundance estimation. Numbers of spawn clumps are taken as a proxy of breeding females whilst the likely sex ratio is often to estimate male numbers. Sex ratios, however, are rarely at parity rendering extrapolation of total population estimates from egg counts dubious. This is a problem when dealing with declining species where accurate population estimation is important for informing conservation. We monitored the breeding activity of the Natterjack toad (*Epidalea calamita*), combining egg string counts and Capture-Mark-Recapture population size and operational sex ratio estimation. Male Natterjack toads were identified by the pattern of natural markings with repeated ID of the same individual confirmed for 10% of the samples using genetic fingerprinting. We identified 647 unique individuals within a closed study population at Caherdaniel, Co Kerry. Population estimates derived from egg string counts estimated a breeding population of 350 females (95%CI 331-369) and Capture-Mark-Recapture estimated a breeding population of 1,698 males (95%CI 1,000-2,397). The female:male sex ratio was conservatively estimated at 1:6 (95%CI 1:3-1:7) where 62 ± 6% of females were assumed to spawn. These substantially departed from any *priori* assumption of 1:1 which could have underestimated the breeding population by up to 83%. Where amphibian absolute population size estimation is necessary, methods should include empirical survey data on operational sex ratios and not rely on assumptions or those derived from the literature which may be highly population and/or context-dependent.

4.2 Introduction

Amphibians are the most threatened vertebrate group with 41% of species threatened with extinction. Population declines have been detected even in common and widespread species (Young *et al.* 2001; Stuart *et al.* 2004; Nyström *et al*. 2007; Adams *et al.* 2013; Grant *et al.* 2016; Petrovan & Schmidt 2016). Potential causes for the observed declines include habitat destruction and fragmentation (Cushman 2006), contamination (Mann *et al.* 2009; Brühl *et al.* 2013), spread of pathogens (Berger *et al.* 1998; Lips 1999; Daszak *et al.* 2003; Martel *et al.* 2013), climate change (Carey & Alexander 2003), invasive species (Johnson *et al.* 2011), illegal harvest and trade (Schlaepfer *et al.* 2005; Chan *et al.* 2014) or interaction among several factors (Pounds *et al.* 2006). Methods supporting monitoring and surveillance are, therefore, particularly important when dealing with threatened and declining species to inform conservation (Schmidt 2004). Most studies on amphibian demographics, distribution and dynamics use indirect count data, for example, counts of egg strings or spawn clumps (for females) or call vocalisations (for males). Such measures are proxies for abundance, are vulnerable to survey bias and errors and are likely imperfect reflections of absolute numbers (Schmidt 2005; Mazerolle *et al.* 2007; Wagner *et al.* 2011). Methodological bias and error, both in field surveys but also subsequent statistical extrapolation, based on assumptions that have not been empirically tested, are likely to yield unreliable abundance estimates generating spurious population trends misinforming conservation management programmes (Kèry & Schmidt 2004; Schmidt 2005). For example, chorus size of male European tree frogs (*Hyla arborea*) taken as an indirect measure has little relationship to abundance estimates derived

from recapture studies and fails to detect significant interannual population change (Pellet *et al.* 2007). Indirect count data have been shown by numerous studies to underestimate true population size (e.g. Mazerolle *et al.* 2007; Dodd & Dorazio 2004) Thus, survey and analytical methods should incorporate variation in detection probability, for example, adopting Capture-Mark-Recapture or Distance-Sampling techniques, to provide more robust estimates of population parameters (Schmidt 2003).

Indirect measures such as egg counts can be a reliable survey technique for estimating the number of reproductive females (Windmiller 1996; Crouch & Paton 2000; Brede & Beebee 2006). For some species, like the Natterjack toad (*Epidalea calamita*), egg string counts can be a good indicator of the number of breeding females as the species usually lays just one egg string per year (Danton & Beebee 1996). However, this method still relies on assumptions regarding the proportion of females that breed annually which depends on weather conditions and availability of breeding sites (Smith & Skelcher 2019). Transferring egg counts into population size estimates can be problematic and highly inaccurate due to extreme variation in sex ratios in amphibians. For instance, the female:male sex ratio of the common toad (*Bufo bufo*) in Great Britain and Sweden are known to vary between 1:2 and 1:8 (Gittins 1983; Reading 1991; Reading 2001; Scrinbner *et al.* 2001). Hence, extrapolating male population estimates from egg counts without knowledge of operational sex ratios is not to be recommended.

Capture-Mark-Recapture (CMR) provides a robust population size estimate with associated margins of error but relies on the reliable recognition of individuals

where the species has individually recognisable markings e.g. natural ventral markings (Mettouris *et al.* 2016), passive DNA collecting allowing the recognition of individuals by genetic fingerprinting e.g. using microsatellite markers (Boersen *et al.* 2003; Ringler *et al.* 2015) or invasive techniques such as toe clipping (Grafe *et al.* 2011) or the use of tags such as Passive Integrated Transponder (PIT) tags (Grant & Raymond 2011; Weber *et al.* 2019). Due to animal welfare implications and potential impacts on behaviour including movements, modern studies tend to favour passive techniques. CMR analyses provide robust estimates of demographic parameters while simultaneously accounting for an imperfect detection. If the assumptions of CMR models are not met, then the magnitude and direction of the bias can be quantified and population estimates statistically adjusted accordingly (Manly *et al.* 1999; Schmidt *et al.* 2002; Schmidt 2004).

The Natterjack toad is the rarest and most range restricted amphibian in Ireland, currently Regionally Red-listed as endangered (King *et al.* 2011). The species is listed under Annex IV of the EU Habitat and Species Directive (92/43/EEC) with EU member states required under Article 17 to report regularly to the European Commission on species' population size and trend. Methods for estimating the species total population size for the assessment of trends (Bécart *et al.* 2007; Sweeney *et al.* 2013; Reyne *et al.* 2019) have relied on egg string counts extrapolated using assumptions for the number of females that spawn annually (following Aubry & Emmerson 2005, derived from Denton & Beebee 1993), and the operational sex ratio typically assumed to be 1:1 (following Denton & Beebee 1996; Buckley *et al.* 2014). Egg counts are known to be a reasonably effective method in monitoring

reproductive females (Crouch & Paton 2000) but amphibian sex ratios are well known to be highly male-biased at breeding sites (e.g. Elmberg 1990; Friedl & Klump 1997; Loman & Madsen 2010) suggesting this may be the greatest source of potential error when estimating the absolute breeding population size.

This study aimed to empirically estimate the operational sex ratio of a (closed) breeding Natterjack toad population in Ireland (with no immigration or emigration due to the study population's geographical isolation). We adopted egg string counts to estimate the female breeding population size while the male breeding population was estimated (with associated confidence intervals) by CMR using passive photo-identification to recognise individuals from their ventral markings verified by molecular genetic fingerprinting using microsatellite markers on DNA recovered from skin swabs. We combine these techniques to estimate the total breeding population size over two years and the sex ratio allowing us to quantify the degree of departure from an assumed 1:1 sex ratio and the error such an assumption might produce. Amphibian sex ratios are difficult to estimate with any degree of accuracy; thus, our approach will not only inform the conservation of the Endangered Natterjack toad in Ireland but also methods for other amphibian demographic studies.

4.3 Methods

4.3.1 Study area

We conducted the study at Caherdaniel, Co. Kerry (51.7592°N, -10.1230°E); the most southerly Natterjack toad population in Ireland (Figure 4.1). The site was selected as it was isolated from all other Natterjack toad populations ensuring it was closed with no immigration or emigration. There are only three breeding ponds (C1= 0.20ha, C2= 0.33ha and C3= 0.03ha) located within 200 m of each other; thus, surveys were able to cover the entire breeding site thoroughly. The habitat was sand dune with a short maritime grass sward.

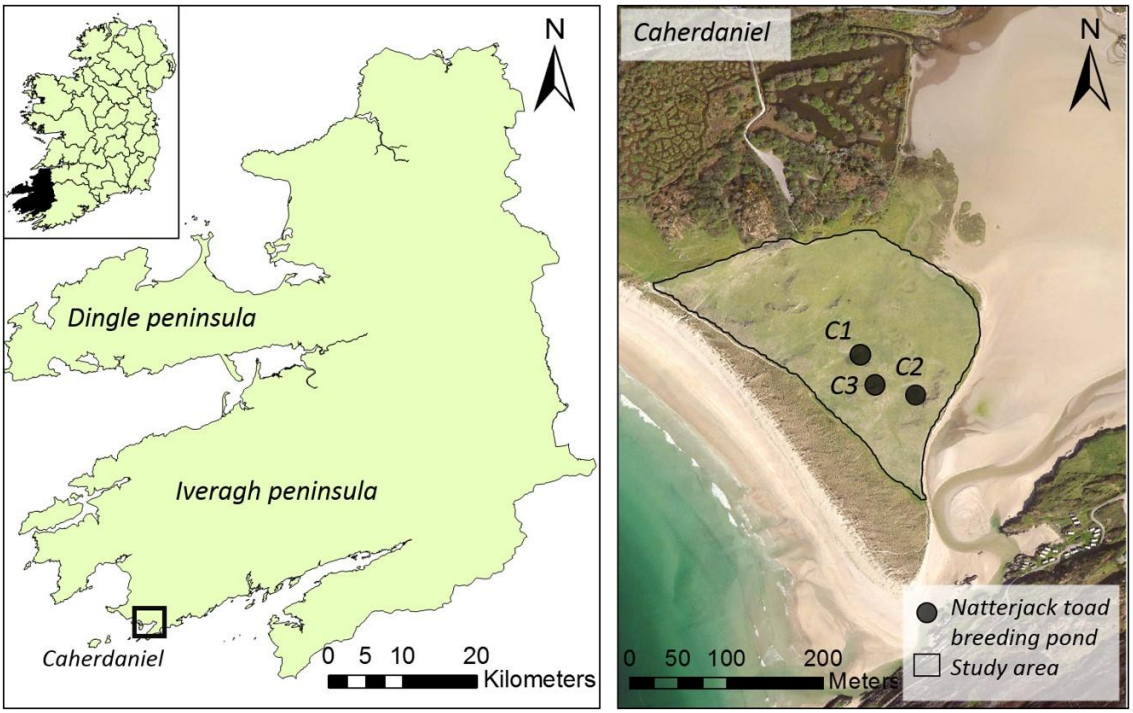

Figure 4.1 Map of the study area and satellite image of Natterjack toad breeding ponds sampled in the study.

4.3.2 Field surveys

We conducted fieldwork in 2017 and 2018 during the Natterjack toad's breeding season (April-July) following Reyne *et al.* (2019). We visited breeding ponds every seven to ten days through the duration of the breeding season. This visit interval was chosen to ensure that egg strings could not be deposited and hatch between visits thus being missed. We recorded the total number of egg strings in each pond by walking the perimeter searching shallow water and aquatic vegetation. Surveys of deeper water away from the perimeter were made using a zigzag transect method. For each pond we mapped egg string locations and stages of development based on Gosner stages (Gosner 1960) in order to avoid double counting during consecutive visits. The earliest stage (Gosner stages 2-6) consisted of two lines of recently laid eggs; the second stage (Gosner stages 7-14) had a single line of eggs; and the third stage near (Gosner stage 16) consisted of well-developed embryos with defined tails. It was assumed that each breeding female deposited one egg string, thus, egg string counts were taken as equivalent to the minimum number of breeding females.

We plotted the cumulative number of egg strings/minimum number of females at each pond visit over the full survey season for each year and the asymptote calculated using a Generalized Additive Model (GAM) which allowed 95% Confidence Intervals to be associated with counts. The lower confidence limit (LCL) accounted for potential double counting where the real number was lower than that counted and the upper confidence limit (UCL) accounted for egg strings potentially being missed (perhaps at depth or within dense vegetation) where the real number was lower.

We conducted a CMR study to estimate the male population size. We sampled breeding aggregations of males at the three breeding ponds by conducting thorough searches for one hour after dusk and capturing by hand all male toads seen (identified by the presence of purple colouration on the neck) or heard calling. We cleaned all toads with water prior to their ventral surface being photographed and swabbed to collect DNA. After sampling, all toads were returned to the breeding site safely and no animals were harmed.

4.3.3 Photo-ID

We placed each male toad in a transparent box and secured them with a light sponge in order to standardize image acquisition. We took photographs using Nikon D3400 and stored them in a digital catalogue. Individuals were identified based on natural ventral markings without the need of invasive techniques such as toe-clipping. Each toad was assigned with a unique numeric identifier. Photographs of captured individuals were compared by single eye matching and a capture history for each individual was constructed.

4.3.4 Genetic fingerprinting

We collected DNA using synthetic sterile cotton swabs (CamLab). Skin swabs have been shown to be an efficient and reliable method for collecting non-invasive samples from amphibian species (Prunier *et al.* 2012). We gently brushed the ventral side of each toad to collect skin cells, avoiding poison glands to prevent contaminating the samples with potential PCR inhibitors. Swabs were stored in 100%

ethanol at ambient temperature. Approximately 10% of the samples were used for genetic fingerprinting in order to verify and provide quality assurance with respect to photo-ID. We used a high salt DNA extraction protocol following Aljanabi and Martinez (1997) as a reliable and cheap alternative to commercial kits (Müller *et al.* 2013). We added a few additional steps to the extraction protocol in order to remove PCR inhibitors (see Appendix D: Protocol CD1 & D.2). We amplified each sample using seven fluorescently labelled microsatellite markers developed by Rowe *et al.* (1997, 2000) and Faucher *et al.* (2016) (Appendix D: Table D.1). Microsatellite markers were chosen based on high levels of polymorphism, low genotyping errors and low probability of the set of molecular markers failing to differentiate between two randomly selected individuals (<0.001). Forward primers were labelled with 6-FAM™, NED™ and VIC™ fluorescent dye (Applied Biosystems, Integrated DNA Technologies). We run 10µl reactions containing 1µl of genomic DNA, 5 µl Type-it Multiplex PCR Master Mix (Qiagen) and primer mix of labelled forward and reverse with 0.1–0.3 µM final concentrations. PCR cycling program had an initial denaturation of 95°C for 15min; 35 cycles of 94 °C for 30sec, annealing temperature 58°C for 90sec, and 72°C for 60sec, and final extension at 60°C for 30min. Samples were randomized during genotyping analysis to avoid bias. Amplification was confirmed for PCR products on 100ml 1% agarose gel stained with ethidium bromide under UV light. PCR product was diluted ten times with ddH_2O. Diluted PCR product (1 µl) together with 8.95µl Hi-Di™ Formamide (Thermo Scientific) and 0.05 µl GeneScan™ 600LIZ ladder (Applied Biosystems) were pooled together for fragment analysis. The microsatellite genotyping was performed on ABI 3730xl Genetic Analyzer (Applied

Biosystems). Alleles were scored with GeneMarker® V1.8 and Peak Scanner™ Software v1.0 (Life Technologies, Inc.). We rounded all genotype calls to an even or odd number based on the respective locus. Differences between assigned and actual allele size were between 0.1 and 1 bp.

We genotyped all samples between three and seven times to identify real genotypes as skin swab samples had a fairly high genotyping error rate. Alleles had to be identical across a minimum two repetitions for heterozygote individuals and three repetitions for homozygotes. We calculated the probability of genotyping errors using PEDANT v10 software (Johnson & Haydon 2007). We used 10,000 search steps to calculate the maximum likelihood error rate of allelic dropout (ADO) and false allele (FA). Contamination with genetic material of a different individual was assigned in cases where additional alleles were present (three alleles per locus). We calculated the percentage of genotyping success over all loci and all repetitions. We calculated the combined non-exclusion identity probability for the chosen set of markers using Cervus v3.07. We used GENECAP, a Microsoft Excel macro (Wilberg *et al.* 2004), to produce a capture history where matching genotypes are considered to belong to the same individual, hence are classified as recaptures. Finally, we compared the number of identified individuals from genetic fingerprinting and photo-ID.

4.3.5 Population size and sex ratio estimation

We used egg string counts to estimate the breeding female population size of the Natterjack toad based on methods used by previous studies in Ireland (Bécart *et al.*

2007; Sweeney *et al.* 2013; Reyne *et al.* 2019). Notwithstanding rare instances of double or multiple clutching (Beebee & Denton 1996), females typically deposit one egg string only (Buckley & Beebee 2004); thus, an accurate egg string count represents the minimum number of females breeding in one year. Denton & Beebee (1993) derived from Denton (1991) studied a Natterjack population in Hampshire (UK) over 5 years and concluded "in any one year only 44-64% of females spawned". Stephan *et al.* (2001) working in Brandberge, Germany from 1992-1999 reported an average estimate of 63% of females spawning annually. Drivers of reproductive activity in females are likely highly context dependent varying between ponds, populations and with weather conditions and invertebrate prey abundance. No empirical data were available for Ireland, however, from the published literature (Table 4.1) we estimated the median ±95%CIs of the percentage of females that breed annually to be 62±6% (95%CIs 56-68%). Thus, to convert egg strings counts into a female population estimate, the lower confidence limit (LCL) was derived as F_{LCL} = S/0.52, the mean estimate by F_{mean} = S/0.58 and the upper confidence limit (UCL) by F_{UCL} = S/0.65, where *F* = the estimated female population and *S* = the total number of egg strings counted. This provided some estimate of the potential error in estimating the female population size only. Initial population size models (following Bécart *et al.* 2007; Sweeney *et al.* 2013; Reyne *et al.* 2019) assumed a sex ratio of 1:1 (following Buckley *et al.* 2014), therefore, the estimated female population size was doubled to estimate the total breeding population.

Table 4.1 The estimated percentage of female Natterjack toads that breeds annually reported by two empirical studies

Source	Location	Year	% females reproductive
Denton & Beebee (1993)	Hampshire, England	1988	55
		1989	64
		1990	44
		1991	63
		1992	61
Stephen *et al.* (2001)	Brandberge, Germany	1992-1999	63
Mean (95%CI)			58 (52 - 65)
Median (95%CI)			**62 (56 - 68)**

We performed CMR analysis using the software program MARK (White & Burnham 1999). The software uses Maximum Likelihood procedures to estimate population parameters (Cooch & Gary 2019). Natterjack toads have a prolonged breeding season (April – July annually). The number of individuals at the breeding sites varies throughout the breeding season with no period when all individuals of the population are present. Even though our study population was closed at the global level (no immigration or emigration), Wagner *et al.* (2011) recommends using an open population modelling approach for amphibians with a prolonged breeding season where individuals can come and go from the breeding site as a better match to their phenology. For our analysis, we used an open population modelling approach based on a Cormack-Jolly-Seber model (Jolly 1965; Seber 1965). We performed analyses using the computer package POPAN (sub-module of MARK) developed by Schwarz & Arnasan (1996). Parameterization included N representing the size of a super-population, that is the total number of individuals in the area, φ was the survival rate, p was the probability of capture and b was the probability of an animal from the super-population entering the sub-population (i.e. toads accruing at the

breeding site). In the model, *(t)* and *(.)* represent time-dependant and constant parameters respectively. The first step in the analysis was to verify if our data met the two main model assumptions: all marked individuals have the same probability of capture and survival between *i* and *i+1* sampling events. We used the RELEASE function to run a Goodness-of-Fit (GOT) test for a fully time dependant model *p(t) φ(t) b(t).* In case the model assumptions were not met, we calculated a *post-hoc* Variance Inflation Factor (VIF) to adjust for the lack of fit, resulting in a Quasi-Akaike Information Criterion corrected for small sample size ($QAIC_C$) value. We constructed models based on different combinations of time-dependant and consistent parameters (*φ, p* and *b*). The single best appropriating model (model with the lowest $QAIC_C$ value) was used to estimate the breeding male population size (with associated 95% Confidence Intervals).

The estimated breeding female and male populations were summed to get the total breeding population. The operational sex ratio was expressed as the ratio of females:males. The total population derived from combined egg string counts and genetically verified photo-ID CMR was contrasted with a traditional population model derived from egg string counts and assuming a 1:1 sex ratio expressed as the percentage difference between the models (propagating 95%CIs to obtain likely ranges).

4.4 Results

4.4.1 Egg string counts / minimum no females

In total, we conducted 30 pond visits throughout the Natterjack toad breeding season during 2017 (*n*=17) and 2018 (*n*=13). We recorded a total of 231 egg strings/minimum number of females during 2017 and 224 egg strings/minimum number of females during 2018. There was a significant sigmoidal accumulation of egg strings (Figure 4.2) during both 2017 ($F=5674$, $p<0.001$, $r^2=0.906$) and 2018 ($F=9439$, $p<0.001$, $r^2=0.960$) resulting in ±4.1% error during 2017 (95%CI 212 – 250) and ±1.2% error during 2018 (95%CI 218 - 229).

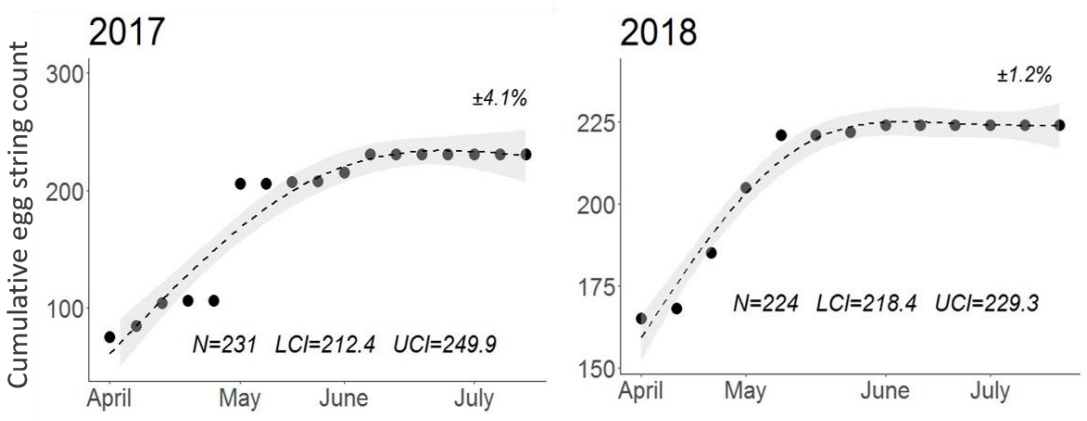

Figure 4.2 Cumulative egg string counts/minimum number of females at Caherdaniel, Co. Kerry from weekly surveys from April-July during 2017 and 2018. Dashed lines represent a Generalized Additive Model (GAM) and shading its 95% Confidence Intervals.

4.4.2 Photo-ID

Over the course of nine survey nights (four during 2017 and five during 2018), we collected, photographed and swabbed a total of 884 male toads. We identified 647 unique individuals based on photographs of ventral marking comparisons (Figure 4.3) enabling recapture histories to be constructed for each individual and the total population (Table 4.2). The recapture rate was 26%. The individual accumulation curve showed no sign of asymptote increasing linearly throughout the study i.e. a roughly similar number of new male individuals were identified at each successive capture event (Figure 4.4).

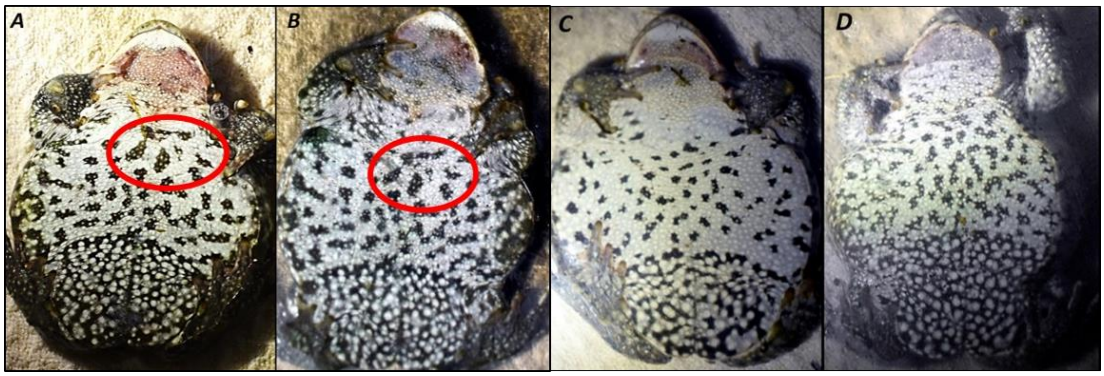

Figure 4.3 Individual variation in male Natterjack toad ventral markings used for photo-ID A-B) shows the same individual caught in 2017 and 2018 with identical markings highlighted by a red circle and C-D) show two different individuals caught during 2018.

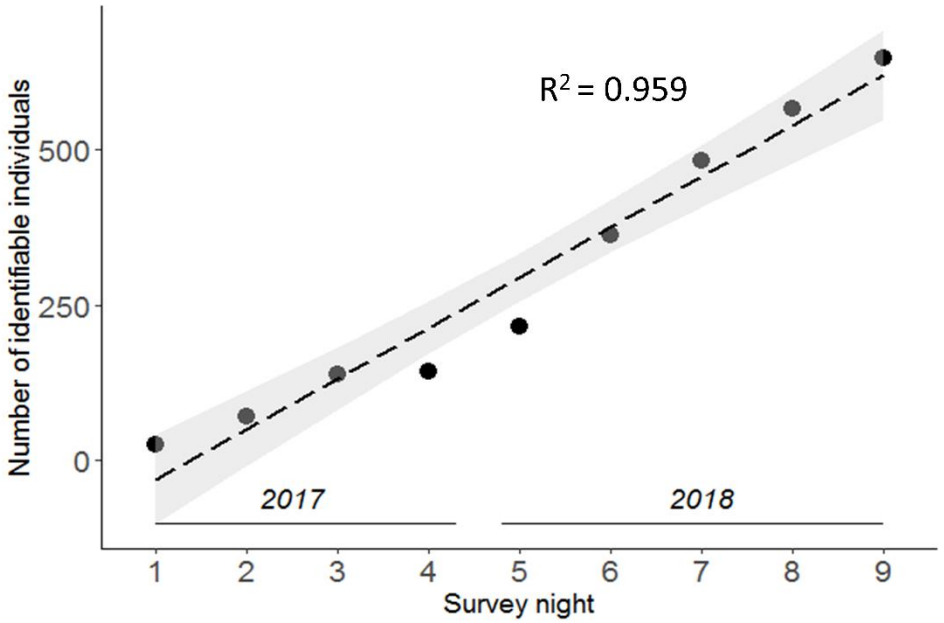

Figure 4.4 Individual accumulation curve (black) for discovery of new male Natterjack toads from photo-ID fitted with a linear regression (grey dash).

Table 4.2 Study-level recapture history of the Natterjack toads where *N* number of captures per dated sampling event.

		Recapture history							
Survey night	***N***	20-May-2017	10-Jun-2017	30-Jun-2017	14-Apr-2018	21-Apr-2018	05-May-2018	19-May-2018	02-Jun-2018
06-May-2017	26	4	4	1	4	4	3	3	2
20-May-2017	50		9	0	5	10	5	7	7
10-Jun-2017	81			1	7	14	7	8	5
30-Jun-2017	5				0	0	1	2	0
14-Apr-2018	89					20	17	12	10
21-Apr-2018	189						50	23	17
05-May-2018	186							32	20
19-May-2018	137								18
02-Jun-2018	121								
TOTAL	**884**	**4**	**13**	**2**	**16**	**48**	**83**	**87**	**79**

4.4.3 Genetic fingerprinting

In total, we genotyped 65 samples (equating to a 10% sample of individual males). Final DNA concentration ranged between 1.1 and 32.4 ng/µl (mean ± SE: 10.3 ± 8.2). The genotyping success rate overall was low with an average of 33% (Appendix D: Table D.2). Genotyping success for one of the markers (BC22) was 8%, hence we excluded the marker from further analysis. The average probability that the final set of microsatellite markers failed to differentiate between two randomly chosen individuals was 0.014, suggesting that the selected assay had an adequate power for individual discrimination. Average rate of genotyping error per allele due to ADO and FA was 0.023 and 0.001 respectively (Appendix D: Table D.2). We discarded samples that either contained DNA from more than one individual (5%) or DNA that failed to amplify for two or more loci (37%) as the probability of successful identification of individuals based on four or fewer markers was low. Hence, we retained 39 samples to create a capture history. Results suggested a total of 45 alleles with an average of 6.4 alleles per locus. GENECAP identified 16 individual recaptures and 25 unique genotypes that matched the individual recapture identities assigned from photo-ID by comparison of images by eye. Thus, male toads recognised as different individuals by their ventral markings were also identified as different individuals by their microsatellite genotypes and *vice versa* providing a measure of quality assurance for photo-ID recapture histories.

4.4.4 Male population size

For the mark-recapture analysis we tested eight different models. Three models failed to achieve numerical convergence: 1) *p(t) φ(t) b(.)*, 2) *p(t) φ(.) b(.)* and 3) *p(.) φ(t) b(.).* The Goodness-of-fit test for the fully time dependant model suggested the presence of overdispersion violating one of the main assumptions of the model i.e. equal survival of all individuals from *i* to *i+1* sampling events (TEST2 + TEST3, Appendix D: Table D.3). We calculated and applied a *post-hoc* VIF of $\hat{c}$ = 1.62. Based on $QAIC_C$, the model we selected was *p(t) φ(.) b(t)* where detection probability and survival were constant while capture probability varied with time (Table 4.3). The single best approximating model estimated the breeding male population at 1,698 (95%CI 1,000 - 2,397) individuals. Separate models were not fitted for each year as the number of capture events per year was low resulting in the failure of the Goodness-of-test for 2017 whilst the model for 2018 yielded 95%CIs of low utility given their width.

Table 4.3 Candidate model selection and abundance estimates for the male Natterjack toad breeding population size where *p* = capture and recapture probability, *φ* = survival probability, *b* = probability of first appearance (population entry), *(t)* = parameter changes over time, *()* = constant over time, *ω* = the Akaike weight of each model, *K* = the number of parameters, *N* = is the estimated male population size, LCI and UCI provide the lower and upper limit of the 95% CI. The single best approximating model is marked with an asterisk.

Model	**Model selection criteria**				**Abundance estimate**			
	$QAIC_C$	$\Delta QAIC_C$	ω	K	*N*	SE	LCI	UCI
*p(t) φ(.) b(t)**	914.1	0.0	0.973	18	1,698	356	1,000	2,397
p(t) φ(t) b(t)	921.3	7.2	0.027	25	1,320	213	902	1,737
p(.) φ(t) b(t)	946.3	322	0.000	17	1,358	95	1,173	1,543
p(.) φ(.) b(t)	972.9	58.8	0.000	6	1,251	80	1,095	1,408
p(.) φ(.) b(.)	22846.6	21932.4	0.000	3	971	29	913	1,028
p(t) φ(t) b(.)	*Numerical convergence not reached*							
p(t) φ(.) b(.)	*Numerical convergence not reached*							
p(.) φ(t) b(.)	*Numerical convergence not reached*							

4.4.5 Total population size

Combining egg string counts to derive a female population estimate and photo-ID verified by genetic fingerprinting to derive a male population estimate (Table 4.4), we estimated the operational sex ratio (propagating and compounding the 95%CIs associated with both) at 1:7 (95%CI 1:5 - 1:10) where egg strings numbers were taken as the minimum number of females. Adjustment for the percentage of females likely to spawn (62 ± 6%) estimated the sex ratio at 1:5 (95%CI 1:3 - 1:7). Both substantially departed from an *a priori* assumption of 1:1 which would have underestimated the total breeding population by 76% (95%CI 68 - 82%) where egg string numbers were taken as the minimum number of females or by 78% (95%CI 69 - 83%) where 62% of the female population was assumed to spawn annually.

Table 4.4 Population estimates for female and male breeding populations at three ponds at Caherdaniel, Co. Kerry derived from egg string counts (confidence intervals from GAM plus those associated with the assumed percentage of females that breed) and photo-ID verified by genetic fingerprinting (confidence intervals from CMR). Note that Confidence Intervals associated with each total population estimate are compound**.**

Year	Abundance estimate			Sex ratio		
	N	LCI	UCI	F:M	LCI	UCI
a) Egg string count / Min no females						
2017	231	212	250			
2018	224	218	229			
Mean	228	215	240			
b) Estimated no females (assuming 62 ± 6% spawning)						
2017	373	379	368			
2018	361	389	337			
Mean	368	384	353			
c) Total pop estimate[1] (Min no females + 1:1 Sex ratio)						
2017	462	424	500	1:1	1:1	1:1
2018	448	436	458	1:1	1:1	1:1
Mean	456	430	480	1:1	1:1	1:1
d) Total pop Estimate[2] (62 ± 6% females spawning + 1:1 sex ratio)						
2017	746	758	736	1:1	1:1	1:1
2018	722	778	674	1:1	1:1	1:1
Mean	736	768	706	1:1	1:1	1:1
e) Estimated no males (Mark-Recapture)						
2017	1,698	1,000	2,397			
2018	1,698	1,000	2,397			
Mean	1,698	1,000	2,397			
f) Total pop estimate[3] (min no females + estimated males)						
2017	1,929	1,212	2,647	1:7.4	1:4.7	1:9.6
2018	1,922	1,218	2,626	1:7.6	1:4.6	1:10.5
Mean	1,926	1,215	2,637	1:7.4	1:4.7	1:10
g) Total pop estimate[4] (62 ± 6% females spawning + estimated males)						
2017	2,071	1,379	2,765	1:4.6	1:2.6	1:6.5
2018	2,059	1,389	2,734	1:4.7	1:2.6	1:7.1
Mean	2,066	1,384	2,750	1:4.6	1:2.6	1:6.8

4.5 Discussion

Population size estimates for the Natterjack toad population at Caherdaniel, Co. Kerry, Ireland derived from egg spawn counts and CMR data differed substantially. Our results demonstrated clearly that assuming a 1:1 sex ratio could underestimate the population by up to 83%. Such method provides a weak basis for understanding population dynamics and could easily fail to detect temporal changes in the population trend and would, therefore, be highly unreliable when developing conservation and management strategies.

Egg spawn counts are widely used in amphibian studies (e.g. Buckley & Beebee, 2004; Loman & Anderssson 2007; Hartel 2008) and can be an effective mean for monitoring reproductive females (Crouch & Paton 2000). However, the variability in probability of detecting spawns is largely unknown. It is likely influenced by a variety of biotic and abiotic factors (e.g. cloud cover, aquatic vegetation and wind affecting visibility through the water surface). Grant *et al.* (2005) examined the spawn detection probability among different observers and breeding pool characteristics (size, depth and vegetation). Detection varied spatially and temporally where no consistent sets of variables were able to explain the observed variation. Hence, assessment of variables that may influence the detection probabilities associated with specific sites or observers are essential in reducing bias in population size estimates. Another source of uncertainty when using spawn counts is the number of clutches deposited annually. Females of some species, like the Natterjack toad, deposit a single egg string, thus the ratio between the number of breeding females and number of egg strings is 1:1. However, a second smaller egg string can be

produced leading to overestimation of the number of breeding females, though this is rare (Denton & Beebee 1996). Many temperate and tropical species deposit between 2 and 12 clutches annually (Wells 2007) making it even more difficult to quantify population size and long-term trends.

Spawn counts represent variation of the proportion of females choosing to breed each year and strongly depends on availability of suitable breeding ponds and weather conditions, especially for species like the Natterjack toad that breeds in ephemeral ponds (Banks & Beebee 1988). A 31-year study of a Natterjack toad population in north-west England found a strong positive relationship between spring rainfall and annual spawn counts. Hence, large variation in the number of egg strings was observed between years as a result of differences in rainfall with some females skipping breeding seasons (Smith & Skelcher 2019). A study of the common toad (*Bufo bufo*) suggested that on average 41% of the females skipped breeding in any particular year, while the corresponding estimate for males was less than 5% (Loman & Madsen 2010). Hence, the total spawn counts represent annual breeding effort and not the total number of females in a population *per se.*

Errors in estimating breeding female number are likely to be less than errors in sex ratio assumptions when calculating population size. A male-biased sex ratio is often observed at amphibian breeding sites (e.g. Reading 2001; Loman 2010). This can be explained by lower female survival rates (Elmberg 1990; Friedl & Klump 1997) and females maturing at a later age (Reading 1991; Miaud *et al*. 1999). However, this is not always the case as large variation has been observed among populations. For instance, the female:male ratio for the Natterjack toad has been observed to vary

between 1:0.3 and 1:11 (Table 4.5). Such is the likely variation between ponds, populations and years that sex ratio is likely the greatest source of biases and error in estimating total population size. The estimate from the current study ranged from 1:3 to 1:11 (depending on assumptions) encompassed most of the variation from previous studies whilst our average of 1:5 was substantially more male-biased than the mean or median from previous studies (Table 4.5). Thus, empirical estimates of sex ratio are essential for obtaining reliable demographic data for rare amphibian species.

Table 4.5 Published Natterjack toad sex ratios as reported in the literature.

Source	Location	Year	Sex ratio			Comments
			Female	**:**	**Male**	
Denton & Beebee (1993)	Hampshire, England	1988	1	:	0.30	All sightings
			1	:	0.56	Known individuals only
		1989	1	:	0.33	All sightings
			1	:	0.47	Known individuals only
Stephan *et al.* (2001)	Halle, Germany	1992-1999	1	:	1.30	Newly hatched
Gunther & Meyer (1996)	Various	-	1	:	0.84	Lowest reported
			1	:	11.20	Highest reported
Mean (95%CI)			1	:	2.14 (000 – 5.11)	
Median (95%CI)			**1**	**:**	**0.56 (000 – 3.53)**	

CMR data and associated statistical models are robust methods for estimating confidence around population estimates. CMR requires identification of individuals; but not all species have natural unique markings. Toe clipping has been widely used as a marking technique for amphibians (Waichaman 1992; Halliday 1996), however, concerns have been raised regarding the ethical treatment of animals and its impact on survival (McCarthy & Parris 2004). Marking techniques widely used with other taxa

like banding and PIT tags are often not suitable for amphibians (Halliday 1996; Funk *et al.* 2005). Over the past decades non-invasive genetic sampling has emerged as an alternative option for Mark-Recapture sampling (e.g. Petit & Valiere 2006; Solberg *et al.* 2006; Mondol *et al.* 2009). Despite its great potential, many challenges still exist regarding low amplification success rate, high genotypic errors and shadow effects (lack of power to distinguish individuals) leading to low reliability in obtaining genotypes. Collected DNA is often degraded, low quantity and contaminated with PCR inhibitors (Idaghdour *et al.* 2003; Lampa *et al.* 2013). Skin swabbing is a preferred method as it is easier and less invasive than buccal swabs, however, skin samples can have a high degree of contamination, especially if samples are collected during the breeding season when amphibians aggregate (Müller *et al.* 2013). In the current study, almost half of the genotyped samples (40%) were discarded due to cross-contamination and poor amplification success rate despite a high number of replications per sample (n=7). Thus, collecting high numbers of genetic samples is necessary while genotyping costs should be considered when planning study designs. Other issues that can arise when working with Mark-Recapture data is low recapture rate especially for animals that are hard to detect and capture, temporary emigration, uneven survival and detectability. In this study, the discovery curve increased linearly throughout the study with new individuals being regularly identified. Perhaps, it can be explained, by the duration of toad searches (one hour after dusk) and high number of males at the breeding site. If the searches had continued throughout of hours of darkness (prohibited by logistics) we would have captured a greater proportion of the male population.

Previous estimates of the Natterjack toad population size in Ireland relied solely on egg string counts with subsequent extrapolations (Becart *et al.* 2006; Sweeney *et al.* 2013). As demonstrated in the current study, such estimates are likely to be unreliable with numbers underestimated when failing to account for detection probability, annual variation in proportion of breeding females and operational sex ratio. Given the scale of the amphibian extinction crisis and the need for robust population monitoring and surveillance techniques, there is need to promote study designs that account for differences between meta-populations including spatial and temporal variation in describing population declines, extinction probabilities and as a guide when informing conservation planning (Stephan *et al.* 2001; Minin & Griffiths 2011). Our results suggest spawn counts cannot be extrapolated with any reliability to produce precise population estimates but they do provide insights into variation in the breeding effort likely reflecting the effective (rather than absolute) population size. From an evolution perspective only individuals contributing to the gene pool of the next generation are important (Wright 1931), hence conservation management goals should be focused on maintaining genetic diversity within the reproductive population (Hedrick 2001). In this case, the effective population size of the Natterjack toad population at Caherdaniel, Co. Kerry Ireland is likely limited by the number of breeding females given they are the rarer sex. Hence, simply taking egg string counts as a measure of changes in the reproductive effort and population health is probably the easiest solution for annual monitoring rather than rolling out sex ratio estimation to produce absolute population estimates.

Chapter 5

Population genetic structure of the Natterjack toad (*Epidalea calamita*) to inform conservation management

A manuscript based on this chapter has been submitted for publication and is *in print* as:

Reyne M, Kara D, McFarlane C, Aubry A, Emmerson M, Marnell F, Reid N, Helyar S. (*in print*) Population genetic structure of the Natterjack toad (*Epidalea calamita*), regionally Red-Listed as Endangered in Ireland, to inform conservation management*. Conservation Genetics.*

5.1 Abstract

Molecular methods can play a crucial role in species management and conservation. Despite the utility of genetic approaches, they are often not explicitly included as part of species recovery plans and conservation practises. The Natterjack toad (*Epidalea calamita*) is regionally Red-Listed as Endangered in Ireland. The species has a declining population trend and is now present at just seven sites within a highly restricted range. This study used thirteen highly polymorphic microsatellite markers to analyse the population genetic diversity and structure. Genetic diversity was high with expected heterozygosity between 0.55 - 0.61 and allelic richness between 4.77-5.92. Effective population sizes were small (N_e <100 individuals), but not abnormal for pond breeding amphibians. However, there was no evidence of historical or contemporary genetic bottlenecks or high levels of inbreeding. We identified a positive relationship between N_e and breeding pond surface area, suggesting that environmental factors are a key component in population genetics. Significant genetic structuring was detected throughout the species' range, and we identified four genetic entities that should be considered in the species' conservation strategies. Management of population declines should be focused on preventing future loss of genetic diversity overall and within genetic entities while maintaining adequate effective population size through site-specific protection and human-mediated translocation and head-start programs. The apparent high levels of genetic variation give hope for the conservation of Ireland's rarest amphibian if appropriately protected and managed.

5.2 Introduction

Given the current global 'amphibian crisis' (IUCN 2020), comprehensive understanding of declining and threatened species genetic structure is essential for effective conservation strategies and population management (Emel & Storfer 2012). Small population size promotes loss of rare alleles, increased homozygosity and accumulation of detrimental recessive alleles (Westemeier *et al*. 1998; Madsen *et al*. 1999), resulting in local declines and elevated extinction risk (Frankel & Soule 1981; Shaffer 1990; Hedrick 2001; Emel & Storfer 2012). Amphibians are particularly at risk to genetic depletion due to their sensitivity to environmental change, low dispersal capability and strong natal philopatry (Beebee & Griffiths 2005; Beebee 2005; Kraaijeveld *et al.* 2005).

Low genetic variability in amphibians has been linked to an increased presence of physical abnormalities (Hitchings & Beebee 1998) and negative impacts on oxygen consumption (Mitton *et al.* 1986), immune response (O'Brien & Evermann 1988; Altizer *et al.* 2003; Cabido *et al.* 2010; Cabido *et al.* 2011), ability to compete for resources (Rowe & Beebee 2005), clutch size (McAlpine 1993), hatching success (Blaustein 1994) and growth rates (Rowe & Beebee 2004). For example, loss of genetic diversity in the Italian agile frog (*Rana latastei*) has been related to higher susceptibility to Ranavirus (Pearman & Garner 2005). Such findings are a cause for concern given the major role disease plays in global amphibian declines (Daszak *et al.* 2003; Skerrat *et al.* 2007; Wake & Vredenburg, 2008). Thus, omitting genetics in species management plans may lead to inappropriate allocation of resources,

ineffective conservation strategies and further population declines (Frankham 2003; Cushman 2006; Noel *et al.* 2007).

Species genetic management is often overlooked in conservation programmes due to lack of resources or expertise (Taylor *et al.* 2017). Understanding of the genetic structure of endangered populations can inform a targeted management approach. Genetic analysis potentially permits assignment of individuals to genetic clusters independent of the sampling location and identifies discrete units for management. Below the species-level, conservation priorities focus on intraspecific diversity for evolutionary plasticity and adaptability in response to environmental change (Shaffer *et al.* 2015). For instance, genetic analysis of the Australian agamid lizard (*Diporiphora nobbi*) identified several Evolutionary Significant Units (ESUs), emphasising the importance of local populations of a widespread species in harbouring intraspecific genetic diversity (Driscoll & Hardy 2005). Conversely, where genetic diversity is critically low conservation managers may need to actively consider genetic rescue by deliberately maximising assisted gene flow between distinct units (Godoy *et al.* 2004; Beauclerc *et al.* 2010; Whiteley *et al.* 2015). An isolated population of adders (*Vipera berus*) in Sweden dramatically increased in number after the introduction of new genes to avoid severe inbreeding averting local extirpation (Madsen *et al.* 1999, 2004). Thus, the approach taken to genetic management is species-, population- and context-dependent.

Effective population size (N_e) plays a crucial role in predicting a population's extinction risk and is often more valuable than estimating absolute census size (Beebee 2005). It is defined as the size of an ideal population that meets all Hardy-

Weinberg assumptions showing the same rate of loss of genetic diversity as the target population (Wright 1931). Effective population size can be derived from genetic data with no additional life-history information (Schwartz *et al.* 1998). Furthermore, molecular makers allow historical patterns of population decline or expansion to be described, informing contemporary management. There are many factors that can influence N_e, including census size, sex ratio and past declines, breeding strategy (polygyny), habitat carrying capacity and connectivity between populations (Waples & Gaggiotti 2006; Mills 2007). Wang *et al*. (2011) found that suitability of breeding habitats can contribute to variation in N_e among populations of the California tiger salamander (*Ambystoma californiense*). Thus, understanding the relationship between demographic parameters, key habitat features, and effective population size is essential for a comprehensive conservation approach.

This study aimed to assess the impact of small population size and recent declines on the genetic structure of the Natterjack toad (*Epidalea calamita*), to inform species conservation management. In Ireland, the species is at its northwesternmost range edge margin (Gasc *et al.* 1997) and is highly range restricted, occurring in seven isolated populations on the coast of Co. Kerry (Figure 5. 1; see Appendix E). One of those seven populations (Caherdaniel) was established in the 1990s as a result of introduction of individuals from the Magharees in order to increase the species distribution range. Regardless of conservation efforts, the species is in decline and is regionally Red-Listed as Endangered in Ireland (King *et al*. 2011).

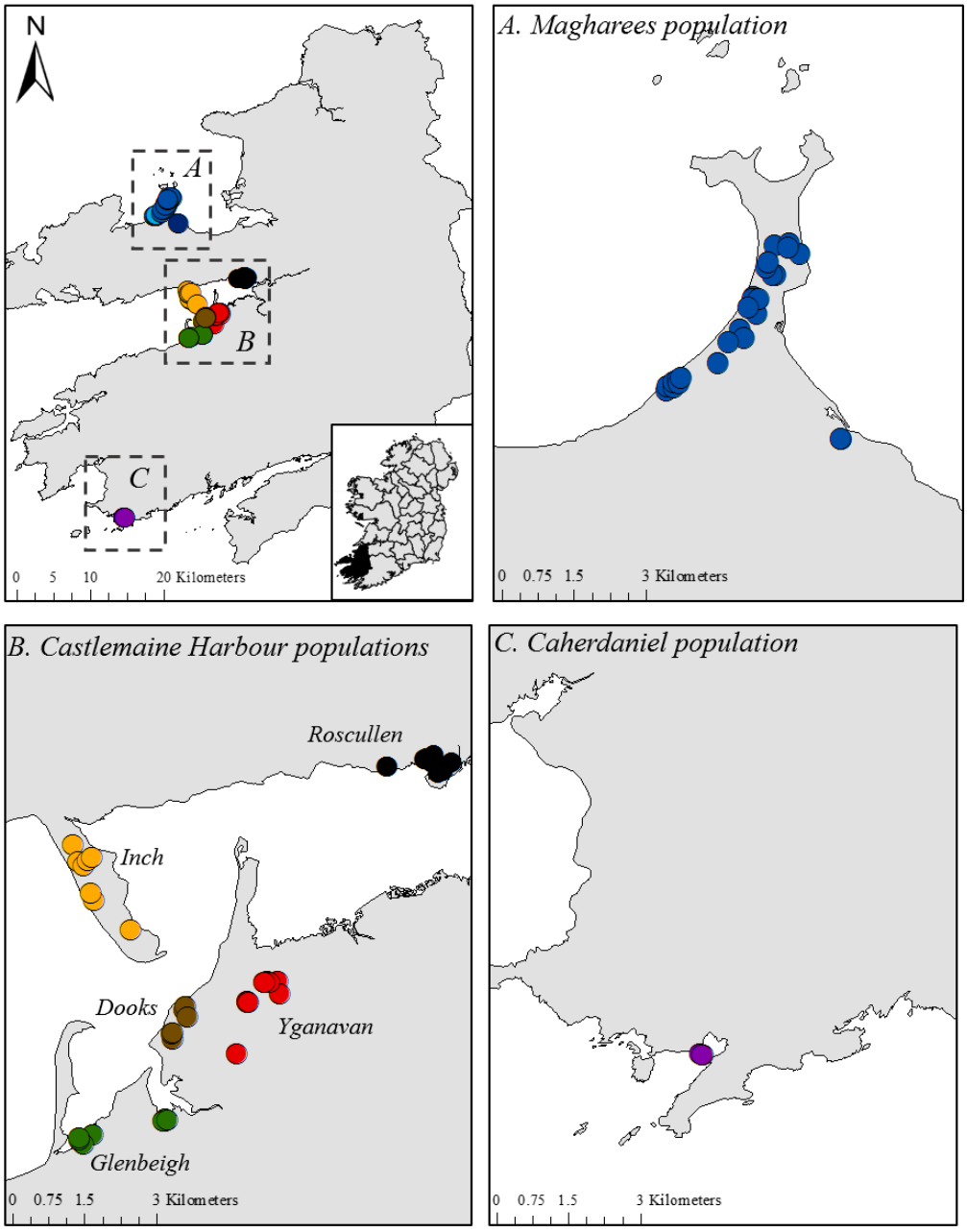

Figure 5.1 Map showing the location of the Natterjack toad breeding ponds for the seven populations in Co Kerry, Ireland. Colours represent different populations.

The Natterjack appears native to Ireland and, despite reporting an apparent bottleneck, May & Beebee (2008) suggested its genetic diversity was "reassuringly high" despite a lack of gene flow. However, low levels of polymorphism in the selected microsatellite markers may have contributed to their low reported allelic

richness (1.86 - 2.89). Since then, Natterjack toad populations have continued to decline, with > 90% loss of spawning in some populations (Reyne *et al.* 2019). In an effort to improve habitat availability, over 100 new breeding ponds were created as part of an agri-environment scheme since 2008 by the National Parks & Wildlife Service (NPWS). NPWS now manages a Head-start and Translocation Programme by which eggs strings and tadpoles are collected annually, raised in captivity and metamorph toadlets released back into the wild, supplementing existing populations as part of assisted migration and translocation to newly created ponds. Since 2016 thirteen translocations were performed to aid colonisation of artificial ponds (Reyne *et al.* 2019). Currently, there are no data available by which to assess the Head-start and Translocation Programme success, as breeding will occur 4 to 5 years post release when toads have reached sexual maturity and return to the ponds for breeding (Beebee 1979). A primary goal of ex-situ conservation is to preserve maximum intraspecific genetic variability to ensure long-term survival (Pelletier *et al.* 2009). However, inbreeding is a major problem in many ex-situ breeding programs (Ralls *et al.* 1979; Bouman 1977), with success often determined by the appropriate selection of founders from source populations (Tzika *et al.* 2009). Selecting individuals and populations for ex-situ breeding and translocations should, therefore, be informed by empirical genetic data ensuring provenance and/or appropriate management of genetic lineages (Witzenberger & Hochkirch 2011; IUCN/SSC 2013). Hence, the specific objectives of this study were to: (i) provide genetic characterisation of each extant population, (ii) reconstruct parentage of offspring samples, (iii) estimate the effective and census population size, (iv) evaluate the impact of pond characteristics

on effective population size, (v) detect any genetic bottleneck effect(s) due to historical or recent population decline, (vi) quantify genetic differentiation between populations, (vii) assign genetic entities and (viii) provide management recommendations.

5.3 Methods

5.3.1 Field surveys and sample collection

We conducted fieldwork in 2017 during the Natterjack toad's breeding season (April-July). Breeding ponds were visited every seven to ten days throughout the duration of the breeding season. We recorded the total number of egg strings in each pond and measured the size (surface area) of each breeding site. For full details of sampling methods see Reyne *et al*. (2019). We collected DNA samples from well-developed Natterjack toad egg strings and tadpoles (tadpole tails or entire small tadpoles) from each of the seven populations, aiming for at least 30 individuals per population. Samples were collected from different breeding ponds within a population to avoid pseudoreplication associated with analysing full siblings. We did not sample any egg strings or tadpoles part of the Head-start and Translocation Programme i.e. translocated between sites. Samples were preserved in 100% ethanol at ambient temperature until extraction.

5.3.2 Genotyping and data validation

Genomic DNA was extracted following a high salt extraction protocol (Appendix F: Protocol F1). Thirteen highly polymorphic fluorescently labelled microsatellite markers were amplified (Table 5.1). PCR reactions were performed in two multiplexes, and forward primers were labelled with 6-FAM™, NED™ and VIC™ fluorescent dye (Applied Biosystems, Integrated DNA Technologies). Multiplex PCR reactions had a total volume of 10µl, and contained 1µl of genomic DNA, 5 µl Type-it

Multiplex PCR Master Mix (Qiagen) and primer mix of labelled forward and reverse with 0.1 – 0.3 µM final concentrations. PCR conditions were: an initial denaturation of 95°C for 15min; followed by 35 cycles of 94 °C for 30sec, annealing temperature of 55°C or 58°C for 90sec for multiplex one and two respectively, and 72°C for 60sec, with a final extension at 60°C for 30min. Samples were randomized over populations during the genotyping analysis to avoid bias and negative controls used throughout. PCR products were diluted ten times with ddH_2O and 1 µl diluted PCR product, was added to 8.95µl Hi-Di™ Formamide (Thermo Scientific) and 0.05 µl GeneScan™ 600LIZ ladder (Applied Biosystems). Fragment analysis was performed on ABI 3730xl Genetic Analyzer (Applied Biosystems). An alternative method was used for labelling PCR product with a fluorescent dye. See Appendix G were a three-primer tail method is discussed.

Alleles were scored with GeneMarker® V1.8. We rounded all genotype calls to an even or odd number based on the respective locus. Differences between assigned and actual allele size were between 0.1 and 1 bp. To calculate potential genotyping errors (Bonin *et al.* 2004; Pompanon *et al.* 2005), twenty-eight samples (approx. 10%) were selected randomly, genotyped three times, and genotyping error rates (allelic dropout and false allele) were calculated using PEDANT v.1.0 software (Johnson & Haydon 2007). In order to calculate error rates from three repeated genotypes, we compared each repeat and averaged the error estimates, as recommended by Johnson (2007).

5.3.3 Genetic diversity

To assess genetic diversity, we estimated allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IT}) for each locus and population using FSTAT version 2.9.4 (Goudet 1995) and Genepop 4.2 (Raymond & Rousset 1995). We also calculated rarefied private allelic richness (A_P) in HP-rare (Kalinowski 2004, 2005). Compliance with Hardy Weinberg equilibrium (HWE) and linkage disequilibrium (LD) was tested among loci using chi-square statistics. Statistical significance was adjusted for multiple comparisons using Bonferroni correction. Data were analysed using the package *HardyWeinberg* in R.

Table 5.1 Description of thirteen microsatellite markers used in the study.

Locus	Repeat structure	Primers (5'- 3')	Ta (C°)	Allelic size range (bp)	Multiple x	Final concentration (µM)	Dye	GenBank	Reference
BC01	$(TAC)_{16}$	F: TCCATAATCAGGCGCTCATA R: TCTATTCTCTTAAACCGGAGAGG	55	85 - 127	1	0.3	FAM	KX237581	Faucher *et al.* 2016
BC09	$(TAGA)_{11}$	F: GGTGGTGGCACATTTCTTTT R: GTAGTTTGCCAGCAATGCCT	55	237 0 273	1	0.1	FAM	KX237593	Faucher *et al.* 2016
BC11	$(GATA)_{11}$	F: AGCCTTCTTTGCATCACTGC R: TAGCGGGAAGAGATGTACGC	55	128 - 158	1	0.1	VIC	KX237575	Faucher *et al.* 2016
BC37	$(ATCT)_{9}$	F: TCACCTGTACCCCTCTGGG R: CCATCCATGACACAGACCAG	55	87 - 116	1	0.1	VIC	KX237591	Faucher *et al.* 2016
BC39	$(TCTA)_{8}$	F: TCTGTCCTTCTGTCCAATCTG R: GCACCTTTGTTCAGGATGGT	55	167 - 195	1	0.3	FAM	KX237592	Faucher *et al.* 2016
BC45	$(TAGA)_{8}$	F: CCCTTGCAGCCAAAATAAAA R: TAACAGGAAACGGATTTGGG	55	118 - 156	1	0.3	NED	KX237594	Faucher *et al.* 2016
BC02	$(GATA)_{14}$	F: TTGCTTGAGAAAAGTCCAACA R: ACTTGCCAACTCTCCCAGAA	58	191 - 218	2	0.3	VIC	KX237585	Faucher *et al*. 2016
BC08	$(TAGA)_{11}$	F: CTCTTGTGCAAGATCTCTGGG R: TACTGACTGCTGCCCTCTCC	58	241 - 279	2	0.1	FAM	KX237574	Faucher *et al.* 2016
BC22	$(ATCT)_{9}$	F: TGCAGATTGCCAGCAGTTTA R: CACTTCCTCAAGGTGGTGCT	58	314 - 339	2	0.1	FAM	KX237578	Faucher *et al.* 2016
Bcalµ1	$(AT)4(GT)_{22}$	F: TGGGAATCCTTAGTGGTGAGCC R: TGAACCCATCTTGTAAATGGCC	58	122 - 138	2	0.1	VIC	X99281	Rowe *et al.* 1997
Bcalµ3	$(TC)_{21}$	F: TGGGTGTCATGTTAGATTCCC R: TGGACACTATTTGGGACTTGC	58	109 - 129	2	0.3	FAM	X99283	Rowe *et al.* 1997
Bcalµ8	$(CT)_6GT(CT)_4GT$ $(CT)_{24}ATAC(AT)_7$	F: TGCTAGGGAATAACTGGAGAGC R: GTGAACAGAAATGGTTTAGGGC	58	153 - 179	2	0.3	NED	X99288	Rowe *et al.* 1997
Bcalµ11	$(AG)_{14}$	F: TCATAGGTCAGTGGAAAGAGCA R: CGTCAACTTCAATTCGCTCA	58	165 - 193	2	0.1	FAM	AF267240	Rowe *et al.* 2000

5.3.4 Polygyny

Polygyny is a common anuran breeding strategy. While multiple paternity of egg clutches as a result of polyandrous mating or clutch piracy have been observed in several amphibian species (e.g. Vieites *et al.* 2004; Knopp & Merilä 2009; Byrne & Roberts 2012), it is unlikely for the Natterjack toad due to species segregated-pair breeding behaviour (May *et al.* 2011). Polygyny was estimated following Ficetola *et al.* (2010). We used the computer software COLONY (Jones & Wang 2010) to estimate half-sibling groups among the sampled eggs and tadpoles. We assumed that all eggs and tadpoles had different mothers based on the sample collection method (i.e. samples were collected from different egg strings or different breeding ponds), but that one male could fertilise more than one egg string. The reconstructed sibships were used to assess the number of egg strings fertilized by each male. We identified all half-siblings and calculated the degree of polygyny at a population level as the average number of egg strings fertilized by reproductive males. Eggs and tadpoles without half-siblings were treated as the offspring of males that fertilized only one egg string. Variance in reproductive success at a population level was calculated as the variance in the number of egg strings fertilized by breeding males. We performed Spearman's rank correlation to investigate relationships among polygyny, variance in reproductive success and number of egg strings. Analysis were performed in R using the *stats* package (R Core Team 2019).

5.3.5 Population measures

We used egg string counts as a measurement to monitor population size, a widely used method by previous Natterjack toad surveys in Great Britain (Smith & Skelcher 2019) and Ireland (Bécart *et al.* 2007; Sweeney *et al*. 2013; Reyne *et al.* 2019). Females typically deposit one egg string per year (Buckley & Beebee 2004), thus the number of egg strings can be used as a proxy for the female breeding population size. We plotted the cumulative number of egg strings at each pond visit for each population and the asymptote was calculated using a Generalized Additive Model (GAM) which allowed 95% confidence intervals to be associated with counts. The lower confidence limit (LCL) accounted for potential double counting and the upper confidence limit (UCL) accounted for egg strings potentially being missed.

We calculated effective population size (N_e) using LDNe 1.0 software (Waples & Do 2008). Any bias caused by a small sample size is corrected by implementing the bias-correction method of Waples (2006). We ran the model with three different critical values (*Pcrit*) of allele frequency. *Pcrit* was set at 0.05, 0.02 and 0.01 which means that all alleles with frequency lower than the critical value were excluded from analysis (Waples 2008). We used linear regression to evaluate the role of number of breeding females in explaining N_e. Analysis were performed in R using the *stats* package (R Core Team 2019).

5.3.6 Breeding area

Total available area to breed at each population was calculated as a sum of pond surface areas where breeding activity was recorded. We performed linear regressions between available breeding surface area and each of the following response variables: effective population size (N_e), mean observed heterozygosity (H_O), mean allelic richness (A_R) and polygyny to examine the relationship between breeding pond characteristics and genetic diversity. Regression analysis was performed in R using the *stats* package (R Core Team 2019).

5.3.7 Bottlenecks

BOTTLENECK v.1.2.0.2 was used to identify populations that recently had undergone a reduction in effective population size through testing for significant deviations from the mutation-drift equilibrium. The program was run under two mutational models for microsatellite data: two-phased (TPM) and stepwise mutation (SMM). The TPM model was run with 95% single-step mutations, 5% multiple-step mutations and variance among multiple steps of 12, as recommended by Piry *et al.* (1999). A Wilcoxon single-rank test was used to test for heterozygosity excess. Significant results of heterozygosity excess indicate evidence of a recent reduction in the effective population size (Piry *et al.* 1999). We also performed a two-way ANOVA between mean observed heterozygosity and mean allelic richness across markers and populations to compare genetic diversity between stable and declining populations. Analysis was performed in R using *stats* package (R Core Team 2019).

5.3.8 Population genetic structure

We performed discriminant analysis of principal components (DAPC), a multivariate analysis which is free from inherent assumptions and has been identified as demonstrating greater resolution of population structure when levels of genetic differentiation are low (Tang *et al.* 2009; Jombart *et al.* 2010). Analyses were carried out in the *adegent* package in R following the procedure suggested by Jombat (2008) We retained ten principal components of PCA in the data transformation step. The optimal number of clusters to retain in the analysis was identified using Akaike Information Criterion (Akaike 1998) and Kullback Information Criterion (Cavanaugh 1999) where lower values indicate better fit. As a complementary approach, we used *snapclust* clustering algorithm to identify optimal number of genetic clusters (K) and assign individuals to panmictic populations. This new method is comparable to STRUCTURE while being computer-efficient and more flexible in generating complex models (Beugin *et al.* 2018). Data were analysed using *adegent* package in R.

F_{ST} (Weir & Cockerham 1984) was used to investigate patterns of differentiation among populations and as an indirect measurement of historical gene flow. We tested for significance across these comparisons using 10,000 permutations. Statistical significance was adjusted for multiple comparisons using Bonferroni correction. Data were analysed using R package *hierfstat* (Goudet 2005) and FSTAT v2.9.4 (Goudet 1995).

Population structure was also assessed using Analysis of Molecular Variance (AMOVA, Excoffier *et al.* 1992) which calculates the proportion of the total genetic

variance originating within and between populations. Permutation tests were performed at three hierarchical levels: among populations, among individuals within populations and within individuals. Analysis were conducted in Arlequin v3.5.2.2. (Excoffier & Lischer 2010).

5.4 Results

In total, 316 samples from all seven Irish populations of Natterjack toad were collected and successfully genotyped. Sample size varied between 32 and 58 individuals (Table 5.2). All 13 microsatellite loci amplified successfully. Genotyping error rate varied among loci ranging from 0 to 0.10 for allelic dropout and from 0 to 0.03 for false alleles (Appendix F: Table F.1). All microsatellite markers were polymorphic with allelic richness ranging from 4.77 to 5.69 (Table 5.2). Mean observed and expected heterozygosity per locus were 0.54 and 0.59 respectively (Appendix F: Table F.2). Linkage-disequilibrium analysis performed 78 pairwise comparisons between loci, identifying 28 linked pairs (36%) (Appendix F: Table F.3). At a population level, Glenbeigh had the lowest (0.49) and Caherdaniel the highest (0.59) observed heterozygosity (Table 5.2). The mean inbreeding coefficient across all populations was low (ranged 0.04 - 0.16; Table 5.2). Overall, the Natterjack toad population in Ireland was not in Hardy-Weinberg equilibrium (p=0.01).

The number of egg strings fertilized by individual males was between 1 and 8. The degree of polygyny at a population level varied between 2.13 and 3.73 (Table 5.2). Variance of male breeding success differed strongly among populations and ranged from 1.98 to 13.22 (Table 5.2). There was no significant relationship between polygyny, variance of breeding success and number of egg strings i.e. breeding females (p>0.05).

Table 5.2 Summary statistics of the seven Natterjack toad populations in Ireland. Sample size (*N*), total number of alleles (A_T), allelic richness (A_R) independent of sample size, private allelic richness (A_P), observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), polygyny and variance in breeding success (Var. success) are given for each population.

Population	*N*	A_T	A_R	A_P	H_O	H_E	F_{IS}	*Polygyny*	*Var. success*
Caherdaniel	41	77	5.69	0.55	0.59	0.61	0.06	3.27	13.22
Dooks	43	74	5.23	0.34	0.57	0.59	0.04	3.73	9.62
Glenbeigh	42	71	5.13	0.26	0.49	0.57	0.16	3.42	6.27
Inch	49	73	5.27	0.40	0.54	0.60	0.09	2.44	3.60
Magharees	58	71	4.79	0.27	0.54	0.57	0.05	2.55	3.59
Roscullen	32	62	4.77	0.28	0.51	0.55	0.07	2.38	2.26
Yganavan	51	66	4.82	0.21	0.56	0.60	0.06	2.13	1.98

In total, we recorded 1,457 egg strings. There was a significant sigmoidal accumulation of egg strings for all populations resulting in narrow 95% confidence intervals (Appendix F: Figure F.1, Table 5.3). The narrow 95% Confidence Intervals for most populations are indicative of reasonably precise estimates. Based on the number of egg strings, the most productive population was at the Magharees sand dune and the least productive at Inch (Table 5.3). Estimates of N_e were low, ranging from 19 at Caherdaniel to 519 at Yganavan but typically <100 individuals at most sites (Table 5.3). There was no significant relationship between the number of breeding females and N_e regardless of the critical value of allele frequencies used to calculate N_e (p>0.05)

Table 5.3 Number of breeding females ($N_{females}$) and effective population size (N_e) calculated for three critical values of allele frequency (0.05, 0.02 and 0.01) with 95% confidence intervals for seven Natterjack toad populations in Ireland.

Population	$N_{females}$	*CI 95%*	$N_{e0.05}$	*CI 95%*	$N_{e0.02}$	*CI 95%*	$N_{e0.01}$	*CI 95%*
Caherdaniel	231	218-248	36.0	23.8 - 62.3	26.6	20.4 - 35.9	18.6	15.0 – 23.0
Dooks	54	50-59	35.5	23.8 - 58.6	40.7	29.0 - 62.2	22.4	18.1 - 28.0
Glenbeigh	59	55 - 62	27.0	18.7 - 41.4	37.1	27.2 - 53.9	49.6	35.3 - 76.3
Inch	18	17 - 19	47.6	30.7 - 86.4	45.0	33.0 - 65.6	40.7	30.7 - 56.9
Magharees	884	836 - 971	145.3	61.7 - ∞	75.6	46.8 – 156.0	50.2	36.5 - 73.9
Roscullen	56	54 - 57	45.2	25.0 - 124.1	36.8	22.9 - 72.2	26.8	18.8 - 41.4
Yganavan	155	147 - 163	519.4	95.7 - ∞	61.0	38.7 - 119.6	118.8	61.9 - 571.0

Linear regression suggested no relationship between available breeding surface area and mean allelic richness A_R ($F_{df=1,5}$=1.658, p=0.254, R^2=0.249), mean observed heterozygosity H_O ($F_{df=1,5}$=0.383, p=0.563, R^2 =0.071) or degree of polygyny ($F_{df=1,5}$=3.037, p=0.142, R^2 = 0.378). There was a positive relationship between breeding surface area and N_e when the critical value of allele frequency was 0.05 and 0.01 ($N_{e\ 0.05}$: $F_{df=1,5}$=136.195, p<0.001, R^2 =0.965; $N_{e\ 0.01}$: $F_{df=1,5}$=37.430, p=0.007, R^2 =0.882) but the relationship was less clear with an allele frequency of 0.02 ($N_{e\ 0.02}$: $F_{df=1,5}$= 4.921, p=0.077, R^2 =0.496).

BOTTLENECK analysis suggested significant deviations from mutational drift equilibrium for some populations (Table 5.4). However, there is no evidence for significant heterozygosity excess in any population. In addition, there was no evidence of mode-shift in allele frequencies as all distributions were L-shaped suggesting no recent reduction in the effective population size. Additionally, a two-way ANOVA showed no significant difference in the allelic richness ($F_{df=6,84}$=0.594, p=0.735) and observed heterozygosity ($F_{df=6,84}$=0.490, p=0.814) among populations.

Table 5.4 BOTTLENECK results for two-step (TPM) and stepwise (SMM) mutational models. L-shape distribution of allele frequency indicates lack of a bottleneck. Statistical test shows deviations from the mutational-drift equilibrium. Significant values are marked with an asterisk.

Population	Mutation model	Sign test	Wilcoxon test			Allele frequency distribution
			H **deficiency**	***H*** **excess**	***H*** **excess & deficiency**	
Caherdaniel	TPM	0.000*	0.000*	1.000	0.000	L - shaped
	SMM	0.000*	0.000*	1.000	0.000	
Dooks	TPM	0.115	0.108	0.905	0.216	L - shaped
	SMM	0.041*	0.034*	0.971	0.068	
Glenbeigh	TPM	0.002*	0.003*	0.998	0.005	L - shaped
	SMM	0.000*	0.001*	1.000	0.001	
Inch	TPM	0.009*	0.024*	0.980	0.048	L - shaped
	SMM	0.009*	0.0118	0.996	0.021	
Magharees	TPM	0.033*	0.020*	0.984	0.040	L - shaped
	SMM	0.035*	0.004*	0.997	0.009	
Rossculen	TPM	0.057	0.040*	0.966	0.080	L - shaped
	SMM	0.015*	0.024*	0.980	0.048	
Yganavan	TPM	0.115	0.188	0.830	0.376	L - shaped
	SMM	0.041*	0.095	0.916	0.191	

Cluster analysis suggested an optimal number of K=5 using AIC and K=4 using KIC (Appendix F: Figure F.2). KIC approach indicated a better fit (sharp decrease in the values followed by a sharp increase), hence the number of cluster retained in the analysis was four. DAPC indicated that the Magharees population differed from the rest of the populations with no overlap (Figure 5.2a). The Caherdaniel population was also distinct from all other populations, although it had similar DAPC axis 1 scores (which carried most of the observed variation) to the Magharees population from which it was derived. The Castlemaine Harbour populations (Glenbeigh, Yganavan, Inch, Dooks and Rossculen) clustered together but re-analysis after the exclusion of

the Magharees and Caherdaniel suggested Rossculen was distinct from the rest whilst Glenbeigh had minimal overlap (Figure 5.2b). *Snapclust* analysis revealed similar pattern where Magharees and Caherdaniel populations were different from the rest of the populations, however it failed to clearly differentiate among the Castlemaine Harbour populations (Appendix F: Figure F.3). Based on our analysis, we identified four main genetic entities: 1) Magharees, 2) Caherdaniel, 3) Roscullen and 4) the remainder of the Castlemaine Harbour populations i.e. Dooks, Glenbeigh, Yganavan and Inch.

AMOVA analysis suggested most molecular variation (96.43%) originated from within individuals while variation between populations accounted for 4.58% (Table 5.5). AMOVA detected significant (p<0.001) differentiation among populations. The pairwise F_{ST} values suggested higher levels of gene flow between Glenbeigh, Yganavan, Dooks and Inch with F_{ST} ranging between 0.016 and 0.020 (Table 5.6). Higher F_{ST} values (>0.05) were observed between Magharees and the rest of the populations apart from Caherdaniel.

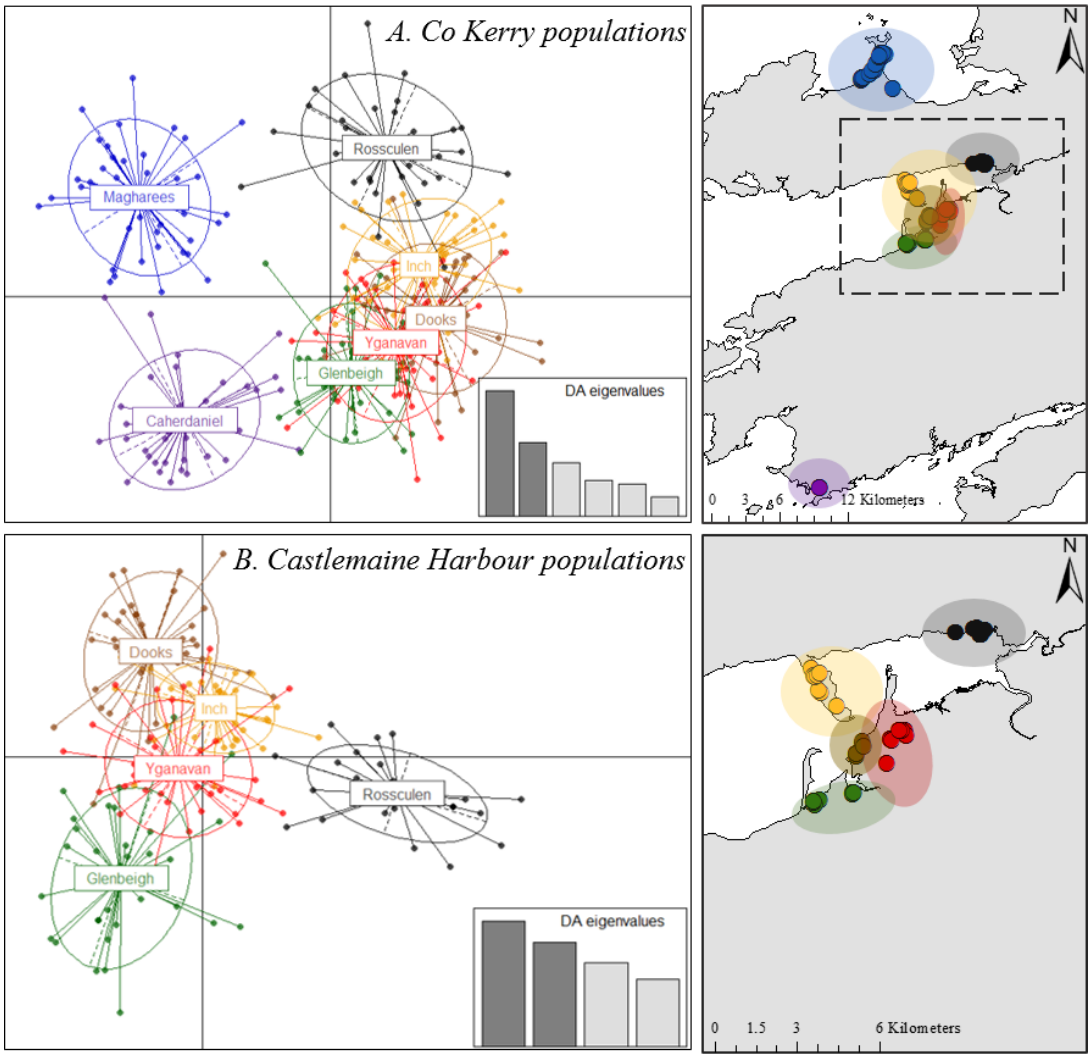

Figure 5.2 Discriminant Analysis of Principal Component (DAPC) of Natterjack toad populations. Ordination plots shows the first two principal components of the DAPC for A) all seven populations and B) separate for the five Castlemaine Harbour populations. Dots on the DAPC graphs present individuals. Populations are shown by different colours and inertia ellipses that correspond to spatial locations on the map. Labels are placed at the centre of dispersion for each population. Eigen values suggest that the first two components explain the biggest genetic variation in the dataset.

Table 5.5 Analysis of molecular variance (AMOVA). Significant values are marked with an asterisk.

	d.f.	**Sum of squares**	**Variance components**	**% variation**	***p***
Between populations	6	52.84	0.11	4.58	<0.001*
Individuals within populations	204	459.69	-0.02	-1.00	0.705
Within individuals	211	485.5	2.30	96.42	0.045*
Total	421	998.02	2.39	-	-

Table 5.6 mPairwise F_{ST} values between seven Natterjack toad populations. Tests were performed with 10,000 permutations. Significance after Bonferroni correction (adjusted α threshold = 0.05) are marked with an asterisk.

	Magharees	Caherdaniel	Rossculen	Dooks	Glenbeigh	Inch
Caherdaniel	0.033*	-	-	-	-	-
Roscullen	0.050*	0.047*	-	-	-	-
Dooks	0.081*	0.046*	0.040*	-	-	-
Glenbeigh	0.062*	0.029*	0.042*	0.029*	-	-
Inch	0.067*	0.039*	0.026*	0.020*	0.024*	-
Yganavan	0.059*	0.028*	0.027*	0.017*	0.018*	0.016*

5.5 Discussion

The regionally Red-Listed Irish Natterjack toad population, whilst having undergone substantial declines in census population size (Reyne *et al.* 2019), exhibited no evidence of genetic bottlenecks or inbreeding with relatively high genetic diversity (allelic richness and heterozygosity), despite low effective population sizes. Analysis of population structure suggested four genetic entities to be considered in species conservation programmes.

All seven Natterjack toad populations in Ireland were polymorphic at the thirteen microsatellite loci. Expected heterozygosity (0.55 - 0.61) and observed heterozygosity (0.49 - 0.59) were higher than previous estimates for the same populations in Ireland and higher than most estimates for Natterjack toad populations throughout Europe (Appendix F: Table F.4). However, estimates of heterozygosity and allelic richness are not directly comparable due to the use of a different set of microsatellite markers. Pond breeding amphibians are predisposed to lower levels of genetic variation compared to other taxa resulting from high amplitude fluctuations in population size (Alford & Richards 1999; Newman & Squire 2001). For example, the Natterjack toad typically breeds in ephemeral ponds where breeding success depends on stochastic climatic variation i.e. wet warm years (Beebee & Griffiths 2000; Baker *et al.* 2011; Smith & Skelcher 2019). However, small, declining amphibian populations have been shown to retain high genetic diversity, for example, the black toad (*Bufo exsul*, Wang 2009), the critically endangered Montseny brook newt (*Calotrina arnoli*, Valbuena-Urena *et al.* 2017) and the Hula

painted frog (*Latonia nigriventer*, Perl *et al.* 2018), considered to be one of the world's rarest amphibians.

One of the key factors determining population genetic diversity is effective population size, a more valuable tool than census size alone for population management. Low N_e values can potentially indicate loss of genetic variability within a population (Frankham *et al.* 2003; Ficetola *et al.* 2007; Palstra & Ruzzante 2008). Our estimates of the effective population size were low for all seven remaining populations (mainly <100 individuals) despite observed high genetic diversity. The N_e for pond breeding amphibians is typically in tens rather than hundreds or thousands of individuals regardless of large census sizes (Beebee & Griffiths 2005). Similar estimates of the effective population size were reported for ten Natterjack toad populations in Europe (Faucher *et al.* 2017) and other amphibian species including the marsh frog (*Rana redibunda*, Zeisset & Beebee 2003), Italian agile frog (*Rana latastei*, Ficetola *et al.* 2010) and common frog (*Rana temporaria*, Brede & Beebee 2006). This generally reflects the most common anuran breeding strategy of scramble competition where large males dominate breeding ponds and available females resulting in only a few males contributing to the gene pool of the next generation (Ficetola *et al.* 2010). Higher levels of polygyny and variance of male breeding success were recorded at Caherdaniel, Dooks and Glenbeigh where few, small breeding ponds are available. Variation in breeding habitat has previously been associated with differences in effective population size (Wang *et al.* 2010), and so too in this study. Natterjack toad N_e was significantly associated with the breeding surface area

available. The highest N_e was calculated for the Yganavan population where toads breed along the shore of a large lake, suggesting less competition among males (i.e. low polygyny). Populations with small available breeding habitat had smaller N_e, but it did not result in lower genetic diversity. Similar results were found for the California tiger salamander (*Ambystoma califroniense*, Wang *et al.* 2011) highlighting the need for a more complete understanding of the parameters influencing N_e.

Despite small N_e and substantial declines in egg sting numbers (breeding females) since 2004 for populations at Rossculen, Yganavan, Dooks and Glenbeigh (Reyne *et al.* 2019), our analysis did not provide evidence of recent genetic bottlenecks. Dooks and Yganavan populations have declined by over 70% (Reyne *et al.* 2019); however, migration between these sites may decrease the number of rare alleles, consequently masking any H_E excess (Cornuet & Luikart 1996). The BOTTLENECK program has been reported to fail to detect known demographic bottlenecks (Whitehouse & Harley 2001). However, measures of allelic diversity have been shown to be good indicators of bottlenecks when comparable data on demographically stable populations are available (Spencer *et al.* 2000; Whitehouse & Harley 2001). The Magharees had the largest Natterjack toad population in Ireland accounting for 90% of the recorded egg strings deposited annually which has remained largely stable over time (Reyne *et al.* 2019). However, allelic richness for the Magharees was among the lowest (4.79) for Irish populations, though there was no significant difference in allelic richness among populations. This would support the assertion of no decline in the effective population size consistent with a lack of

evidence of a genetic bottleneck. Similarly, the Australian bell frog (*Litoria aurea*) has undergone an 80% population decline over the past 40 years with no apparent genetic bottleneck with levels of allelic richness not significantly different for nineteen out of twenty-one populations when compared to a large demographically stable population (Burns *et al.* 2004).

The Natterjack toad in Ireland exhibited a high level of genetic structuring throughout its range with all sampled locations being significantly different. DAPC results suggest that Natterjack toad populations in Ireland can be divided into fourgenetic entities: 1) Magharees; 2) Caherdaniel; 3) Roscullen; 4) Dooks, Glenbeigh, Yganavan and Inch. Amphibians exhibit strong site fidelity and limited dispersal and migration, resulting in low levels of gene flow among populations (Reading *et al.* 1991; Kusano *et al.* 1999; Pittman *et al.* 2008). Several studies suggested that highly structured populations are often typical of amphibians with distinction at scales less than 5km (e.g. Shaffer & Breden 1989; Routman 1993; Driscoll 1998) questioning the theory of metapopulation. For amphibian species with highly structured populations site-specific protection and human-mediated translocations/reintroductions may be critical management tools to preserve intraspecific genetic diversity (Shaffer *et al.* 2000).

Conservation recommendations should focus on maintaining high genetic diversity as well as protection of the genetic integrity of identified genetic entities. This can be achieved by maintaining adequate effective population size (Storfer *et al.* 2007; Wang *et al.* 2009) especially in small and fragmented populations (Wang *et al.*

2011). The National Parks & Wildlife Service (NPWS) pond creation scheme is not currently creating new ponds but should future measures to incentivise farmers to create new potential breeding ponds, and these should be clustered within and around each identified management unit. Furthermore, our results suggest that having large breeding ponds or high numbers of small ponds in close proximity can be particularly valuable for ensuring a large N_e associated with large breeding surface area and higher number of successfully mating male toads. The ongoing NPWS Head-start and Translocation Programme should be cognisant of our identified genetic entities when releasing toadlets back into the wild respecting their genetic provenance, not translocating individuals between entitiesd and selecting the source populations from geographically proximate sites within the same unit. The only exception is Caherdaniel, as the population is likely distinct due to founder effects, subsequent gene drift and lack of gene flow after its establishment using translocated individuals from the Magherees. Thus, should further translocations be required in the future to maintain the population these can be drawn from the original source population. The lowest observed heterozygosity and highest inbreeding coefficient were recorded for the Glenbeigh population. Inbred Natterjack toad's tadpoles have slower growth rates and lower survival rates (Rowe & Beebee 2005). These findings, therefore, pose conservation concerns about the long-term survival of the Glenbeigh population. Consideration should be given to population supplementation at Glenbeigh with translocated individuals ideally from Yganavan as it is genetically the closest population, but alternatively from any population within the management

unit. It is important to note that while genetically most relevant population is preferable as a source population, it becomes unfavourable if removal of those individuals might put the source population at risk. Hence, other factors like population size and trend, potential threats and pressures should be considered when selecting source populations.

There may be an aspiration that the Natterjack toad's range in Ireland is enlarged beyond its current highly restricted range, for example, reintroducing animals to sites in the southwest occupied historically or introducing animals to suitable habitat in the west more generally which have never been occupied (for example, assisted migration of populations northward to track climate change). In these cases, donor populations should either be the geographically closest population or, if a new location is distinct from existing genetic entities and isolated by barriers to dispersal, the Magharees population can be considered as donors due to the high genetic diversity and large population size. An experimental approach could be taken by mixing individuals from each of the genetic entity to create artificially high genetic diversity buffering any new population against local extirpation due to small initial effective population size (Beauclerc *et al.* 2010; IUCN/SSC 2013). Monitoring such populations would be warranted.

Our findings have important conservation implications for the management of the Natterjack toad, which is regionally Red-Listed as Endangered in Ireland. Populations appear to have no deficiency in allelic richness or heterozygosity despite low effective population sizes and there is no evidence of genetic bottlenecks despite

declines in census size. We identifiedfour genetic entities, which we urge species conservation programmes to consider when undertaking population supplementation, translocation or assisted migration. Apparent high levels of genetic variation gives hope for the conservation of Ireland's rarest amphibian.

Chapter 6

Landscape genetics identifies barriers to Natterjack toad metapopulation dispersal

A manuscript based on this chapter has been submitted and is in review as:

Reyne M, Kara D, Flanagan J, Nolan P, Aubry A, Emmerson M, Marnell F, Helyar S, Reid N (*in review*) Landscape genetics identifies barriers to Natterjack toad metapopulation dispersal. *Molecular Ecology.*

6.1 Abstract

Habitat fragmentation and loss restrict gene flow among populations leading to loss of genetic diversity. Functional connectivity is key for species persistence in human-modified landscapes. To inform species conservation management, we investigated spatial genetic structure, gene flow and inferred dispersal between twelve metapopulations of the Natterjack toad (*Bufo calamita*); regionally Red-Listed as Endangered in Ireland. Spatial genetic structure was determined using non-Bayesian clustering analysis of 13 polymorphic microsatellite loci genotyping 247 individuals. We tested the influence of geographic distance, climate, habitat, geographical features, and anthropogenic pressure on pairwise genetic distances between metapopulations using Isolation-by-distance and Isolation-by-resistance based on least-cost path and circuit theory models of functional connectivity. There was clear spatial structuring with genetic distances increasing with geographic distance. Gene flow was best explained by Isolation-by-resistance models with coniferous forestry plantations, bog, marsh, moor and heath, scrub, anthropogenic presence (Human Influence Index) and rivers (riparian density) identified as barriers to gene flow while metapopulation connectivity was enhanced by coastal habitats (beaches, dunes, sand and salt marshes) and coastal grassland. Despite substantial declines in census numbers over the past 15 years and its regional status as Endangered, the Natterjack toad population in Ireland retains high genetic diversity. If declines continue, maintaining habitat connectivity to prevent genetic erosion by management of coastal grasslands, pond construction and assisted migration through translocation will be increasingly important.

6.2 Introduction

Habitat degradation and fragmentation are key drivers of the current global biodiversity crisis (Foley *et al.* 2005). Habitat fragmentation, through habitat loss and habitat patch isolation changes animal behaviour including dispersal (Janin *et al.* 2012), movement patterns (Poessel *et al.* 2014), mortality rates (Pinto *et al.* 2018), population growth (Bascompte *et al.* 2002), population structure (Haag *et al.* 2010) and population viability (Newman *et al.* 2013). Regions experiencing habitat loss have a greater proportion of species in decline than regions of intact habitat (Donovan & Flather 2000), with the degree of landscape fragmentation key to population persistence (Clobert *et al.* 2001). Unconstrained animal movements are important in foraging, breeding, dispersal, (re)colonization and essential for responding to environmental change (Zeller *et al.* 2012). These factors are particularly important in metapopulations where a species distribution is disjunct and separated in discrete, dispersed populations. Factors limiting dispersal rates increase isolation and are thus likely to limit gene flow and genetic differentiation between populations, potentially accelerating inbreeding and vulnerability to the extinction vortex (Fahrig 2002).

The global amphibian crisis, whilst predominately driven by disease (fungal Chytridiomycosis), has been exacerbated by habitat destruction and degradation such that 41% of species are threatened with extinction (IUCN 2020). Amphibians have poor dispersal abilities and are particularly vulnerable to habitat fragmentation (Graeter *et al.* 2008) due to small effective population size (Funk *et al.* 1999), high site fidelity (Joly *et al.* 2003) and a complex terrestrial-freshwater life cycle which necessitates two distinct environments narrowing their habitat tolerances (Houlahan

& Findlay 2003). Lack of connectivity between breeding sites and suitable terrestrial habitats can lead to high mortality during breeding migrations and low recruitment of dispersing individuals (Janin *et al.* 2012). Amphibian metapopulations are highly dynamic and susceptible to local extirpations and turnover, hence habitat connectivity is crucial for recolonization (Brown & Kodric-Brown 1977) and population persistence (Trenham *et al.* 2003). Pond breeding amphibians are notably sensitive to isolation due to habitat fragmentation between breeding sites, which is a key issue in their conservation (Cushman 2006). Understanding dispersal, population genetic structure and the impact of landscape features on habitat connectivity are crucial for developing targeted and species-specific conservation management approaches at the landscape level. Moreover, such information can be used to predict the impact of proposed land use changes and/or infrastructural developments helping shape mitigation strategies such as the creation of dispersal corridors (Storfer 2007).

Monitoring dispersal through direct observations can be costly, difficult and time consuming (Broquet & Petit 2009), where it is possible at all. Recent development in geospatial information technology, molecular biology and the resolution of several statistical problems in spatial genetics such as nonindependence among samples (Peterman 2018) facilitate the indirect study of movements using gene flow and Global Information Systems (GIS) (Balkenhol *et al*. 2009). Landscape genetics integrates population genetics, spatial analysis, biogeography and landscape ecology to detect genetic discontinuities to quantify the effects of geographical distance and landscape permeability on metapopulation genetic structure (*Manel et*

al. 2003; Storfer *et al.* 2007). As an alternative to a null model where no spatial genetic structure is assumed, an isolation-by-distance (IBD) approach assumes that observed genetic differences between populations are a function of geographic distance i.e. isolation alone. IBD is expected for populations inhabiting continuous habitats where the main factor contributing to the genetic differentiation is the distance between populations and the dispersal capabilities of the studied species. Habitat fragmentation creates dispersal barriers limiting gene flow. An isolation-by-resistance (IBR) approach assumes that observed genetic differences between populations are a function of landscape resistance to dispersing individuals (McRae 2006). IBR is expected for populations inhabiting heterogenous landscapes of discontinuous habitats. Resistance surfaces represent the cost to a dispersing individual in crossing a landscape where low resistance values represent ease of movement and high resistance values represent restricted movement due to the presence of barriers along a permeability gradient (Zeller *et al*. 2012). IBD indicates lack of population isolation due to habitat fragmentation while IBR indicates population isolation due to limited dispersal explained by habitat fragmentation and loss of connectivity (Kobayashi & Sota 2019).

This study aimed to investigate the role of geographic distance, climate, habitat, geographical features, and anthropogenic pressure in determining the spatial genetic structure of the Natterjack toad (*Epidalea calamita*) in Ireland, regionally Red-listed as Endangered (King *et al*. 2011). Ireland has lost most of its freshwater ponds (Reid *et al.* 2012), with loss of breeding sites identified as the single most important driver of Natterjack toad population declines (Beebee 2002). The

National Parks and Wildlife Service (NPWS) initiated a Pond Creation Scheme in 2008 installing over 100 artificial ponds throughout the Natterjack toad range, though <25% had been colonised by 2018 (Reyne *et al.* 2019). Consequently, the NPWS initiated a Natterjack toad Head-start and Translocation Programme in 2016 by collecting egg strings/tadpoles annually to raise toadlets in captivity before release back into the wild as part of assisted migration and translocation to newly created ponds. The objectives of this study were to: i) use microsatellite markers to estimate genetic structure and quantify pairwise genetic distances between remaining breeding sites, ii) quantify climatic and habitat landscape variability, and iii) relate genetic distance to a) geographic distance and b) landscape dispersal resistance explicitly testing isolation-by-distance (IBD) and isolation-by-resistance (IBR) models. We hypothesised that due to the Natterjack toad's affiliation with coastal habitats in Ireland, inland climate and habitat factors would limit dispersal and explain landscape genetic structure better than geographic distance alone. Our results should inform ongoing species conservation management particularly in respect to pond creation and assisted migration through translocation.

6.3 Methods

6.3.1 Study area and sampling

The Natterjack toad is highly range restricted in Ireland, found only in County Kerry, Ireland (Figure 6.1a-b). We investigated gene flow between twelve breeding sites (Figure 6.1c), with field surveys conducted during April to July 2017. We collected samples for DNA extraction consisting of well-developed egg strings and tadpoles from different breeding ponds to avoid pseudoreplication by analysing siblings. Samples collected in the field were stored in 100% ethanol at ambient temperature until extraction.

6.3.2 DNA extraction and genotyping

DNA extraction and genotyping followed those described in Chapter 5. We checked for null alleles with the R package PopGenReport v3.0.0 (Adamack & Gruber, 2014). Approximately 10% of the samples were randomly selected and genotyped three times to calculate the rate of genotyping errors (allelic dropout and false alleles) using PEDANT v 1.0 software (Johnson 2007).

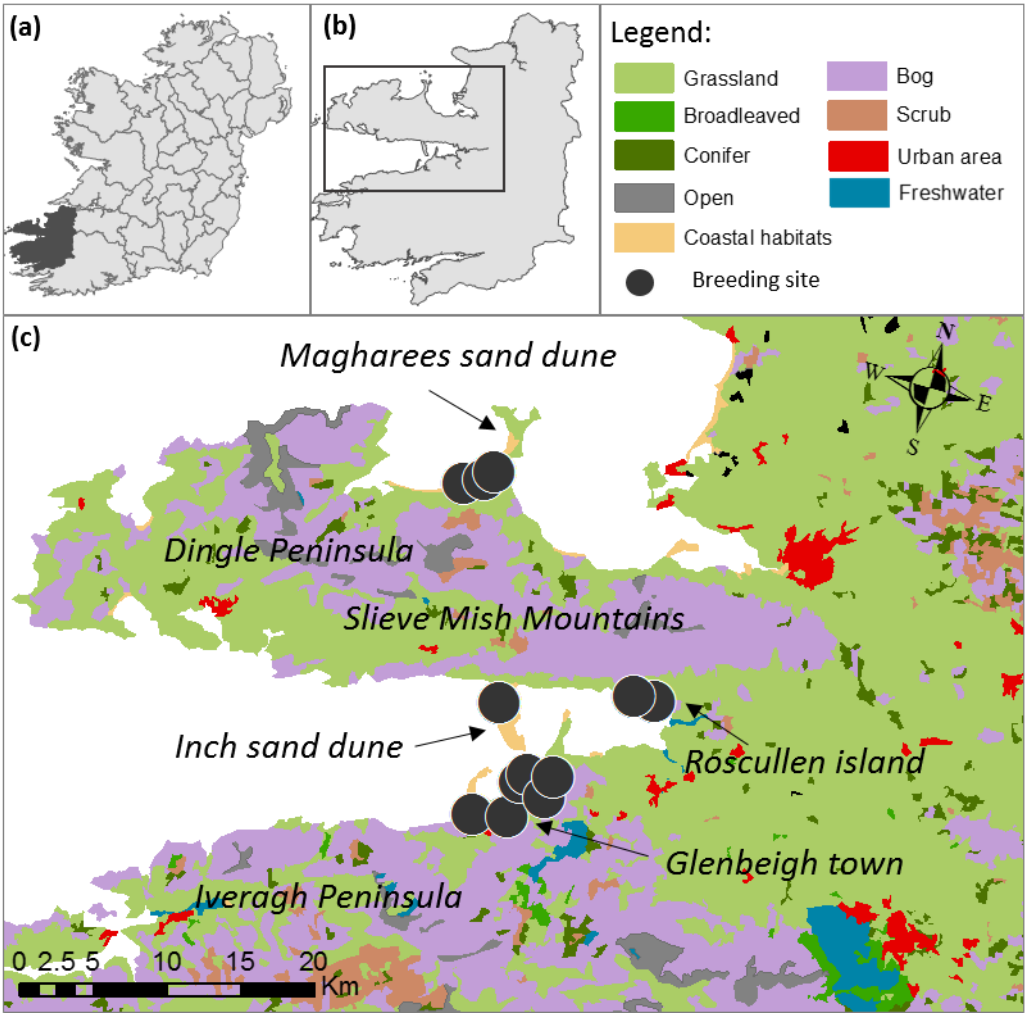

Figure 6.1 The location of the study area (grey shading) in Ireland, (b) within Co. Kerry including (c) breeding sites (dots) and habitat categories based on CORINE2018

6.3.3 Genetic diversity

Analyses of genetic diversity were performed at the breeding site level. We estimated the number of alleles per locus, allelic richness, observed (H_O) and expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) using the adegent package (Jombat 2008) in R (R Core Team 2019).

6.3.4 Population structure

We performed two non-Bayesian clustering analyses to infer population structure which are not dependent on Hardy-Weinberg or linkage equilibrium. Principal Coordinate Analysis (PCoA) is a multivariate technique that allows major patterns within a multivariate dataset to be identified based on algorithms developed by Orloci (1978). We used two measures of genetic distances to build PCoA biplots, F_{ST} (Weir & Cockerham 1984) and its unbiased estimate G''_{ST} (Meirmans & Hedrick 2011) calculated in FSTAT v2.9.4. (Goudet 1995) and GenAlEx 6.5 respectively. To investigate cryptic genetic patterns as a result of isolation, we performed a spatial Principal Components Analysis (sPCA; following Jombart *et al.* 2008). The method maximized the variance in individual allele frequencies while simultaneously accounting for spatial autocorrelation estimated using Moran's I (Moran 1948, 1950) using 9,999 randomized Monte-Carlo permutations to test for differences in allelic frequencies between neighbouring breeding sites. Data were analysed by sPCA using two multivariate statistical tests (global and local tests each with 9,999 randomized Monte-Carlo permutations) to assess if there were significant patterns at either scale. Presence of a global pattern indicates positive spatial autocorrelation i.e. neighbouring breeding sites tend to be similar, while presence of local structure indicates negative spatial autocorrelation i.e. neighbouring sites are dissimilar. All connection networks were defined using the Delaunay triangulation (Upton & Fingleton 1985). The minimum distance was set at 0 km and the maximum distance was set at 12 km, based on amphibian dispersal estimates reported by Smith & Green (2005). Genetic structure was analysed hierarchically, initially using the entire dataset

then using sample subsets based on detected genetic clusters. Data were analysed using the package ade4 for multivariate analysis (Dray *et al.* 2007), spdep for spatial methods (Bivand 2013, 2018) and adegenet for sPCA and multivariate tests (Jombart 2008) in R.

6.3.5 Environmental parameters

The Irish Centre for High End Computing (ICHEC) provided five spatially explicit climate variables derived from the COSMO-CLM5 ensemble model (averaging five different climate models: CNRM-CM5, EC-EARTH, HadGEM2-ES, MIROC5 and MPI-ESM-LR) downscaled specifically for Ireland at a 4km cell resolution (Table 6.1, Appendix H: Figure H.1). Variables were selected for their perceived relevance to Natterjack toad biology, specifically, temperature (air and surface) influencing activity levels and tadpole development and precipitation, humidity and surface evaporation influencing the permanency of breeding ponds. Climate variables accounted also for differences in the altitude across the study area.

A total of eleven land cover/habitat, geographical feature or anthropogenic pressure variables were captured (Table 6.1, Appendix H: Figure H.1). Land cover/habitat was extracted from CORINE2018 (EEA 2018) with Simpson's Diversity Index derived to quantify habitat diversity. Geographical features included distance to coast and riparian corridor density derived from an Ireland-specific GIS line shapefile of freshwater watercourses. Anthropogenic pressure was captured by the Human Influence Index (HII) downloaded from the Last of the Wild Project (Wildlife

Conservation Society 2005) aggregating human population density, land use, infrastructure and human access. All surfaces were mapped at 4km cell resolution to match the available climate data. Spatial data were extracted using ArcMap v 10.5 (ESRI, California, USA).

Table 6.1 Description of 16 environmental predictors including a) climatic variables obtained from the Irish Centre for High End Computing (ICHEC) and b) Habitat land cover derived from CORINE, c) geographical features and d) anthropogenic pressure.

Environmental parameter	Description including units and habitat ID codes
a) Climatic (ICHEC)	
Evaporation	Surface water evaporation (kg/m^2)
Humidity	Relative humidity at 2m (%)
Precipitation	Precipitation (kg/m2)
Temperature	Air temperature measured 2m above ground (^{o}C)
Surface temperature	Surface temperature (^{o}C)
b) Habitat (CORINE)	
Grassland	Pastures (231), Natural grasslands (321) and Land occupied by agriculture with significant natural vegetation (243)
Broadleaved	Broadleaved forest (311) and Mixed forest (313)
Conifer	Coniferous agroforestry plantations (312)
Scrub	Transitional woodland-shrub (324) and Fruit trees and berry plantations (222)
Bog, marsh, moor & heath	Peat bogs (412), Burnt areas (334), Moors & heathland (322) and Inland marshes (411)
Open	Bare rocks (332), Sparsely vegetated areas (333) and Burnt areas (334)
Coastal habitats	Beaches, dunes, sand (331) and salt marshes (421)
SDI	Simpson's Diversity Index of habitats
c) Geographical features	
Dist. to coast	Distance to coast: shortest perpendicular distance from each 4km cell centroid to the high tide mark (m)
Riparian dens.	Riparian density: density of linear freshwater features i.e. streams/rivers (km/km2)
d) Anthropogenic pressure	
HII	Human Influence Index

6.3.6 Landscape genetics analysis

We assessed three main models: (a) isolation-by-distance (IBD), where gene flow was a function of the distance between breeding sites; (b) isolation-by-resistance (IBR) where gene flow was a function of landscape resistance between breeding sites; and (c) a null model where gene flow was not characterised by spatial structuring between breeding sites.

IBD analysis related pairwise genetic distances between populations using linear mixed effects models with a maximum likelihood population effects (MLPE) parameterization (following Clarke *et al.* 2002) performed in the package ResistanceGA (Peterman 2018). Three measures of distance were used: i) Euclidian distance which was the shortest distance between each pair of sites as the-crow-flies, ii) geographic distance which was the distance between each pair of sites taking into account marine areas and including 3D topography and iii) historical distance which was geographic distance modified to account for a potential connection in the past between populations at Inch sand dunes and Iveragh Peninsula (Figure 6.1c). Distances were calculated using ArcMap. The dependent variable in each MLPE model was a genetic distance matrix (F_{ST} and G''_{ST}), breeding site was fitted as a Random Factor to account for non-independence of values in the pairwise matrix, and each measure of geographic distance was fixed as covariate in three separate models.

IBR analysis related connectivity with climate, habitat, geographic features and anthropogenic pressure variables using the package ResistanceGA. Pairwise resistance distances were calculated between breeding sites by implementing

genetic algorithms to optimise surface resistance based on pairwise genetic and resistance distances. Two IBR scenarios were tested: i) least-cost path and ii) circuit theory distances (following Kivimäki *et al.* 2014) using the costDistance and commuteDistance functions (Peterman 2018). Least-cost path distances estimated the optimal path with least resistance between two breeding sites, while circuit theory distance estimated commute-time distances performed in ResistanceGA equivalent to Circuitscape simultaneously considering all possible routes between the breeding sites (McRae 2006). IBR analyses were performed in two steps. The first step optimised the resistance of a single environmental surface by testing all 16 environmental parameters separately. Surface optimizations were performed twice as recommended by Peterman (2018) to check for convergence supporting parameter estimates. Model fit for each resistance surface was assessed based on corrected Akaike Information Criterion (AICc) values. The fit of the two measurements of genetic distances was evaluated based on marginal R^2 values (Kimmig *et al*. 2020). The second step combined the most relevant environmental variables into a multiple resistance surface. MLPE models were performed using the same structure as described above. Environmental variables were selected based on low AICc values from single surface optimisation. Analyses were performed using 1,000 bootstrap iterations to assess the relative support of each optimised surface and the robustness of model selection. The method used performed a pseudobootstrap where genetic and resistance distance matrices were sub-sampled without replacement for each bootstrap. While the resistance surfaces were not re-optimised, the MLPE model was refitted and the AICc scores re-calculated. We

performed Mantel tests between both genetic distances and all generated landscape distances using the package vegan (Oksanen *et al.* 2018).

6.4 Results

6.4.1 Genetic diversity

We successfully genotyped 247 samples at 13 microsatellite loci. Null alleles were observed for all loci but Bcalμ1. For these loci, null allele frequency estimates ranged between 0.01 and 0.13 (Appendix H: Table H.1). Mean error rate, determined by repeat genotyping, in the 13 microsatellite loci was 5% for allelic dropout and 0.2% for false alleles (Appendix H: Table H.1). All microsatellite markers were polymorphic. The total number of alleles per breeding site ranged from 40 to 68 alleles with mean allelic richness ranging from 3.0 - 3.8. Expected heterozygosity was the lowest (H_E = 0.50) for Castlegregory Golf Course (CGC) and highest (H_E = 0.60) for Yganavan and Quarry. No strong evidence of inbreeding was detected at population level (Table 6.2).

Table 6.2 Genetic diversity of twelve Natterjack toad breeding sites in Ireland. *N* is number of samples, A_N is total number of alleles, A_R is mean allelic richness, H_O is mean observed heterozygosity and H_E is mean expected heterozygosity, F_{IS} is inbreeding coefficient.

Breeding site	*N*	A_N	A_R	H_O	H_E	F_{IS}
Glenbeigh	22	54	3.1	0.53	0.59	0.13
Quarry	19	68	3.78	0.6	0.59	0.16
DGC	17	56	3.26	0.57	0.52	0.01
Dooks	17	56	3.26	0.57	0.52	0.04
Nambrackdarrig	14	47	3.18	0.59	0.59	0.02
Yganavan	26	62	3.50	0.60	0.57	0.08
Inch	43	73	3.65	0.60	0.57	0.09
Roscullen	21	50	3.00	0.52	0.56	0.09
Killeen	10	52	3.41	0.59	0.53	0.03
CGC	26	60	3.27	0.58	0.50	0.12
Lough Gill	7	40	2.89	0.56	0.55	-0.19
Magharees	18	48	2.95	0.54	0.53	0.01

6.4.2 Population structure

The Natterjack toad metapopulation in Ireland exhibited significant genetic structuring with clear differentiation between populations (Appendix H: Table H.2) with genetic structure reflecting clustering and proximity of breeding sites (Figure 6.2). sPCA results shed additional light on genetic structure. Moran's I derived from the full dataset suggested significant spatial autocorrelation ($I = 0.614$, $p = 0.001$). The global test confirmed positive spatial autocorrelation (global $R = 0.012$, $p = 0.004$) indicating presence of global structure i.e. neighbouring breeding sites tend to be similar (Figure 6.3a). sPCA analysis based on the first and second principal component axes scores suggested three genetic clusters (Figure 6.3b). Hierarchical analysis of those three clusters identified additional structure. Positive spatial autocorrelation

was detected among breeding sites at Inch sand dune and Iveragh Peninsula (global R = 0.013, p = 0.006) with three clusters: (i) Inch (ii) Dooks, Dooks Golf Club, Yganavan and Nambrackdarrig (iii) Quarry and Glenbeigh (Figure 6.3c). No spatial autocorrelation was detected between the three breeding sites at the north of the Dingle Peninsula (global R = 0.032, p = 0.319; local R = 0.034, p = 0.108) or between the two breeding sites at Roscullen Island (global R = 0.0519, p = 0.215; local R = 0.045, p = 0.838).

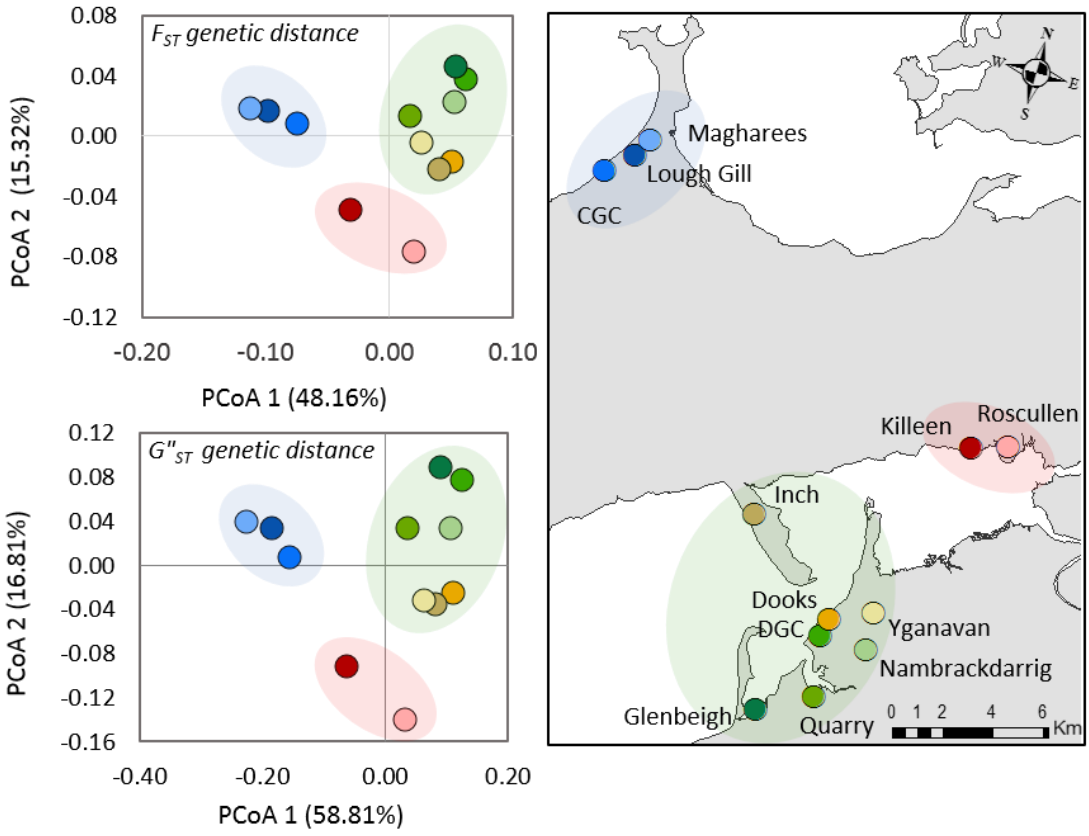

Figure 6.2 Principal Coordinates Analysis (PCoA) of Natterjack toad microsatellite genotypes using two measures of genetic distance. Colours correspond to spatial locations on the map.

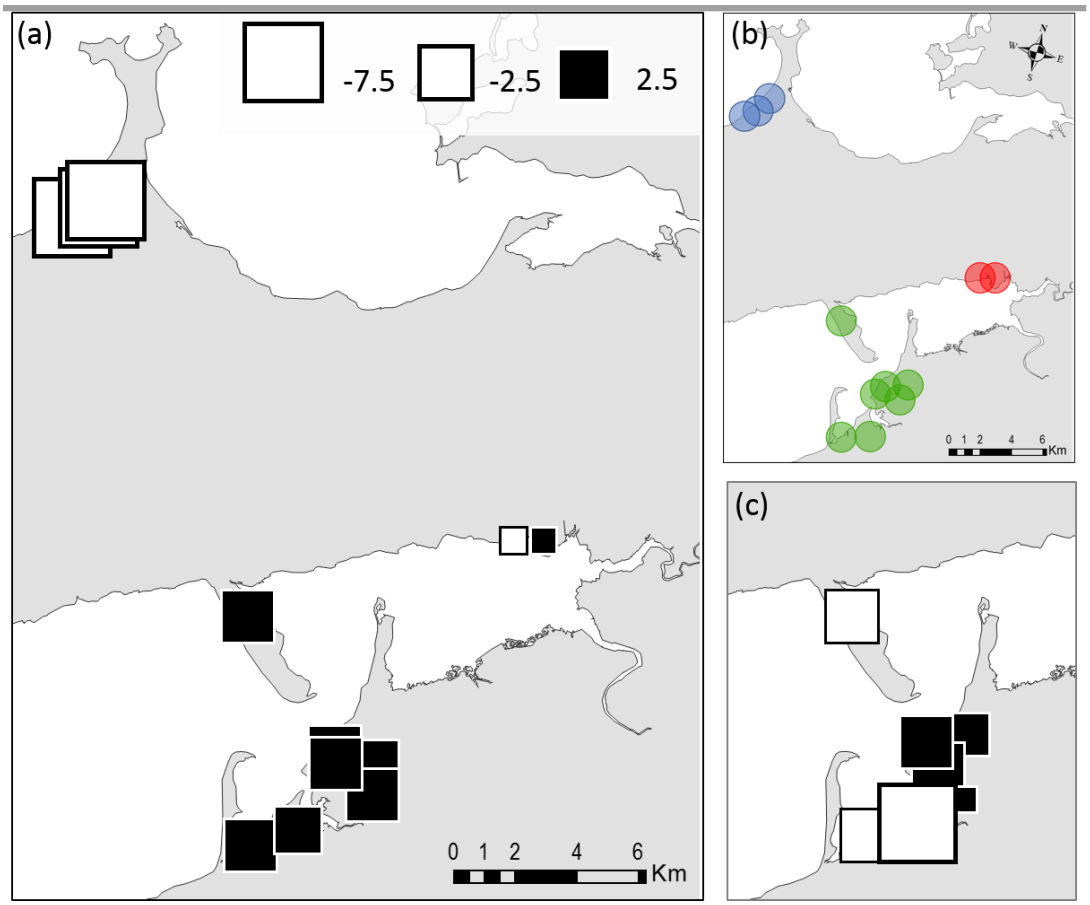

Figure 6.3 sPCA results of Natterjack toad genetic variability and spatial structure. Square size indicates the magnitude of variance. Negative scores (white squares) represent local patterns of spatial autocorrelation and positive scores (black squares) represent global patterns. (a) Map of the entire study area showing genetic variability between breeding sites. (b) Three genetic clusters analysed based on first and second sPCA axes scores. (c) Hierarchical analysis of breeding sites at Inch sand dune and Iveragh Peninsula.

6.4.3 Landscape genetics analysis

Genetic distance increased with geographic distance regardless of the genetic and distance measures used (Figure 6.4). Mantel correlation coefficients whilst highest for Euclidian distance were similarly high for historical distance suggesting existence of a possible past connection between Inch sand dune and the Iveragh Peninsula as current geographic distance had notably lower coefficients. Euclidian distance

showed the best correlation with genetic distance and was used in all subsequent analyses.

Natterjack toad spatial genetic structure was not explained by IBD alone (Euclidean distance), but was also strongly influenced by all resistance surfaces, which were strongly correlated with genetic distance regardless of the metric (Mantel's r >0.7, p <0.05) using both least-cost path and circuit theory scenarios (Table 6.3 and Table 6.4). For the least-cost path scenario, marginal R^2 values were similar regardless of the genetic distance measure used (0.737 - 0.850) with relatively small differences in the predictive power between environmental resistance surfaces. Riparian density was consistently identified as the single most significant barrier to gene flow >2 ΔAICC units away from all other parameters (Table 6.3, Appendix H: Table H.4). For the circuit theory scenario, marginal R^2 values were also similar regardless of the genetic distance measure used (0.768 - 0.807), however, the two measures of genetic distance did not converge on the same model parameters. Euclidean distance was identified as the most significant predictor for gene flow when using F_{ST} as the genetic distance measure though conifer ranked as the second-best model <2 ΔAICC units away from the single best model suggesting comparable predictive power. Distance to coast was identified as the single most significant predictor for gene flow >2 ΔAICC units away from all other parameters when using G''_{ST} as the genetic distance measure (Table 6.4, Appendix H: Table H.5). After single surface optimisation, the relevant variables were combined into a composite resistance surface. The four top models for both scenarios were selected to test whether models with several environmental predictors are better supported than

models with a single environmental predictor. None of the climate variables were identified as predictors of gene flow, thus were not selected for further analysis. Ranking of the sixteen single surface optimisation models for both scenarios and genetic distances remained identical between the two independent optimisation runs (Appendix H: Table H.4 and Table H.5). We applied four different data transformations to generate resistance surfaces for multiple resistance surface optimisations (Appendix H: Figure H.2).

Results of the multiple surface optimisation indicated that gene flow was better explained by a combination of habitats rather than by IBD alone (Euclidean distance) or by IBR using a single predictor (Table 6.5, Appendixx H: Table H.6). The difference between the IBD model (Euclidean distance) and the best multiple resistance surfaces IBR model was large regardless of the scenario or genetic distance measure (ΔAICC > 119). Models using least-cost path performed considerably better in comparison to circuit theory distances and identified more areas with high resistance representing restricted movement (Table 6.5, Figure 6.5). Landscape genetic analysis of multiple resistance surfaces using both IBR scenarios (least-cost path and circuit theory) suggested that Natterjack toad gene flow between metapopulations in Ireland was positively influenced by coastal habitats (beaches, dunes, sand and salt marshes) and grassland whilst barriers to gene flow included coniferous forestry plantations, bog, marsh, moor and heath, scrub, anthropogenic presence (Human Influence Index) and rivers (riparian density).

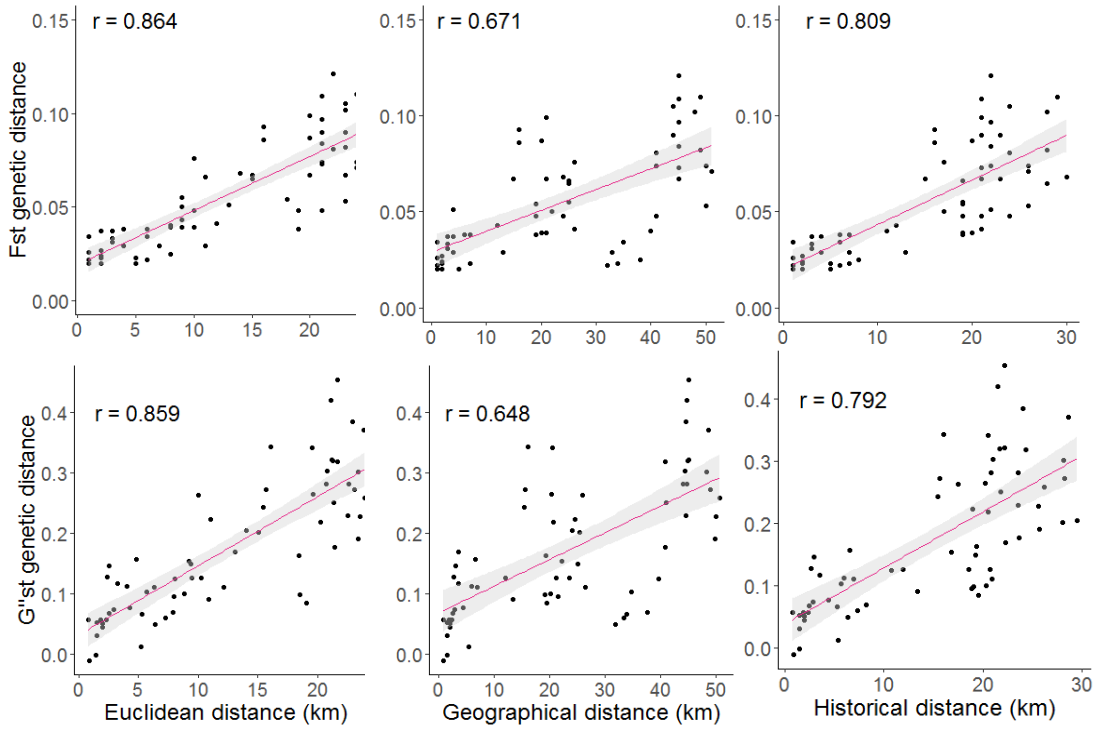

Figure 6.4 Relationship between two measures of genetic distance (y-axes) and three measures of geographic distance (x-axes). Euclidian distance was the shortest distance between each pair of sites as the-crow-flies. Geographic distance was the distance between each pair of sites including 3D topography. Historical distance was geographic distance modified to account for the existence of a sand spit previously connecting the population at Inch sand dunes and those on the Iveragh Peninsula. Correlation coefficients were calculated using Mantel test. Regression lines with 95% confidence intervals are based on fitted values of linear mixed-effects model with maximum likelihood population effects parameterization (MLPE).

Table 6.3 Single surface optimisation for *least-cost path* isolation-by-resistance (IBR) scenarios. Mantel correlations and maximum likelihood population effects (MLPE) are given for the relationship between two genetic distances and resistance **(a-b)**. Environmental predictors are ranked in ascending order of AIC_c values. K is number of fitted parameters; R^2m and R^2c are the marginal and conditional R^2 values; LL is the log likelihood.

Model	Mantel test		Maximum likelihood population effects (MLPE)					
	r	*p*	K	AICc	ΔAIC_c	R^2m	R^2c	LL
(a) F_{ST} genetic distance								
Null	-	-	1	-173.640	104.725	0.000	0.187	88.020
Isolation-by-distance								
Euclidean dist.	0.864	<0.001	2	-265.200	13.164	0.782	0.819	135.267
Isolation-by-resistance								
Riparian dens.	**0.834**	**0.001**	**4**	**-278.365**	**0.000**	**0.847**	**0.895**	**146.039**
Dist. to coast	0.877	0.001	4	-269.545	8.819	0.823	0.877	141.630
HII	0.841	0.001	4	-267.464	10.900	0.824	0.838	140.589
Coastal habitats	0.862	0.001	4	-262.393	15.972	0.811	0.853	138.053
(b) G''_{ST} genetic distance								
Null	-	-	1	7.449	111.51	0.000	0.208	-2.524
Isolation-by-distance								
Euclidean dist.	0.859	<0.001	2	-84.039	20.022	0.771	0.832	44.686
Isolation-by-resistance								
Riparian dens.	**0.892**	**0.001**	**4**	**-104.061**	**0.000**	**0.850**	**0.915**	**58.888**
Dist. to coast	0.918	0.001	4	-94.282	9.779	0.826	0.899	53.998
HII	0.885	0.002	4	-84.317	19.744	0.816	0.849	49.016
Scrub	0.862	0.001	4	-83.123	20.938	0.791	0.875	48.418

Table 6.4 Single surface optimisation for *circuit theory* isolation-by-resistance (IBR) scenarios. See table 5.3 legend for explanation.

Model	Mantel test		Maximum likelihood population effect (MLPE)					
	r	*p*	K	AICc	ΔAIC_c	R^2m	R^2c	LL
(a) F_{ST} genetic distance								
Null	-	-	1	-173.640	85.3314	0.000	0.187	88.020
Isolation-by-distance								
Euclidean dist.	**0.864**	**<0.001**	**2**	**-258.971**	**0.000**	**0.773**	**0.776**	**132.152**
Isolation-by-resistance								
Conifer	**0.931**	**0.001**	**4**	**-257.933**	**1.038**	**0.796**	**0.797**	**135.824**
Dist. to coast	0.932	0.002	4	-255.474	3.497	0.798	0.840	134.594
Grassland	0.918	0.001	4	-252.196	6.775	0.779	0.781	132.955
Bog	0.907	0.001	4	-252.190	-6.781	0.779	0.780	132.952
(b) G''_{ST} genetic distance								
Null	-	-	1	7.449	-85.156	0.000	0.208	-2.524
Isolation-by-distance								
Euclidean dist.	0.859	<0.001	2	-75.216	-2.491	0.768	0.791	40.275
Isolation-by-resistance								
Dist. to coast	**0.937**	**0.001**	**4**	**-77.707**	**0.000**	**0.802**	**0.866**	**45.711**
Conifer	0.928	0.001	4	-75.127	2.580	0.800	0.820	44.421
HII	0.910	0.001	4	-69.593	8.114	0.785	0.821	41.654
Coastal habitats	0.919	0.001	4	-69.577	-8.130	0.776	0.824	41.646

Table 6.5 Multiple surface optimisation for **(a)** least-cost path and **(b)** circuit theory distances. Model evaluation metrics were produced using 1,000 bootstrap iterations: Avg.AIC_C is an averaged AICc value; avg.weight is the averaged AIC_C weights; avg.mR^2 is the averaged marginal R^2.

Model	k	avg.AICc	ΔAIC_C	avg.weight	avg.mR^2	avg.LL
(a) Least-cost path						
F_{ST} genetic distance						
Dist. to coast + Riparian dens. + Coastal habitats	**10**	**-263.459**	**0.000**	**0.274**	**0.837**	**80.729**
Dist. to coast + HII + Riparian dens.	10	-263.196	0.263	0.229	0.835	80.598
HII + Riparian dens. + Coastal habitats	10	-263.390	0.069	0.268	0.836	80.695
Dist. to coast + HII + Coastal habitats	10	-259.187	4.272	0.229	0.819	78.593
Dist. to coast + HII + Riparian dens. + Coastal habitats	13	-225.408	38.051	0.000	0.833	80.304
HII Riparian dens.	4	-147.261	116.198	0.000	0.858	82.630
G''_{ST} genetic distance						
Scrub + HII + Riparian dens.	**10**	**-166.716**	**0.000**	**0.319**	**0.833**	**32.358**
HII + Riparian dens. + Dist. to coast	10	-167.340	0.624	0.354	0.843	32.670
Dist. to coast + Riparian dens. + Scrub	10	-166.867	0.152	0.267	0.837	32.434
Dist. to coast + HII + Scrub	10	-161.473	5.242	0.059	0.818	29.737
Dist. to coast + HII + Riparian dens. + Scrub	13	-127.845	38.871	0.000	0.830	31.522
Riparian dens.	4	-47.185	119.531	0.000	0.843	32.592
HII	4	-43.546	123.170	0.000	0.842	30.773
(b) Circuit theory distance						
F_{ST} genetic distance						
Grassland + Conifer + Bog	**10**	**-258.845**	**0.000**	**0.420**	**0.817**	**78.422**
Dist. to coast + Conifer + Bog	10	-257.606	1.239	0.236	0.814	77.803
Dist. to coast + Grassland + Bog	10	-257.404	1.441	0.295	0.816	77.702
Dist. to coast + Conifer + Grassland	10	-253.342	5.503	0.049	0.787	75.671
Dist. to coast + Grassland + Conifer + Bog	13	-222.351	36.494	0.000	0.820	78.775
Euclidean dist.	2	-137.780	121.065	0.000	0.762	73.890
Conifer	4	-134.113	124.732	0.000	0.790	76.057
G''_{ST} genetic distance						
Dist. to coast + HII + Conifer	**10**	**-156.989**	**0.000**	**0.291**	**0.804**	**27.494**
Coastal habitats + HII + Conifer	10	-155.932	1.057	0.285	0.805	26.966
Dist. to coast + Coastal habitats + Conifer	10	-155.636	1.353	0.220	0.795	26.818
Dist. to coast + HII + Coastal habitats	10	-155.301	1.687	0.204	0.794	26.651
Dist. to coast + HII + Coastal habitats + Conifer	13	-117.522	39.466	0.000	0.792	26.361
Euclidean dist.	2	-37.168	119.821	0.000	0.752	23.584
Dist. to coast	4	-34.340	122.649	0.000	0.786	26.170

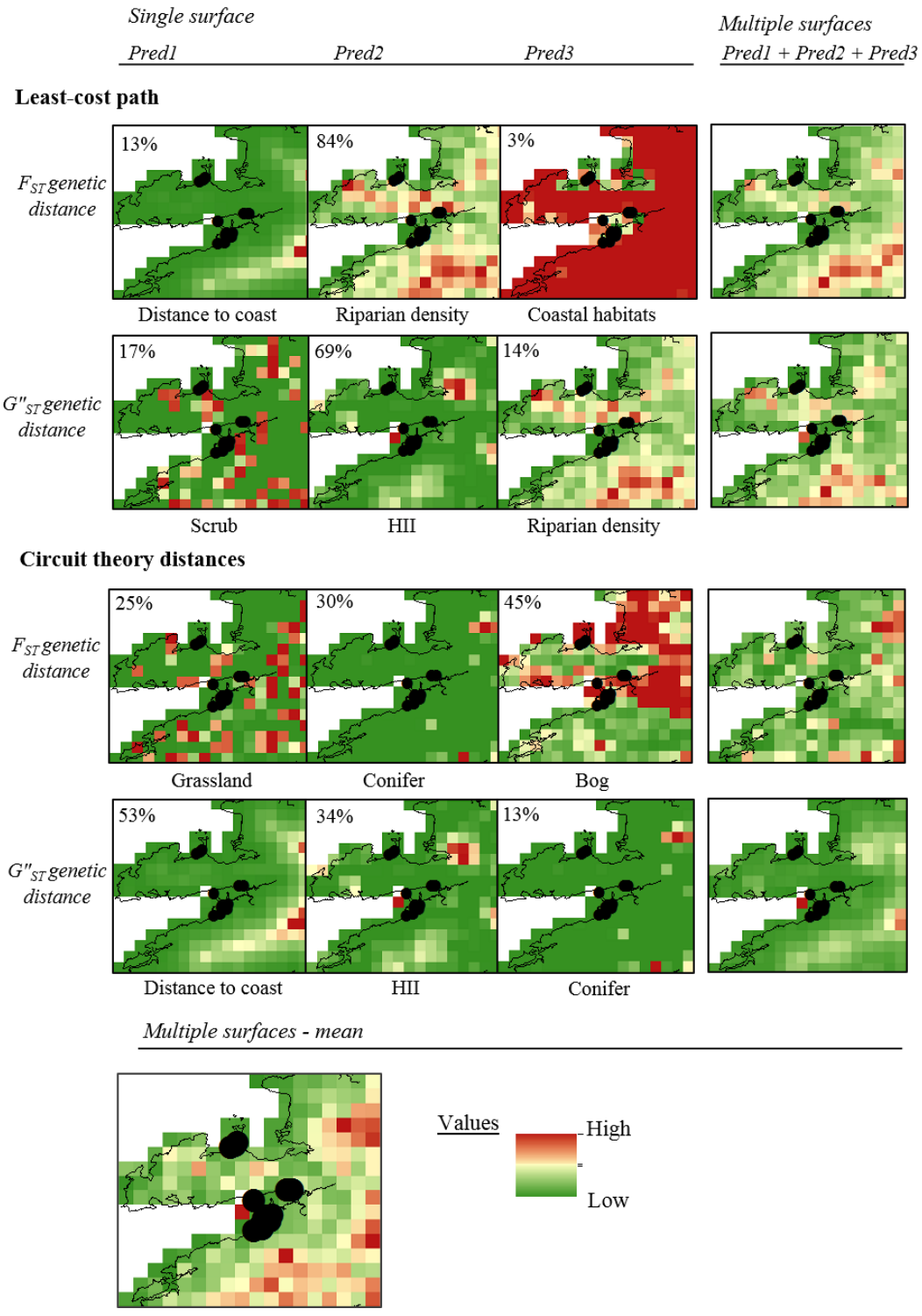

Figure 6.5 Maps illustrating habitat connectivity between Natterjack toad metapopulations in Ireland based on single and multiple surface optimisations. High values represent high resistance to movement, and low values represent low resistance. Percent contribution of each environmental predictors in multiple surface optimisation models is shown. Black circles indicate breeding sites.

6.5 Discussion

The Natterjack toad in Ireland, regionally Red-listed as Endangered, is not a single panmictic population. Our results suggest a high level of genetic differentiation and population structure with restricted dispersal and gene flow between metapopulations associated with environmental factors related to habitat, proximity to the coast and anthropogenic pressure.

One of the most important traits affecting population genetic structure is species dispersal capability, as this is essential in maintaining gene flow between breeding sites and populations. The Natterjack toad is a highly mobile amphibian with annual migrations to breeding sites of 3-4 km (Sanuy *et al.* 2000; Sinsch *et al.* 2012), thus a high rate of gene flow might be expected between breeding sites within the toad's dispersal ability. However, significant genetic differentiation was found between breeding sites in Ireland separated by less than 3km, so geographic distance and dispersal ability were not the principal factors limiting gene flow. This was confirmed by our modelling work, where resistance distances based on habitat explained Natterjack toad genetic structure and gene flow better than Euclidian distance alone. Analysis suggested spatial genetic structuring with three main clusters separated by distance and unfavourable habitat: (i) Magharees, Castlegregory Golf Course (CGC) and Lough Gill; (ii) Killeen and Roscullen; (iii) Inch, Dooks, Dooks Golf Club (DGC), Nambrackdarrig, Yganavan, Quarry and Glenbeigh.

Results suggested recent exchange of individuals between the three breeding sites at the north of the Dingle Peninsula (Magharees, Castlegregory Golf Course and Lough Gill). The main habitats in this area are sand dunes and grasslands both

identified in the present study as facilitating dispersal and gene flow. Cox *et al.* (2017) demonstrated that dispersal of the Natterjack toad is facilitated by sand dunes and beaches, and the largest Natterjack toad population in Ireland is in the Magharees sand dune system, but breeding depends on the formation of ephemeral breeding pools in sand dune slacks (Reyne *et al.* 2019). Access to permanent ponds at the nearby Castlegregory Golf Course (CGC) and suitable sites along the lakeshore of Lough Gill provide a valuable alternative in dry years, thus maintaining connectivity between the breeding sites is essential. Keeping sand dune and coastal grassland vegetation open in structure to support toad dispersal may require cattle grazing and control of scrub encroachment, particularly of invasive sea buckthorn*, Hippophae rhamnoides* (Plassmann *et al.* 2010). The three breeding sites at the north of the Dingle Peninsula were isolated from those of Castlemaine Harbour by the Slieve Mish Mountains dominated by peat bogs, marsh, heath and moor with patches of scrub and coniferous forest plantations. Such habitats were identified by landscape genetic analyses as barriers to dispersal with a high resistance, which, along with the distance between populations north and south of the Dingle peninsula, suggests the Slieve Mish Mountains are impassable. Peatland and their associated marshy wetlands are acid, negatively affecting local distribution and abundance of amphibians (Freda 1986) whilst upland heath and moor tend to be drier habitats and at higher elevation both being suboptimal for amphibians. Frei *et al.* (2016) also found forest to negatively affect Natterjack toad gene flow and population size in Switzerland, yet woodland was preferred to pastures and agricultural fields by Natterjack toadlets in Belgium (Stevens *et al.* 2004, 2006). Natterjack toad habitat selection varies notably

across its range throughout Europe and regional idiosyncrasies in ecology remain poorly studied. Coniferous forestry in Ireland usually consists of tightly planted non-native sitka spruce (*Picea sitchensis*) with highly acidic pine needle leaf litter supporting fewer invertebrates compared to native broad-leaved woodland (Pedley *et al.* 2014). Such conditions are likely to be avoided by toads. Additionally, roads extending east to west along the Dingle peninsula probably represented further barriers to gene flow relevant to the Human Influence Index in this landscape.

The genetic cluster at Killeen and Roscullen breed entirely in artificial ponds constructed on grassland <200m from the coast as part of the NPWS Pond Creation Scheme. Grasslands and proximity to the coast facilitated Natterjack toad dispersal and gene flow with the species known to inhabit and forage even on intensively managed agricultural lands most notably during summer (Miaud & Sanuy 2005; Schweizer 2014). A further 20 artificial ponds that have been created in proximity (within 4km) to Killeen and Roscullen have not yet been colonised naturally up to 10 years after their creation (Reyne *et al*. 2019). Most adult toads have high breeding-site fidelity with dispersal usually by juveniles (Stevens *et al.* 2004). Toadlets may avoid agricultural areas (Stevens *et al.* 2004, 2006), or suffer high rates of mortality due to its management practices there e.g. grass harvest by silage cutting which may represent an ecological trap for wildlife more generally (e.g. Reid *et al.* 2010). Thus, colonisation of new ponds at the limits of Natterjack dispersal capability may be slow. Other demographic parameters such as low local breeding success may also contribute to lower recruitment and dispersal. Certainly, Natterjack toad population at Killeen and Roscullen have exhibited substantial census size declines over the past

15 years (Reyne *et al.* 2019). We propose including Killeen and Roscullen in the Natterjack toad NPWS Head-start and Translocation Programme with occupied breeding sites used as a source population, with assisted migration and translocation to adjacent artificial ponds. More generally, any Natterjack toad translocations in Ireland should utilise the closest breeding sites as the source population to maintain genetic provenance and spatial genetic structuring; existing genetic diversity precludes the need for population admixture between genetic clusters.

Our results suggest gene flow between Natterjack populations at Inch sand dunes and those of the Iveragh Peninsula. As these populations are separated by open sea, gene flow presumably occurred historically when dispersal between the sites might have been possible during low tides and in presence of sand spits since eroded by strong winds and storms (Orford *et al.* 1999). In this study, we estimated high genetic diversity and no signs of inbreeding at Inch sand dune. However, complete isolation, lack of long-term population data and dependence of breeding success on ephemeral ponds formation in sand dune slacks raise concerns for the future viability of this population. Close monitoring is required whilst ongoing inclusion in the NPWS Head-start and Translocation Programme will ensure continued recruitment at the site even in dry years when all breeding sites evaporate before metamorphosis.

Genetic connectivity was detected between breeding sites at Dooks, Dooks Golf Club (DGC), Nambrackdarrig and Yganavan where the main habitat was grassland interspersed by bog and scrub. Abandonment of agricultural grassland management results in rank vegetation and scrub encroachment which are threats to Natterjack

toads in the area (Reyne *et al*. 2019). Scrub was also identified as a barrier to dispersal thus local management should focus on preventing further deterioration in habitat quality. Construction of additional artificial ponds between existing breeding sites may be beneficial (Cox *et al.* 2017).

We detected restricted gene flow between Glenbeigh and Quarry and the rest of the Iveragh Peninsula breeding sites likely because of two barriers: Glenbeigh town and the Caragh River and estuary. Increased traffic density due to the N70 road that crosses Glenbeigh town, part of the high traffic 'Ring of Kerry' tourist route and local urbanisation (captured in the Human Influence Index) may also present barriers to movement. Riparian density i.e. rivers and streams also had a negative impact on gene flow. Toads are poor swimmers and waterways such as the Caragh River and its associated saltwater tidal estuary are likely barriers to dispersal. Recent development in the area of Glenbeigh and Quarry breeding sites may increase population isolation, and whilst these sites had good genetic diversity, they also have a small number of breeding females (Reyne *et al.* 2019) and the highest inbreeding coefficient for any population in Ireland raising concerns about future viability. Translocation of toadlets as part of the NPWS Head-start and Translocation Programme sourced from Yganavan and Nambrackdarrig (breeding sites in the same genetic cluster with high numbers of egg strings and tadpoles) to Glenbeigh and Quarry may increase population numbers and mitigate any risk of future genetic erosion. Consideration should also be given to the creation of new artificial breeding ponds in this area.

Understanding endangered amphibian movement and connectivity between breeding sites and metapopulations is key to their conservation. We show clear

spatial structuring of the Natterjack toad in Ireland explained by Isolation-by-resistance with coniferous forestry plantations, bog, marsh, moor and heath, scrub, anthropogenic presence (roads) and rivers identified as barriers to gene flow while metapopulation connectivity was enhanced by coastal habitats (beaches, dunes, sand and salt marshes) and coastal grassland. Substantial population declines over the past 15 years necessitate increased conservation management efforts by the Government: principally artificial pond creation and assisted migration through translocation. Our results are invaluable in informing planned improvements in connectivity by suggesting sites where corridors of new ponds may be beneficial and the clusters of sites that can be used as source populations for translocation while maintaining genetic provenance. Moreover, we identify barriers to dispersal and suggest site management measures to mitigate their effects, for example, prevention of scrub encroachment. Thus, this study is valuable in understanding how landscape effects dispersal, gene flow and habitat connectivity of a declining pond-breeding amphibian. The same approach could be used in other regions to direct and inform conservation managers.

Chapter 7

Will predicted positive effects of climate change be enough to reverse declines of the regionally Endangered Natterjack toad in Ireland?

A manuscript based on this chapterwas published as:

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7.1 Abstract

The global amphibian crisis is driven by a range of stressors including disease, habitat loss and environmental contamination. The role of climate change remains poorly studied and is likely to influence environmental suitability, ranges, reproduction and phenology. This study aimed to characterize the bioclimatic-habitat niche space of the Natterjack toad (*Epidalea calamita*) throughout its distribution range and to assess the impact of climate on the toad's environmental suitability, number of laid egg strings and spawning behaviour. The European range of the species was found to be limited by winter temperatures whilst its bioclimatic niche varied throughout its range. Species Distribution Models suggested projected climate change will increase environmental suitability for the species throughout its range, most notably in Scandinavia and the Baltic. Egg string production in Ireland was greatest during the cool temperatures of spring than warmer conditions later in the species' prolonged breeding season, and after wet winters associated with ephemeral breeding pool availability. Warm, dry summers in the preceding year influenced egg string production the following spring indicative of carryover effects. Initiation of spawning was driven by spring temperatures, not rainfall. Projections suggested future climate change may increase the number of egg strings in Ireland while spawning may commence earlier throughout the 21st century especially under a high greenhouse gas emission scenario. Despite recent range contraction and population declines due to habitat deterioration, the Natterjack toad, if subject to a suitable species conservation strategy, has the potential to be a climate change winner,

notwithstanding unpredictable habitat and land use change, sea level rise inducing coastal erosion, changes in invertebrate prey abundance and disease.

7.2 Introduction

Amphibians are the most endangered vertebrate group with 41% of species threatened with extinction (IUCN 2020). Population declines have been detected even in common and widespread species (Stuart *et al*. 2004; Adams *et al.* 2013; Petrovan & Schmidt 2016) as a consequence of ongoing stressors including habitat loss (Cushman 2006), contamination (Mann *et al*. 2009; Brühl *et al.* 2013), disease (Berger *et al.* 1998; Lips 1999; Martel *et al.* 2013), invasive species (Johnson *et al.* 2011) and illegal harvest and trade (Schlaepfer *et al.* 2005; Chan *et al.* 2014). Recent studies suggest that climate change poses an additional serious threat to amphibian populations (e.g. Carey & Alexander 2003; Pounds *et al.* 2006; Bombi & D'amen 2009), directly impacting species behaviour and phenology (Semlitsch 1988), availability of suitable habitat (McMenamin *et al.* 2008) or by interacting with other factors such as disease (Bosch *et al.* 2007; Pounds *et al.* 2006; Laurance 2008). However, evidence that climate change is directly causing amphibian declines and extinction is weak and controversial (Carey &Alexander 2003; McCallum 2005; Rohr *et al.* 2008; Yiming *et al.* 2013). Understanding the role of climate in population dynamics and the potential impact of future climate change on population viability and extinction risk of endangered species is crucial for implementing effective species management strategies (Shoo *et al.* 2011).

Amphibia are ectotherms, and all aspects of their physiology, behaviour and life history are strongly dependent on weather and climate, especially for temperate species exposed to clearly defined seasons. Temperature impacts their mechanism of gas exchange (Wood & Glass 1991), metabolic rate (White *et al.* 2006), immune

function (Raffel *et al.* 2006), and phenology like timing of breeding (Beebee 1995) and duration of hibernation (Jørgensen 1986). Activity and breeding migrations are often positively correlated with precipitation (Gibbsons & Bennett 1974; Smith & Skelcher 2019). Decreased precipitation and ambient moisture can alter pond hydroperiods, resulting in early or rapid pond desiccation (McMenamin *et al.* 2008), consequently altering larval development (Reading & Clarke 1999). Climate change is, therefore, likely to have a significant impact on growth, body condition, reproduction, fecundity, and recruitment i.e. population dynamics and trajectory, of amphibians.

Global mean surface temperature has increased by approximately 0.8˚C over the last century and is likely to continue to increase throughout the 21st century by between 2.6 ˚C and 4.1 ˚C, calculated based on different greenhouse gas (GHG) emission scenarios (Sherwood *et al.* 2020). Climate change is generally expected to lead to more variable and intense precipitation with longer periods of drought between precipitation events (IPCC 2007). Distribution of suitable habitats for a wide variety of species may change by the end of the 21st century, resulting in increased extinction risk, especially for those that are range restricted (Thomas *et al.* 2004; Penman *et al.* 2009; Gibson *et al.* 2010; Marini *et al.* 2010). Amphibians are likely to be particularly sensitive to climate change given the high proportion of declining populations, dependence on temperature and humidity, high sensitivity to stressors and low ability to disperse (Blaustein *et al.* 2001; Carey & Alexander 2003). Climate change is likely to cause major shifts in spatial patterns of amphibian diversity, resulting in range contraction and expansion (Zank *et al.* 2014; Duan *et al.* 2016).

Range shifts are the most common response to climate change (Parmesan & Yohe 2003; Root *et al.* 2003) and a species' ability to track its suitable bioclimatic envelope will be essential for survival (Sunday *et al.* 2014).

The Natterjack toad (*Epidalea calamita*) is widespread throughout Europe, ranging from Iberia to the Baltic (Gasc *et al.* 1997). The species is often associated with scrubby, open habitat on sandy substrates or dry heath with shallow seasonally ephemeral ponds (Beebee & Griffiths 2000). In some regions of its range, Ireland for example, the Natterjack toad is regionally Red-Listed as Endangered due to a 50-60% range contraction since the 1970s, driven by loss of aquatic and terrestrial habitats e.g. drainage and agricultural intensification, and deterioration of habitat quality e.g. reed encroachment of ponds and undergrazing of terrestrial habitats around ponds, leading to rank vegetation and poor foraging conditions (King *et al*. 2011). In Ireland, the numbers of egg strings deposited annually has also declined at most metapopulations (Reyne *et al.* 2019) causing concern that population size is declining. The role of climate in changes on range, egg string production, and phenology is unknown.

This study aimed to quantify the impact of climate change on the Natterjack toad throughout its European range and assess changes in a focal range edge population (Ireland). The main objectives were to i) characterize its bioclimatic-habitat niche throughout its range, including Ireland, ii) use Species Distribution Models at different spatial and temporal extents to predict the potential impact of projected climate change on environmental suitability and, potentially, suitable range for the species, and iii) model number of egg strings and initiation of spawning, projecting

potential climate change effects on reproduction. Our goal was to predict the impact of climate change on a range edge population regionally Red-Listed as Endangered to inform species conservation management.

7.3 Methods

7.3.1 Species records and spawning

A total of 470,245 species records for all 84 amphibian species known to occur in Europe, including 37,062 Natterjack toad (*Epidalea calamita*) records, were downloaded from the Global Biodiversity Information Facility (GBIF.org). Ireland represents the extreme north-western range edge margin of the Natterjack toad distribution, where the species is highly range restricted, represented by twelve metapopulations with 91 breeding sites within its native range in Co. Kerry in the south-west, and one introduced population in Co. Wexford in the south-east (Figure 7.1). The species has been monitored intensively by three major projects from: a) 2004-2006 (Bécart *et al.* 2007), b) 2011-2012 (Sweeney *et al.* 2013) and c) 2016-2018 (Reyne *et al.* 2019). In Ireland, the species has a protracted breeding period from April to July, during which each potentially occupied breeding pond was visited every 7 to 10 days of each survey year and the total number of egg strings recorded (for full methodological details see Reyne *et al.* 2019). Thus, the number of ponds that formed annually, the presence/absence of Natterjack toads, their egg string production and spawning dates were known throughout their range in Ireland with a high degree of accuracy for the years surveyed.

7.3.2 Climate data

Climate at the extent of Europe was characterised by data downloaded from Worldclim (worldclim.org) at a 2.5^{o} (~4km) grid cell resolution. Of the nineteen bioclimatic variables available, we selected seven based on their perceived relevance to amphibian biology (Appendix I: Table I.1; Figure I.1). Toads are ectothermic and hibernate during winter, thus mean annual temperature (bio1), the diurnal temperature range (bio2), mean temperature of the warmest quarter (bio10) and mean temperature of the coldest quarter (bio11) were selected as potentially relevant to homeostasis, activity and hibernation. Total precipitation (bio12), precipitation of the wettest quarter (bio17) and precipitation of the driest quarter (bio17) were selected as potentially relevant to breeding pool formation and ephemerality. Future climatological projections used the HadGEM2-ES model which is a coupled Earth System developed by the UK Met Office Hadley Centre for the World Climate Research Programme (WCRP) Coupled Model Intercomparison Project Phase 5 (CMIP5) centennial simulations. HadGEM2-ES was chosen as it incorporated high levels of climatic complexity and a well-resolved stratosphere with atmospheric chemistry, ocean biology and dynamic vegetation. Future projections were downloaded for the mid-century 2050s (average for 2041-2060) and late century 2070s (averaged for 2061-2080) for both an intermediate greenhouse gas emission scenario or Representative Concentration Pathway (RCP4.5 were emissions are assumed to peak around 2040 and then decline) and a high emissions scenario (RCP8.5 were emissions are assumed to continue to increase throughout the 21st century).

Climate at the extent of Ireland has been simulated at high spatial resolution using the Consortium for Small-scale Modelling-Climate Limited-area Modelling (COSMO-CLM; v5.0) Regional Climate Model (Rockel *et al*. 2008; Steppeler *et al.* 2003) by the Irish Centre for High End Computing (ICHEC; ichec.ie). The following Coupled Model Intercomparison Projection Phase 5 (CMIP5; Taylor *et al.* 2012) Global Climate Model (GCM) datasets were dynamically downscaled: the UK Met Office's Hadley Centre Global Environment Model version 2 Earth System (HadGEM2-ES) configuration GCM (Collins *et al.* 2011); the EC-Earth consortium GCM (Hazeleger *et al.* 2011); the CNRM-CM5 GCM developed by the Centre National de Recherches Météorologiques–Groupe d'études de l'Atmosphère Météorologique (CNRM-GAME) and the Centre Européen de Recherche et de Formation Avancée (Cerfacs) (Voldoire *et al.* 2013); the Model for Interdisciplinary Research on Climate (MIROC5) GCM developed by the MIROC5 Japanese research consortium (Watanabe *et al.* 2010); and the MPI-ESM-LR Earth System Model developed by the Max Planck Institute for Meteorology (Giorgetta e*t al.* 2013).

Gridded climate datasets for Ireland, both historical (1976–2005) and future (2021–2100) were generated at temporal and spatial resolutions of 3 hours and 4km, respectively. To account for the uncertainty arising from the estimation of future global emission of greenhouse gases and changing land use, downscaled GCM simulations based on two Representative Concentration Pathways: RCP4.5 and RCP8.5 (van Vuuren *et al.* 2011) were used to simulate the future climate. For a full description of the RCM configuration and an overview of validations and projections see Nolan *et al*. (2017) and Nolan & Flanagan (2020).

Of the twenty-two variables available, we selected four on their perceived relevance to toad biology (Appendix I: Table I.1, Figure I.2). In addition to surface temperature (T_S) and rainfall (TOT_PREC), we selected variables not available via Worldclim including soil temperature 54cm below ground level (T_SO_00540mm) as relevant to hibernation, subsurface runoff (RUNOFF_G) and wind speed <10m (WDSPD_10m) as relevant to pool formation, water levels and pool ephemerality. For Species Distribution Modelling variable historical averages from 1976-2005 were taken to represent 'current' conditions matching the timeframe of most of those downloaded from GBIF though preceding much of the data collected during surveys in Ireland.

The same variables were also available at hourly intervals historically from 1976-2005. Thus, values were averaged for the 6-hourly 9am to 3pm period on each date for which an egg string survey had been performed allowing conditions during each survey day to be quantified ($Survey_t$). We were interested in seasonal lagged effects and calculated average daily (6-hourly 9am to 3pm) values for the focal $Spring_t$ (Mar-Apr-May) of each toad breeding season in the year of survey ($_t$) and seasons in the preceding year ($_{t-1}$): $Spring_{t-1}$, $Summer_t$ (Jun-Jul-Aug), $Autumn_{t-1}$ (Sep-Oct-Nov) and $Winter_{t-1}$ (Dec-Jan-Feb). Future climatological projections used the COSMO-CLM5 ensemble (averaged across five different climate models: CNRM-CM5, EC-EARTH, HadGEM2-ES, MIROC5 and MPI-ESM-LR). Future projections covered the same daily 6-hourly 9am to 3pm windows and were averaged for each season and obtained for the mid-century 2050s (averaged for 2041-2070) and late century 2080s (averaged for 2071-2100) for both RCP4.5 and RCP8.5. Note that the spatial and

temporal resolution and timeframes covered by the COSMO-CLM5 data for Ireland differed from that of Worldclim for Europe or Ireland.

7.3.3 Habitat data

Habitat data were downloaded for CORINE Land Cover 2018 from the European Environment Agency (EEA 2020; https://land.copernicus.eu/pan-european/corine-land-cover/clc2018) and summarised at a 2.5^{o} (~4km) grid cell resolution throughout Europe and a 4km grid cell resolution throughout Ireland to match the two climate datasets. Spatial manipulation of habitat data used ArcMap 10.7.1 (ESRI, California, USA). Individual CORINE land codes were aggregated and collapsed to derive simplified, ecologically relevant habitat classifications (Appendix I: Table I.1): coastal habitats, freshwater, grassland, scrub and sparse vegetation. Habitat categories were selected based on known Natterjack toad habitat preferences (Beebee 1983). As the species is exclusively coastal in Ireland, we calculated the distance of the centroid of each grid cell from the marine high-water mark i.e. distance to coast.

7.3.4 Niche characterisation

The Natterjack toad's core range extends from the Mediterranean coast of Iberia, northward through France, and north and east into Germany and the Netherlands where records become more sporadic (Figure 7.1). Records occur in the Baltic but no GBIF records were available from Poland suggesting either no recording effort, that Poland does not submit records to GBIF, or that the species, whilst it occurs there (e.g. Franz *et al.* 2013), is scarce. Nevertheless, Poland was included in the species

IUCN range polygon (Figure 7.1). Natterjack toads also occur along the southern coast of Sweden and in Great Britain in highly isolated populations. Climatic conditions in each of these regions are very different, thus to characterise spatial variation in the Natterjack toad's niche tolerance, Worldclim bioclimatic and CORINE habitat variables were extracted for each species record and analysed using Discriminant Function Analysis (DFA), fitting region (Ireland, Great Britain, Europe, Scandinavia and the Baltic) as the grouping variable. For each axis with an Eigenvalue >1, the median, interquartile range and 95% Confidence Intervals of axis scores were plotted using a boxplot and differences tested using a one-way ANOVA with pairwise Least Significant Difference *post-hoc* tests used between each region.

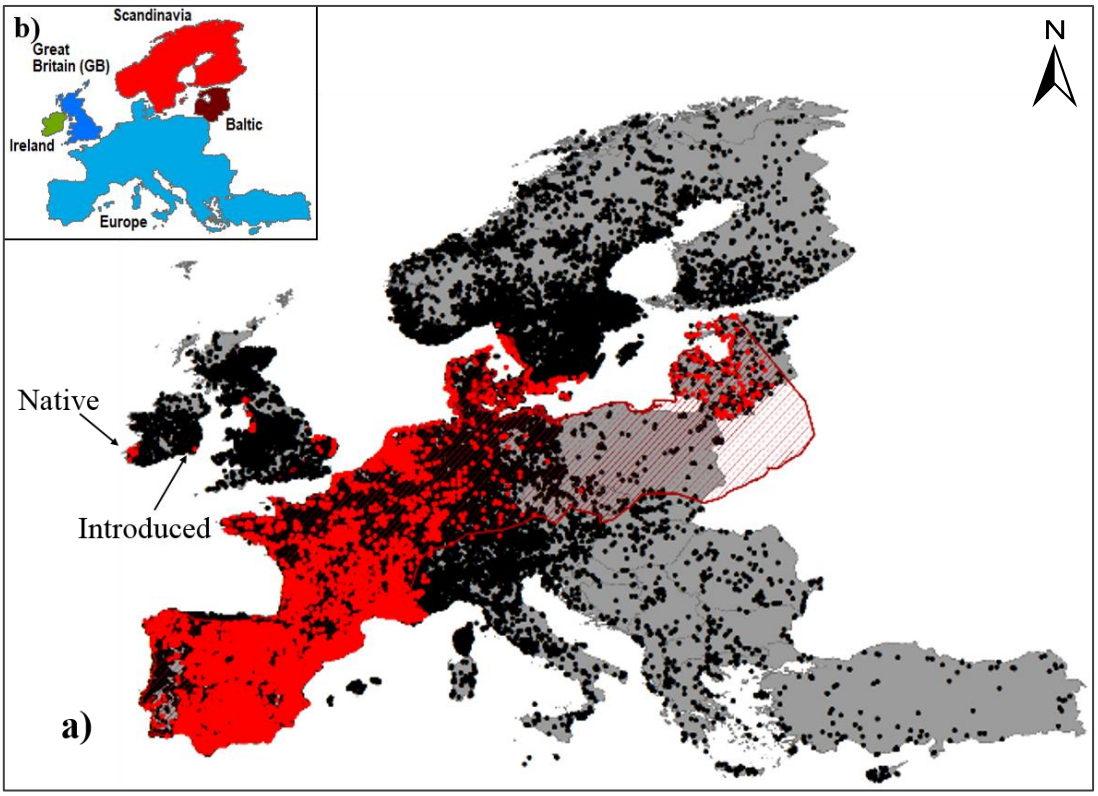


Figure 7.1 a) European distribution of Natterjack toad records (red dots n=37,062) overlaid with the IUCN species range polygon (red hatching) and underlaid with GBIF records for 84 other Amphibian species recorded throughout Europe (black dots n=470,245). The Natterjack toad is highly range restricted in Ireland with its native range in the south-west and an introduced population in the south-east (labels). b) European region names used in analysis for orientation.

7.3.5 Species Distribution Models

Species Distribution Models were constructed using maximum entropy and the programme Maxent 3.4.1 (Phillips *et al.* 2020). To minimise spatial autocorrelation and to prevent drawing duplicate records from the same cell during the same model run, species records were reduced in resolution to match that of input environmental data, decreasing sample sizes (*n*=40,861 Natterjack records across 443,030 grid cells

in Europe, *n*=24 records across 7,037 grid cells in Ireland at 2.5° and *n*=11 records across 5,503 grid cells in Ireland at 4km).

As the Natterjack toad's bioclimatic-habitat niche varied across Europe, three Species Distribution Models (SDMs) were created: 1) at the full extent of Europe using WorldClim climate data, hereafter referred to as the $Europe_{WorldClim}$ model, 2) at the extent of Ireland only using WorldClim climate data, hereafter referred to as the $Ireland_{WorldClim}$ model, and 3) at the extent of Ireland using Ireland-specific downscaled climate variables, hereafter referred to as the $Ireland_{ICHEC}$ model.

Species records represented presence data. To account for some degree of survey effort across Europe, background points (pseudo-absences) were not drawn at random from throughout the full model extent, but instead were confined to cells in which any of the 84 amphibian species that are known to occur in Europe had been recorded i.e. we could be confident an observer predisposed to submitting an amphibian record was present in the cell but failed to report a Natterjack toad sighting. Thus, background points more closely approximated true absence data than if randomly selected from throughout the extent of Europe. For models at the extent of Ireland, background points were drawn from throughout the model extent as the Natterjack toad is known not to occur anywhere outside its recorded range with certainty, thus background points reflected true absences.

Species records were split into model training datasets (75% of records chosen randomly) and test datasets (25% chosen randomly) with four replicate model runs (with bootstrapping) such that every record had a roughly equal chance of being selected once as a test record. Model outputs across the four replicate runs were

averaged. To minimise model overfitting, hinge and threshold responses were excluded with only linear and quadratic curves fitted to create smoothed (ecologically plausible) response curves for each input variable. A Jackknife analysis of variable importance to test gain was used to assess the contribution of variables to model predictive success.

The most used SDM evaluation metric is the Area Under the Curve (AUC) of the Receiver Operating Characteristic (ROC) curve (Merow *et al.* 2013). AUC can be problematic when using presence-only data as it must distinguish between presence and true absence (Allouche *et al.* 2006); though our restriction of background points across Europe, whilst imperfect, will have gone some way to minimising false negatives whilst our models at the extent of Ireland conformed to this assumption. AUC is heavily influenced by the extent of model prediction and can be artificially inflated (Smith 2013). Thus, the models were tested with different validation methods: AUC, sensitivity, specificity, omission rate, percentage correct and True Skill Statistics (Allouche *et al.* 2006).

Heatmaps of the continuous probability of environmental suitability (hereafter, referred to as suitability) were binarized into greyscale maps of likely suitable conditions (hereafter, referred to as the suitable bioclimatic envelope) using the Maximum Test Sensitivity plus Specificity (MaxTSS) threshold, which optimised models using their ability to predict test rather than training data (Smeraldo *et al.* 2020; Nameer 2020). Models were temporally extrapolated into future climatological conditions assuming low (RCP4.5) and high (RCP8.5) emissions scenarios.

Suitability values per cell were compared between current and each future climate scenario using paired t-tests. Change in the suitable bioclimatic envelope (number of suitable/unsuitable cells) was assessed between current and each future climate scenario using 2x2 χ^2 Contingency tests. Percentage change in suitability and the number of suitable grid cells was calculated between current conditions and future conditions (Bosso *et al.* 2017; Wei *et al.* 2018).

7.3.6 Modelling spawning

For Ireland, egg string numbers at each pond visit across all surveys was fitted as the dependent variable in a Generalised Linear Mixed Model (GLMM), fitting Pond_ID as a Random Factor to account for replicate surveys per pond. Linear modelling techniques are vulnerable to model leverage due to collinearity. Climate and habitat variables are highly collinear and could not be fitted separately in the same model. Thus, sets of climate and habitat variables were each reduced in separate Principal Component Analyses (PCA) to create orthogonal axes. Only axes with an Eigenvalue >1 were retained for inclusion in analysis (Kaiser 1960). Climate PCA axis scores at the point of $Survey_t$ and seasonal effects covering $Spring_t$ as well as temporally lagged effects covering $Spring_{t-1}$, $Summer_{t-1}$, $Autumn_{t-1}$ and $Winter_{t-1}$ (those of the preceding year) were fitted. Habitat PCA axis scores were also fitted. Projections were made using future climatic conditions under low (RCP 4.5) and high (RCP 8.5) emission scenarios for each spatially explicit survey location. For prediction, future seasonal averages were used for both current ($_t$) and lagged effects ($_{t-1}$). A similar GLMM was constructed for the first spawning date for each pond each year fitting Julian day as

the dependent variable and Pond_ID as Random Factor. In this case, only climate PCA axes for $Spring_t$ were fitted as independent variables. Predicted egg string numbers and Julian day of first spawning were compared between current and each future climate scenario using a two-way ANOVA fitting Prediod and Emission scenario with median, interquartile ranges and 95% Confidence Intervals plotted as boxplots. Percentage change in predicted egg string numbers was calculated between current and future conditions. At the aggregate level of each survey year, the total number of potential breeding ponds that formed each spring and the cumulative total number of egg strings deposited were related to rainfall during $Winter_{t-1}$ using Spearman's correlation. All analyses were performed using IBM SPSS Statistics v26.

7.4 Results

Discriminant Function Analysis captured the bioclimatic-habitat variation of the Natterjack toad's niche space throughout its European range with DFA1 capturing 85% of variation and the only axis with an eigenvalue >1 (Table 7.1). DFA1 scores varied significantly between regions ($F_{df=4,6154} = 2{,}810$, $p<0.001$) with all pairwise Least Significant Difference *post-hoc* tests significant ($p<0.001$), exhibiting a very strong trend from Ireland in the west to the Baltic in the east (Figure 7.2). DFA1 was characterized predominantly by mean temperature of the coldest quarter (bio11) i.e. average winter temperatures which ranged from 6.3 ± 0.4°C in Ireland to -4.2 ± 0.7°C in the Baltic (Figure 7.2 insert).

SDM predictive performance varied marginally between models at different extents and using different climate data but all models had good predictive success with AUC>0.7, while TSS showed values between 0.524 ± 0.006 ($Europe_{Worldclim}$) and 0.800 ± 0.184 ($Ireland_{Worldclim}$) (Table 7.2; Appendix I: Table I.2). The $Ireland_{ICHEC}$ model performed better than the $Ireland_{Worldclim}$ model which, in turn, was better than the $Europe_{Worldclim}$ model. The $Europe_{Worldclim}$ model test gain was contributed to most by bio11 (mean temperature of the coldest quarter), bio1 (mean annual temperature) and bio10 (mean temperature of the warmest quarter) with likelihood of presence greatest at higher temperatures (Figure 7.3). Habitat (mostly sparse vegetation and scrub) contributed little to the overall model. The $Ireland_{Worldclim}$ model test gain was contributed to most by bio11 (mean temperature of the coldest quarter), bio16 (precipitation of the wettest quarter) and bio1 (mean annual temperature) but also coastal habitats (all positive relationships) and distance to coast (a strong negative

relationship). Unlike Worldclim models, the $Ireland_{ICHEC}$ model test gain was contributed to most, not by climate, but by a negative relationship with distance to coast followed by positive relationships with surface temperature (T_S), and soil temperature (T_SO_00540mm), coastal habitats, rainfall (TOT_PREC), subsurface runoff (RUNOFF_G) and negative relationships with grassland and windspeed (WDSPD_10m) contributing least (Figure 7.3).

The $Europe_{Worldclim}$ model (% correct = 0.682, Table 7.2) predicted suitable conditions for Natterjack toads throughout Europe, including all of Ireland, except for Scandinavia (Figure 7.4). Suitability for Natterjack toads increased significantly (p<0.001) under all future climate scenarios in all European regions (Appendix I: Table I.3) with greatest improvement (% change) in Scandinavia and the Baltic (Appendix I: Figure I.3). Consequently, the suitable bioclimatic envelope (number of suitable grid cells) increased most significantly in both regions but also increased across mainland Europe and Great Britain (Figure 7.4; Appendix I: Table I.4 and Figure I.4). Despite a significant increase in suitability (Appendix I: Table I.3), Ireland was predicted to be 100% suitable (above the MaxTSS threshold) under current conditions and to remain so regardless of the greenhouse gas emission scenario or future timeframe (Figure 7.4, Appendix I: Table I.4 and Figure I.4).

The $Ireland_{Worldclim}$ model (% correct = 0.917, Table 7.2) predicted that only the south-west of Ireland was currently suitable for the Natterjack toad with greatest suitability matching the cells that are currently occupied (Figure 7.4). By 2070 under the high emissions scenario (RCP8.5) a >1,000% increase in suitability (Appendix I: Table I.3 and Figure I.3) and 42% increase in suitable cells (Appendix I: Table I.4 and

Figure I.3) was predicted with virtually the entire Irish coast (Figure 7.4) likely to be suitable.

The $Ireland_{ICHEC}$ model (% correct = 0.966, Table 7.2) predicted that small patches of the Irish coast, mostly in the south and south-west were currently suitable for the Natterjack toad, with these regions expanding throughout the 21st century (Figure 7.4). The suitability of Ireland was projected to increase by 41% and the number of suitable cells by 291% by the 2070s under the high emissions scenario (Appendix I: Tables I.3 and I.4; Figure I.3). When restricted to just those cells currently occupied by Natterjack toads, suitability increased by 27% by the 2070s under the high emission scenario (Appendix I: Table I.3).

Prior to modelling number of egg strings and first spawning dates using GLMM, climate variables were reduced to two principal components: PC1 (eigenvalue 1.959) positively loaded for total precipitation (+0.762), subsurface runoff (+0.817) and wind speed, (+0.843), hereafter referred to simply as 'rainfall' and PC2 (eigenvalue 1.900) positively loaded for surface temperature (+0.975) and soil temperature (+0.975), hereafter referred to simply as 'temperature'. Habitat was captured by a single principal component: PC3 (eigenvalue 1.505) negatively loaded for grassland (-0.868) and positively loaded for coastal habitats (+0.868), hereafter referred to as a grassland-dune gradient.

Natterjack toad egg string production was negatively related to temperature during the $Survey_{t}$ and positively during $Summer_{t-1}$. Number of egg strings was negatively related to rainfall during $Summer_{t-1}$ but positively during $Winter_{t-1}$ (Table 7.3). At the aggregate population level, the number of ponds that formed annually

was significantly positively correlated with rainfall during Winter$_{t-1}$ (r_s=0.778, p=0.023) and the cumulative total number of egg strings deposited throughout the Natterjack toad's Irish range each breeding season (r_s=0.778, p=0.023). Weekly numbers of egg strings deposited was predicted to increase significantly as the century progresses ($F_{df=1,16,946}$ = 7641.089, p<0.001), being significantly higher under the high than low emission scenarios ($F_{df=1,16,946}$ = 12,857.447, p<0.001) and increasing by 104% from 2.7 egg strings per pond visit currently to 5.5 eggs strings during 2050 RCP4.5; by 201% to 8.1 eggs strings during 2050 RCP8.5; by 158% to 7.0 eggs strings during 2070 RCP4.5; and by 425% to 14.2 egg strings during 2070 RCP8.5 (Figure 7.5).

First spawning dates, currently occurring on average by 21st or 22nd April (Julian day 112), were negatively related to Spring$_t$ temperatures but unrelated to rainfall (Table 7.3). Initiation of spawning was predicted to occur significantly earlier as the century progresses ($F_{df=1,1121}$ = 58.497, p<0.001) and earlier under the high than low emission scenario ($F_{df=1,1121}$ = 85.229, p<0.001) with future predictions suggesting advancement by, on average, 6 days to 15th or 16th April (Julian day 106) by the 2070s RCP8.5 (Figure 7.5).

Table 7.1 Discriminant Function Analysis (DFA) of Worldclim bioclimatic and CORINE habitat variables for Natterjack toad species records throughout Europe. *symbolized variables that significantly (p<0.05) contributed to each DFA axis.

	DFA1	**DFA2**	**DFA3**	**DFA4**
Eigenvalue	1.827	0.186	0.104	0.033
% of Variance	85	9	5	2
Cumulative %	85	94	98	100
Variable	**Loadings**			
bio11	**0.380***	0.168	-0.213	0.114
bio2	0.196	**0.652***	-0.296	0.106
bio10	0.149	**0.434***	-0.374	0.173
dist_to_coast	0.039	**0.418***	-0.076	0.063
sparse	-0.043	**-0.345***	-0.174	0.343
bio1	0.273	**0.341***	-0.293	0.120
scrub	0.005	**0.238***	-0.139	0.115
grassland	0.017	**-0.217***	0.163	-0.047
bio16	0.048	-0.098	**0.703***	0.379
bio12	0.058	-0.156	**0.624***	0.302
coastal_habs	0.057	-0.262	**0.591***	-0.351
bio17	0.041	-0.163	**0.360***	0.079
freshwater	-0.060	-0.027	0.016	**-0.095***

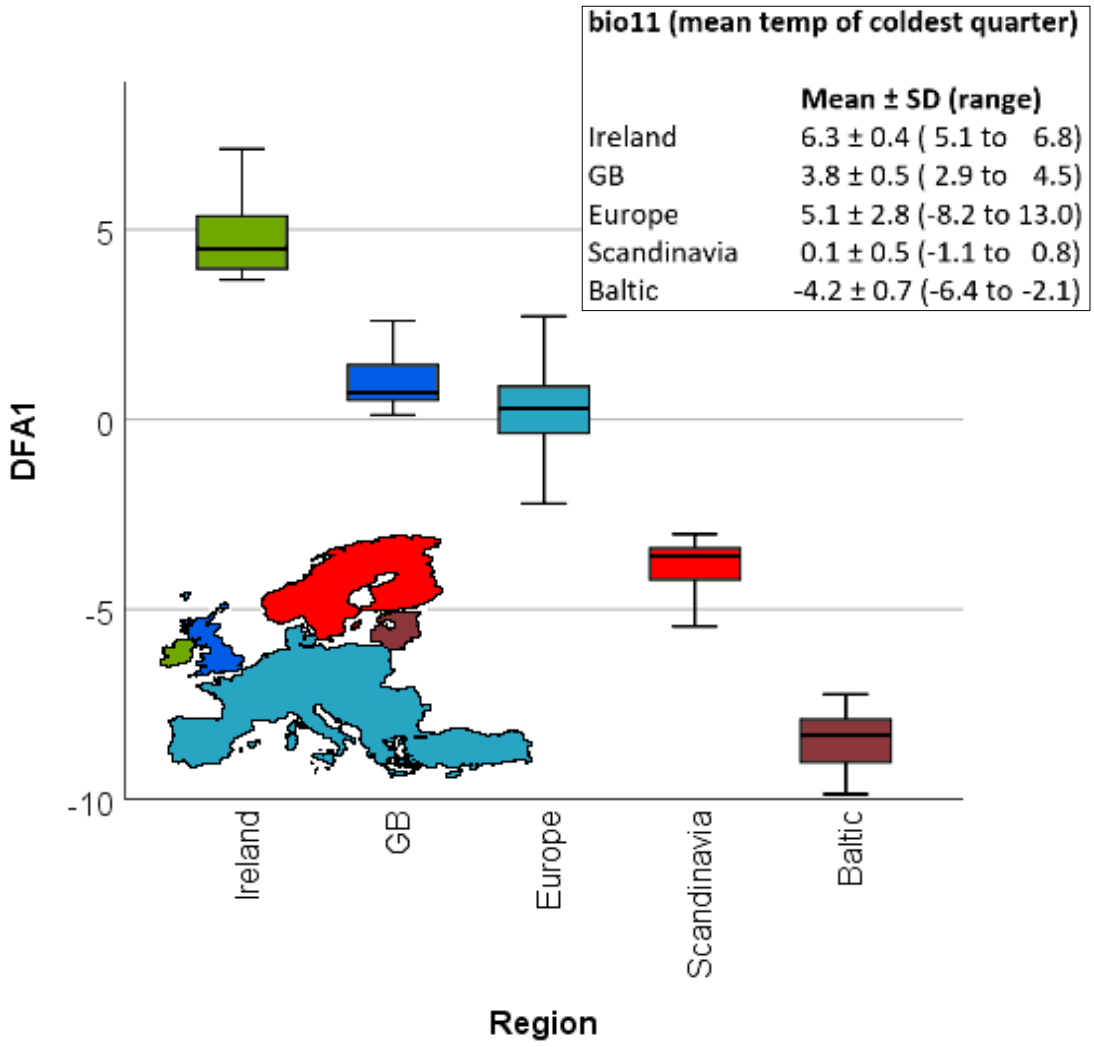
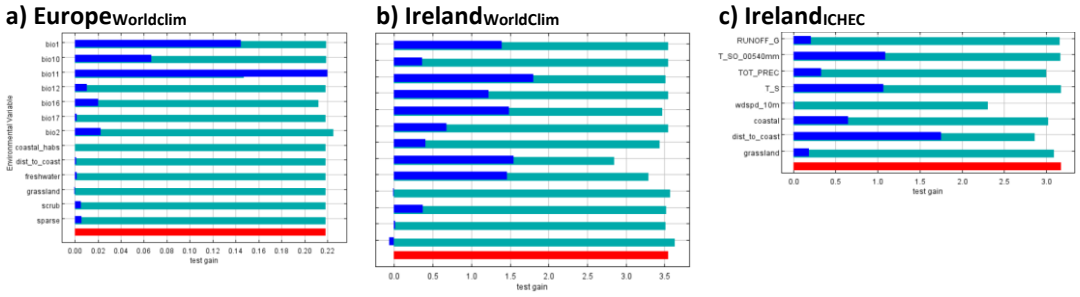


Figure 7.2 Bioclimatic-habitat DFA1 axis scores for Natterjack toad records for each European region with the insert showing mean ± SD in bio11 (mean temperature of the coldest quarter) which was the main contributing variable to DFA1.

Table 7.2 Species Distribution Model average sample sizes (background, training and test) per run (x4 replicate runs) and average model evaluation metrics ± S.E. Subscript MaxTSS = maximum test sensitivity plus specificity, used as a classification threshold for suitable/unsuitable bioclimatic conditions.

		SDM		
Description	**Parameter**	**Europe$_{Worldclim}$**	**Ireland$_{Worldclim}$**	**Ireland$_{ICHEC}$**
Sample size (*n*)	Background	14,514	7,045	5,491
	75% training	4,625	18	9
	25% test	1,541	6	3
Model evaluation *test*	AUC $_{no\ threshold}$	0.832 ± 0.006	0.967 ± 0.021	0.984 ± 0.009
	AUC $_{MaxTSS}$	0.767 ± 0.005	0.896 ± 0.078	0.900 ± 0.092
	Sensitivity $_{MaxTSS}$	0.883 ± 0.018	0.875 ± 0.144	0.833 ± 0.192
	Specificity $_{MaxTSS}$	0.651 ± 0.023	0.917 ± 0.047	0.966 ± 0.017
	Omission rate $_{MaxTSS}$	0.117 ± 0.018	0.125 ± 0.144	0.167 ± 0.192
	Proportion correct $_{MaxTSS}$	0.682 ± 0.018	0.917 ± 0.047	0.966 ± 0.017



d)

		SDMs			
	Data sources	**Extent**		**Data sources**	**Extent**
	variables	**Europe**	**Ireland**	variables	**Ireland**
Climate	**Worldclim**			**ICHEC**	
	bio1 Mean temp.			T_S Surface temp.	
	bio2 Temp. diurnal range			T_SO_00540mm Temp. 53cm belowground	
	bio10 Temp. warmest quarter			TOT_PREC Rainfall	
	bio11 Temp. coldest quarter			RUNOFF_G Subsurface runoff	
	bio12 Precip.			WDSPD_10m Wind speed <10m	
	bio16 Precip. wettest quarter				
	bio17 Precip. driest quarter				
Habitat	**CORINE**			**CORINE**	
	dist_to_coast			dist_to_coast	
	coastal_habitats beaches, sand, saltmarsh			coastal_habitats	
	freshwater				
	grassland			grassland	
	scrub				
	sparse_veg bare rock, sparsely vegetated				

Figure 7. 3 Jackknife test of variable importance to test gain for SDMs built at the extent of **a)** Europe using Worldclim, **b)** Ireland using Worldclim and **c)** Ireland using ICHEC climate variables. Note the *x*-axis varies between models with interpretation based on the relative size of the bars within each plot. **d)** Species response curves showing suitability (line) for species presence (*y*-axes vary from 0 to 1) with variation in each climate and habitat variable. Curves reflect dependence of predicted suitability both on the named variable and dependencies induced by correlations between the named variable and all other variables.

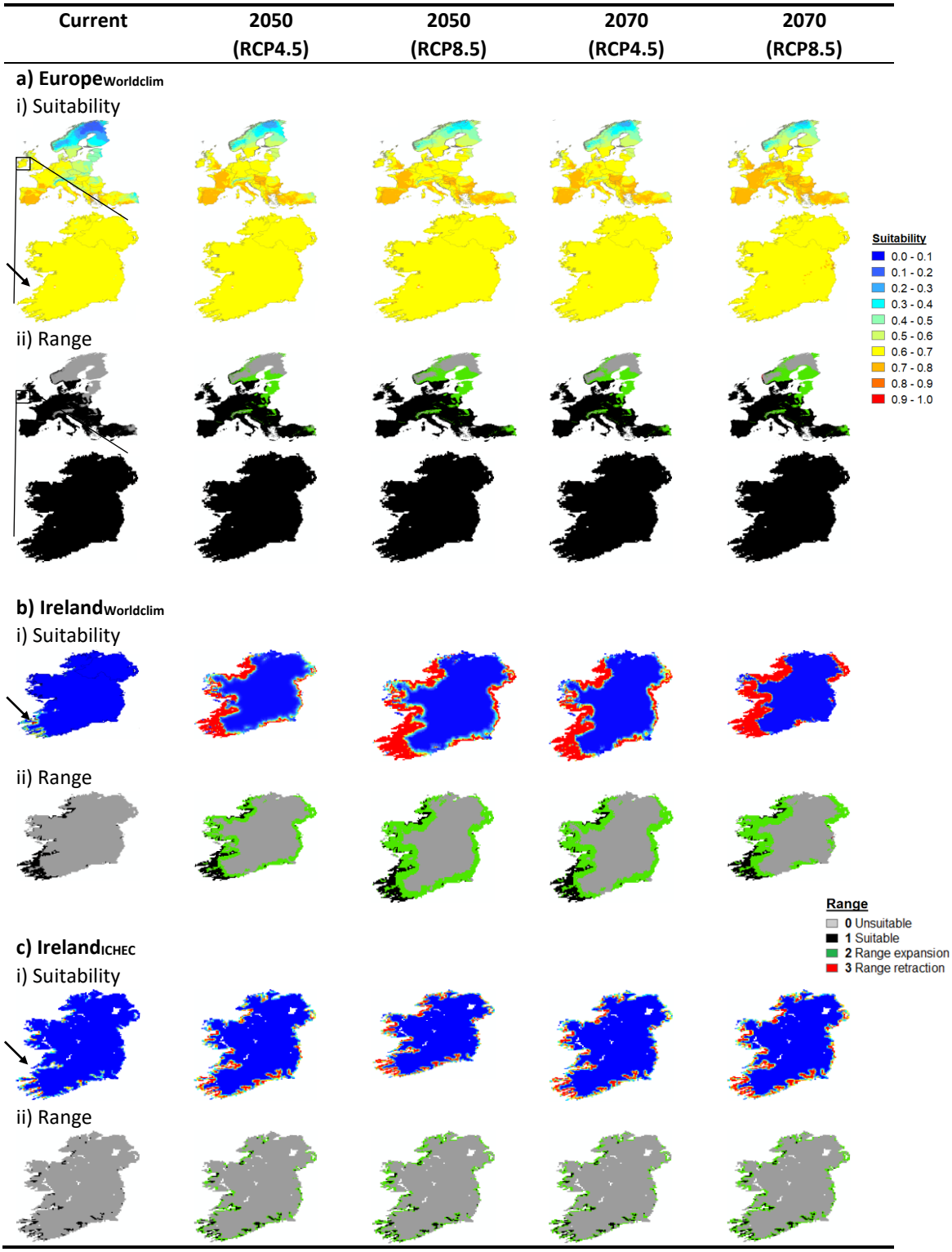


Figure 7.4 Three Natterjack toad SDMs: **a)** Europe using Worldclim, **b)** Ireland using Worldclim and **c)** Ireland using ICHEC climate data showing **i)** suitability (continuous predicted probability) and **ii)** suitable range (binary suitable/unsuitable using the MaxTSS threshold) showing range expansion (green) or retraction (red) from current conditions to future climate scenarios (columns). The native range of the Natterjack toad in Ireland is indicated by the arrow.

Table 7.3 GLMM results for the effects of seasonal temporal lags in climate on Natterjack toad **a)** egg stringsand **b)** first spawning dates.

Model Variables		***F***	**β ± se**	**n.df.**	**d.df.**	***p***
a) Number of egg strings $F_{df=13,3,376}$ = 9.783, p<0.001						
Survey$_t$	temperature	48.113	-2.119 ± 0.306	1	3,376	**<0.001**
	rainfall	0.498	0.207 ± 0.294	1	3,376	0.480
Spring$_t$	temperature	0.543	0.694 ± 0.941	1	3,376	0.461
	rainfall	2.947	1.528 ± 0.890	1	3,376	0.086
Winter$_{t-1}$	temperature	1.745	1.271 ± 0.962	1	3,376	0.187
	rainfall	18.395	3.927 ± 0.916	1	3,376	**<0.001**
Autumn$_{t-1}$	temperature	0.959	-0.621 ± 0.635	1	3,376	0.327
	rainfall	2.682	-1.456 ± 0.889	1	3,376	0.102
Summer$_{t-1}$	temperature	6.746	2.339 ± 0.900	1	3,376	**0.009**
	rainfall	11.996	-3.024 ± 0.873	1	3,376	**0.001**
Spring$_{t-1}$	temperature	0.922	0.814 ± 0.847	1	3,376	0.337
	rainfall	0.114	-0.347 ± 1.030	1	3,376	0.736
Habitat	grassland-dunes	1.087	0.478 ± 0.459	1	3,376	0.297
b) First spawning date (Julian day) $F_{df=2,222}$ = 3.442, p=0.034						
Spring$_t$	temperature	6.452	-3.219 ± 1.267	1	222	**0.012**
	rainfall	0.797	-1.195 ± 1.339	1	222	0.373

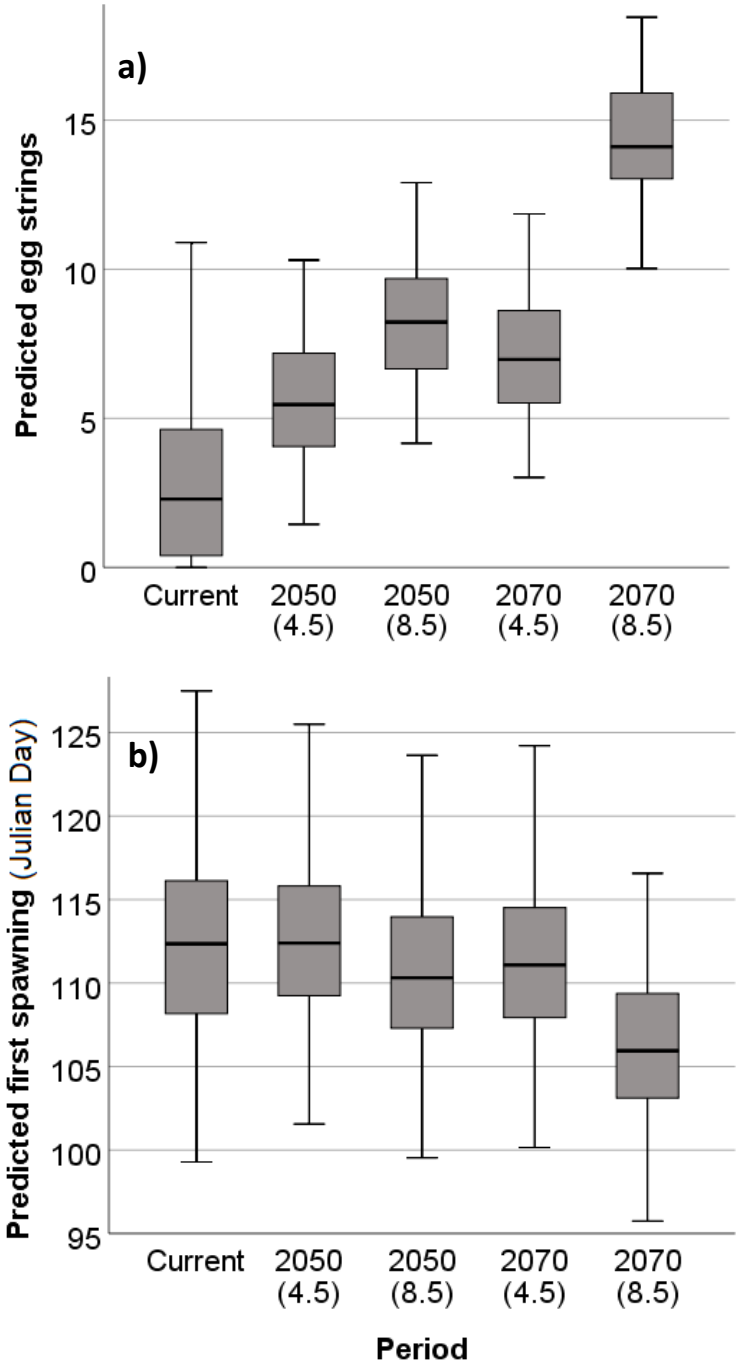


Figure 7.5 GLMM predictions of **a)** number of egg stings and **b)** first spawning dates (Julian day) between current conditions and the mid- to late 21st century under low (RCP4.5) and high (RCP8.5) GHG emissions scenarios.

7.5 Discussion

Models of the Natterjack toad environmental suitability, bioclimatic envelope (potentially suitable cells), egg string production and phenology suggest the species is highly responsive to climate but inhabits a wide range of climatic and habitat conditions throughout Europe. Our results suggest projected climate change may make Europe, including Ireland, more suitable for the species (most notably at northern latitudes in Scandinavia and the Baltic). Number of laid egg strings in Ireland is projected to increase with earlier spawning due to increasingly favourable conditions as the 21st century progresses, especially under a high greenhouse gas emissions scenario.

Niche characterisation and Species Distribution Modelling at the extent of Europe suggested that mean temperature of the coldest quarter (winter temperatures) is most limiting to the range of the Natterjack toad with most of Scandinavia, the Baltic and Eastern Europe currently unsuitable, matching its known range edge margins. In the Northern hemisphere climate change is predicted to lead to milder winter temperatures and a decrease in duration of cold periods and snow cover (Räisänen *et al.* 2004; IPCC 2013). In alpine and boreal habitats, milder winter temperatures have been shown to promote amphibian population viability (McCaffery & Maxell 2010; Üveges *et al.* 2016). Thus, reduced winter severity in the future could be potentially beneficial for the Natterjack toad, especially in Scandinavia and the Baltic, where the species exists at the extremes of its thermal limits. More generally, the suitability of central Europe for the species may also

increase with climate change as conditions more characteristic of its current core range in Iberia and France become more widespread.

The SDM at the extent of Europe failed to predict the species' restricted range in Ireland and Great Britain, and its more widespread range in the Baltic. This is likely due to the extreme range of conditions tolerated by the Natterjack toad at its range edge margins with the model failing to account for colonisation history and local adaptation. Populations have adapted to breed in water with salinity above the species lethal threshold (Gomez-Mestre & Tejedo 2003), in the presence of other amphibian competitors (Gomez-Mestre & Tejedo 2002) or in large lakes (see Appendix E), suggesting a high degree of plasticity and adaptation to less favourable conditions. The impact of climate change can be highly variable among populations with some being more resilient to climatic variation (Griffiths *et al.* 2010; Muths *et al.* 2017), indicating highly context-dependent responses. Our SDMs at the extent of Ireland, whilst failing to capture broader environmental tolerances of the species throughout Europe, accurately reflected the Natterjack toad's highly restricted range in the south-west of Ireland. Both Worldclim and ICHEC climate data, indicated that suitable bioclimatic-habitat conditions currently exist more widely in the south-west of Ireland but also around the Irish coast than are currently occupied. SDMs fail to account for the chronology of biogeographical events after the last glaciation leading to Ireland's colonisation by the Natterjack toad (Rowe *et al.* 2006) and human impacts through ancient landscape modification and modern habitat destruction and deterioration. Natterjack toads, like most amphibians, have limited dispersal capabilities (Smith & Green 2005), suggesting that once historically restricted to the

south-west of Ireland they were unable to subsequently disperse to other suitable regions regardless of their proximity.

A positive impact of climate change on amphibian populations has been projected for other European species (Carey & Alexander 2003; Corn 2005; Dolgener *et al.* 2014). Araújo *et al.* (2006) predicted that 42 amphibian species in Europe will expand their distribution northward by 2050. However, the simple existence of suitable conditions does not necessarily mean that species ranges will expand as expansion will depend on each species' dispersal ability, the existence of vital source populations, suitable habitats and pathways for dispersal (Girardello *et al.* 2010). When comparing the distribution of Iberian amphibian species between two time periods (1901-1990 *vs*. 2000-2015) almost no shifts in distribution were observed, despite changes in climatic conditions (Enriquez-Urzelai *et al.* 2019). Under stressful conditions, like hot and dry weather, amphibians tend to seek refuge and travel shorter distances, thus further restricting their already limited dispersal capabilities (Chan-McLeod 2003; Roe & Grayson 2008). Considering amphibian dispersal limitations, species will likely fail to track shifts in their suitable bioclimatic envelope in the future (Lawler *et al.* 2010). It is important to note that in the current study, species occurrence data represented a snapshot of distribution with toads more likely to be recorded during the breeding season when they and their spawn were more conspicuous. Variation in habitat requirements for breeding, foraging and winter refugia exist (Denton & Beebee 1993), which are not considered in the current analysis. Thus, spatially explicit predictions of models should be treated with caution

with models being indicative of the likely trajectory of the impact of climate change only.

Predictions of number of egg strings and first spawning dates in Ireland, supported SDM predictions of increasing suitability with numbers of eggs strings predicted to increase and initiation of spawning likely to occur earlier in the future, especially under a high greenhouse gas emission scenario. The number of egg strings deposited was most strongly associated with lower temperatures during the breeding season (April to July) as most are deposited in late spring (April to May) when its cooler than during early summer (June to July) when it is warmer. Thus, spring temperature may be more informative by its effect on the initiation of spawning rather than number of egg strings *per se*. Spawning was earlier when spring surface and soil temperatures were warm, which may be linked to earlier emergence from hibernacula, and later when they were cool. Amphibians exhibit the greatest phenological response of any taxa to climate change (Parmesan 2007). Shifts in reproductive timing ave already been observed among various pond breeding amphibians in North America and Europe (e.g. Gibbs & Breisch 2001; Tryjanowski *et al.* 2003; Scott *et al.* 2008; Todd *et al.* 2011). Early breeding can have positive effects like longer development times for tadpoles, more time to accumulate energy reserves and for development of ovaries of recently sexually matured females, enabling spawning in the next breeding season (Jørgensen 1986; Morbey & Ydenberg 2001; Tryjanowski *et al.* 2003), thus increasing fecundity, recruitment and survival. However, advancement of the first spawning date can expose eggs and tadpoles to more variable and unpredictable weather, like freezing, or to inter-specific

competition. For instance, early Natterjack toad breeding can increase the niche overlap with tadpoles of early breeders like the common frog (*Rana temporaria*), thus inducing competition and potential predation (Beebee 2002; Richter-Boix *et al.* 2006). Hence, consequences of breeding earlier on population trends are hard to predict.

Natterjack toad egg string production in Ireland was positively associated with rainfall during the preceding winter, which formed more breeding pools and typically extends pond hydroperiods, especially in sand dune slacks (Reyne *et al.* 2019). Number of egg strings was also influenced by conditions of the summer in the preceding year, suggesting carryover effects may be important. Number of egg strings increased when the previous summer was warm and dry, presumably benefiting invertebrate prey activity, adult toad activity and body condition (toad fecundity is positively correlated with body size; Reading, 1986), and toadlet survival, growth and population recruitment.

The interactions between climate change, range, population, and life histories are complex. Many key factors relevant to amphibian biology have not, or cannot, be parameterised and predicted for the future. Whilst habitat was explicitly included to increase the predictive power of our models, no projections of likely future land cover change are available given the unpredictable nature of coastal habitat development, urbanisation and food production that drives agricultural change. The Natterjack toad in Ireland is regionally Red-Listed as Endangered due to recent range contraction and population decline driven by habitat loss and deterioration (King *et al.* 2011). Regardless of whether climate may become more benign for Natterjack toads in

Ireland, if historical and current threats and pressures continue it seems unlikely that climate will be able to mitigate ongoing declines. Climate change can lead to increased use of pesticides (Kattwinkel *et al.* 2011) and enhanced toxicity of environmental contaminants (Noyes *et al.* 2009). Declines in global invertebrates (van Swaay *et al.* 2013; Winfree *et al.* 2009; Potts *et al.* 2010; Conrad *et al.* 2006) of up to 82% have been recorded in recent decades in some regions of Europe (Hallmannn *et al*. 2017). Changes in prey availability in addition to increased metabolic rate and calorific requirements because of warmer temperatures, can decrease body condition, impacting fecundity and recruitment (Martin *et al.* 2010). In Ireland, the Natterjack toad is found exclusively in coastal habitats. Climate change will cause sea level rise and more frequent and intense storms (IPCC 2007), which may result in saltwater inundation of freshwater breeding ponds or sand dune erosion. Already, reduction in amphibian abundance and diversity has been observed in the USA in areas severely damaged by hurricanes (Schriever *et al.* 2009). Amphibians are highly vulnerable to pathogens and climate change can alter their spread and epidemiology. Kiesecker & Skelly (2001) showed that reduction in water depth leads to concentration of amphibian larvae and trematode-infected snails, leading to significantly increased parasitism of host amphibians. Several hypotheses link climate change to increased chytrid fungus (*Batrachochytrium dendrobatidis*) infection rates, which may be a key factor in global amphibian declines (Pounds & Crump 1994; Pounds *et al.* 1999; Lampo *et al.* 2006; Rohr *et al.* 2008; Rohr & Raffel 2010). Modelling of climate change allows us to estimate its potential impact on some aspect of species biology including environmental suitability, suitable niche space and

reproduction, but without an ability to parameterise or predict other vital aspects of the environment or species adaptation capacity, such predictions may be of limited utility.

This study suggests that the Natterjack toad is a highly adaptable species, inhabiting a wide range of conditions throughout its European range, limited principally by winter temperatures. By the end of the 21st century conditions in Europe may become more favourable for the species, most notably in Scandinavia and the Baltic but also Ireland. Should threats and pressures largely associated with declines in extent and habitat quality be resolved, we might expect that the number of egg strings (and by extension recruitment and population size) may increase with favourable changes in phenology allowing earlier spawning and longer maturation periods. Currently, species conservation strategies in Ireland include the National Parks & Wildlife Service (NPWS) Pond Creation Scheme and Head-start and Translocation Programme aimed at creating artificial farmland ponds as potential new breeding sites, while assisting colonisation (and existing population augmentation) by captive rearing of toadlets for release back into the wild. Our results suggest that, given the wider distribution of suitable conditions outside their current highly restricted range in Ireland, Natterjack toads could be reintroduced to nearby areas where they previously existed and have since been extirpated. In addition, assisted migration could be employed to enable the species to colonise sand dunes and coastal grasslands beyond its historically known range, where suitable climatic conditions occur, forming the basis of a proactive and pre-emptive climate change adaptation strategy. However, careful evaluation of potential

(re)introduction sites is required. For instance, our models predict two sand dune systems (Rossbeigh and Banna Strand) in Co. Kerry have high climatic-habitat suitability, but field surveys report high salinity at ponds in the dune slacks at these sites (see Appendix B) making them unsuitable for breeding. Any conservation (re)introductions should be carefully planned and in accordance with IUCN Species Survival Commission Guidelines (IUCN/SSC 2013). Moreover, as climate change progresses there is a need to reassess its impact on specific populations and adapt conservation practises accordingly.

Chapter 8

General Discussion

8.1 Summary of the main findings

The Natterjack toad population in Ireland has declined, despite substantial conservation efforts over the past decade. Number of egg stringsdecreased by -23% over a 14-year period (-1.6%/yr), but not all populations have negative trajectories. Egg string production of the largest population in Ireland (at the Magharees) increased by +114% but this was insufficient to offset the sum of declines across all populations.

Current habitat restoration practises (pond creation and terrestrial habitat management) failed to provide suitable breeding conditions. Twenty-two of 100 artificial ponds were colonised by 2018, but artificial sites accounted for <10% of all egg strings laid and offset further decline by only +4%. However, habitat restoration practises secured key breeding habitats at two locations (Roscullen Island and Killeen) where action may have prevented local extirpation. The main threat to the species was habitat loss mainly associated with poor water quality at breeding sites and abandonment of surrounding agricultural land leading to unsuitable terrestrial vegetation. Overall, the species was strongly associated with sand dunes, highlighting the importance of protecting these habitats for the persistence of the species at the landscape scale.

The National Parks and Wildlife Service (NPWS) Pond Creation Scheme, launched in 2008, failed to arrest ongoing declines despite some localised success at Roscullen Island and Killeen. Moreover, agri-environment scheme ponds failed to replicate the environment of natural breeding sites differing significantly from

natural ponds in size, pH, conductivity, productivity and surrounding vegetation structure. However, the wider conservation value of artificial ponds should not be underestimated as they had 43% higher aquatic macroinvertebrate species richness and 33% higher macroinvertebrate abundance than natural ponds in adjacent semi-natural habitats.

Previous Natterjack toad population estimates for Ireland relied solely on extrapolations from egg string counts (Becart *et al.* 2006; Sweeney *et al.* 2013). However, such estimates are likely to be unreliable due to survey bias and error (Shmidt 2005; Mazerolle *et al.* 2007; Wagner *et al.* 2011). Population size estimates from Capture-Mark-Recapture suggest that the census size may be underestimated by up to 83% by extrapolating from egg string numbers assuming a 1:1 sex ratio. Empirically estimated sex ratios at one metapopulation (Caherdaniel) suggest there may be up to 7 males to each female. This may suggest that despite substantial declines in the number of egg string, the population size of the Natterjack toad in Ireland may be larger than previously assumed.

The Natterjack toad population in Ireland retains high genetic diversity, despite declines in the number of egg strings and the predisposition of pond-breeding amphibians to lower levels of genetic variation compared to other taxa (Alford & Richards 1999; Newman & Squire 2001). No evidence of genetic bottlenecks or high levels of inbreeding were detected, even for populations at Roscullen and Dooks where egg string production declined by >90%. This suggested that the effective population size (N_e) has not yet dropped below census size (N_C). The Irish Natterjack

toad population exhibits significant spatial genetic structuring with the presence of barriers to inter-metapopulation dispersal. Genetic distance increased with geographical distance, but gene flow was best explained by habitat heterogeneity. Coniferous forestry plantations, bog, marsh, moor and heath, scrub, human influence and rivers were all identified as potential barriers to gene flow while metapopulation connectivity was enhanced by coastal habitats and coastal grassland. If egg string production continues to decline, ensuring habitat connectivity and gene flow between breeding sites to maintain high genetic diversity will be increasingly important.

Models of the environmental suitability for the Natterjack toad, their bioclimatic envelope niche space, egg string productionand phenology suggest the species has the potential to benefit from projected climate change. Under both low and high greenhouse gas (GHG) emissions scenarios, the Natterjack toad is predicted to extend its distribution northward and to higher elevations and increase the number of laid egg strings with spawning initiation likely to occur earlier. However, many key factors important to amphibian biology and species adaptation capacity are not easily parameterised and modelled. Limited dispersal capacity may mean future suitable climate space will go unoccupied while the causes of its recent declines (habitat destruction and deterioration), if unresolved by species conservation strategies, may result in continued declines regardless of potentially improving climatic conditions.

8.2 Conservation recommendations

One of the main aims of the current research was to provide clear data-driven, evidence-based, management recommendations to the Government, in order to promote more-informed and effective decision-making. This was achieved by a close collaboration with the National Parks and Wildlife Service (NPWS) during current research and generation of a Governmental report with specific site-by-site priority actions (Reyne *et al.* 2019) for achieving favourable conservation status for the Natterjack toad in Ireland (see Table 8.1). During 2021, NPWS is planning to initiate a new agri-environmental scheme that will gradually replace the existing pond creation scheme. The new result-based scheme will incorporate our recommendations regarding species surveillance, habitat management and the Head-start and Translocation Programme.

While the Natterjack toad is in decline, the apparent high levels of genetic variation, underestimation of population sizes derived from egg string counts, potential positive impact of climate change and the start of a new Natterjack toad pond creation scheme offer hope for the future of the species in Ireland if appropriately protected and managed.

Table 8.1 Summary of the priority actions recommended to the Irish Government to encourage the conservation of the Natterjack toad in Co Kerry, Ireland.

Recommendations
Continued long-term monitoring and surveillance: - Annual monitoring of the number of egg strings - Conduct regular searches for new breeding sites throughout the whole Inch peninsula and along the shore of Lough Gill, Yganavan and Nambrackdarring - Use of mark-recapture methods for absolute population size estimation
Habitat management: - Collaboration with landowners, farmers and golf courses - Management of cattle grazing - Prevention of scrub encroachment - Maintaining of open habitats (i.e. short sward) - Removing of pond emergent vegetation - Ensure compliance with existing Pond Creation Scheme
Magharees sand dune conservation: - Removal of the invasive sea buckthorn - Management of cattle grazing and access to breeding ponds - Restriction of framing activities in close vicinity of breeding sites during the breeding season - Creation of scrapes
Expansion of the Pond Creation Scheme to include both farmland and natural habitat, creation of additionals pond as stepping stones to facilitate dispersal
Head-start and Translocation Programme: - select source populations from geographically close breeding sites within the same genetic entities - post-release monitoring to estimate survival rates and overall the efficiency of the programme

8.2.1 Monitoring and surveillance

Continued long-term monitoring and surveillance is necessary to differentiate natural interannual fluctuations in breeding activity from consistent temporal trends in the number of egg strings and/or population size. The easiest solution for annual monitoring of reproductive effort and overall population health is egg string counts. However, count data cannot be extrapolated with any reliability to produce precise population estimates. Where absolute population size estimation is necessary (e.g. for Habitats Directive reporting), a combination of egg string counts and Mark-Recapture (using photo ID or genetic fingerprinting) may be appropriate.

8.2.2 Habitat management

Protection of sand dunes is key to the Natterjack toad's survival in Ireland. Removal of invasive sea buckthorn in proximity to breeding ponds and close collaboration with landowners and farmers on cattle management to maximise the benefits of conservation grazing are recommended.

Artificial ponds provided less favourable breeding conditions than natural ponds, due to eutrophication and unsuitable vegetation surrounding the immediate vicinity of the pond. These issues need to be addressed by ensuring compliance with habitat maintenance recommendations of the Pond Creation Scheme (e.g. grazing by livestock, removing of the emergent vegetation) and close collaboration with local stakeholders to reduce agricultural runoff causing poor water quality. Expansion of

any future Pond Creation Scheme to include natural habitat to, for example, create scrapes in sand dunes could provide additional breeding sites for the Natterjack toads where they occur creating more optimal breeding conditions than those of artificial ponds in agriculture grassland.

Habitat management such as preventing scrub encroachment (a barrier to dispersal), maintaining open coastal habitats (facilitating dispersal) and creating additional ponds as stepping stones between currently occupied sites may help improve habitat connectivity between breeding sites. Creating large breeding ponds or a high number of small ponds in close proximity is recommended to ensure a large effective population size which may promote dispersal.

Conservation practises should focus on maintaining high adaptive genetic variationas well as protection of the genetic integrity of identified entities. Ideally, the Head-start and Translocation Programme should select source populations from geographically close breeding sites within the same management unit to maintain genetic provenance and spatial genetic structuring.

8.2.3 Reintroductions and range expansion

There may be an aspiration that the Natterjack toad's range in Ireland is enlarged beyond its current highly restricted range to occupy sites previously extirpated or to establish new sites by assisted migration as part of a climate change impact mitigation strategy. Species Distribution Modelling indicated that suitable bioclimatic-habitat conditions do occurr in areas of Co. Kerry, and more widely in the

south and west of Ireland, which could support Natterjack populations. Assisted migration is a controversial conservation strategy as it involves pre-emptive action in combating the impacts of climate change and is, therefore, less conservative than traditional conservation measures. Should such a strategy be pursued, donor populations should be selected from the geographically closest population or, if a new location is distinct and isolated, the Magharees population may be considered as a potential donor due to its high genetic diversity and large population size.

8.3 Future perspectives

The present research highlights further questions to be addressed. While translocations are becoming increasingly important in species conservation (Seddon *et al.* 2014), post-release monitoring is rarely conducted and failures often remain unknown (Seddon *et al.* 2007; Rojahn *et al.* 2018). Post-release monitoring of the Natterjack toad currently relies on detecting calling males and/or egg strings once individuals reach sexual maturity and return to ponds to breed. The release of early stages (tadpoles and metamorphs) means the success of any translocation remains unknown for many years. Environmental or eDNA protocols developed for the species (see Appendix B) could be used to monitor species occurrence and colonisation of new ponds while further development of quantitative PCR methods to estimate DNA concentrations in pond water could be correlated with breeding population sizes providing an indirect proxy method to monitor changes in

abundance that may be less intensive in terms of person hours, lowering staff input freeing up more resource for other conservation activities.

Another avenue of potential future investigation includes the use of passive acoustic monitoring to capture and quantify the initiation of breeding behaviour by calling males, numbers of calls and intensity of calling. Many studies are now using Artificial Intelligence (AI) to extract soundscape variables enabling the rapid, low cost analysis of 'Big Data'. If initially paired and calibrated with Mark-Recapture methods to estimate the male population at each pond, acoustic monitoring may be another useful low cost indirect method for proxy for population size or reproductive effort providing a low cost indirect method for monitoring and surveillance.

Natterjack toad terrestrial habitat preferences outside the breeding season and the availability of suitable hibernation sites remain unknown. Most amphibian studies are conducted during the breeding season when animals aggregate at high density. However, the terrestrial environment contributes greatly to amphibian population dynamics (Marsh & Trenham 2000; Joly *et al.* 2001; Regosin *et al.* 2003) and knowledge on the use of terrestrial habitats is important for species management. Radio-telemetry studies could yield insights into post-breeding movement, habitat preferences and overwintering locations (Miaud & Sanuy 2005).

The Natterjack toad appears to be resistant to chytridiomycosis with no population declines throughout its range associated with the disease thus far (Cunningham & Minting 2008; May *et al.* 2011). Even though *Batrachochytrium dendrobatidis* (*Bd*) has not been detected in Ireland (Gandola & Hendry 2013),

continued surveillance for the pathogens presence is important, given the major role disease plays in the global amphibian extinction crisis. This should include monitoring all three of Ireland's amphibian species (*Epidalea calamita*, *Rana temporaria* and *Lissotriton vulgaris*) for clinical signs or collection of skin swabs and molecular testing for both *Bd* and *Batrachochytrium salamandrivorans* (*Bsal*). Further investigation into the Natterjack toad immune defence might shade light on amphibian susceptibility to chytridiomycosis.

8.4 Conclusion

The Natterjack toad population in Ireland, like many other amphibian species around the world, is declining. Finding solutions to counter the global amphibian extinction crisis is one of the greatest challenges in conservation biology, which has serious implications for the health of ecosystems. Although scientific research has led to a better understanding of the amphibian decline, many questions remain unanswered. A major challenge lies in breaching the boundaries between academic research, Government and conservation management decision making and practical on-the-ground conservation action by various stakeholders (for example, landowners and farmers) to make conservation programmes more effective and efficient.

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Appendices

10.1 Appendix A

Chapter 2. Conservation efforts fail to halt the decline of the regionally endangered Natterjack toad (*Epidalea calamita*) in Ireland

Table A.1 The total number of egg strings and toadlet abundance (i.e. density in toadlets/m^2 multiplied by the area surveyed around each pond based on pond circumference) recorded at all sites and areas during the breeding season in 2016-18.

Breeding site	Egg strings			Toadlet abundance		
	2016	2017	2018	2016	2017	2018
Magharees	2,261	386	1,519	479,372	5,280	487,684
Castlegregory Golf Course	354	495	405	24,960	86,880	53,777
Tullaree	1	3	16	0	0	20,924
Inch	17	18	392	3,440	0	0
Killeen	12	6	8	0	0	0
Roscullen island	79	50	64	720	1,120	2,732
Dooks Golf Course	50	54	22	0	160	0
Lough Yganavan	155	146	23	16,080	13,280	178
Nambrackdarrig	1	9	1	320	0	7
Glenbeigh	44	59	11	160	1,440	64
Caherdaniel	242	231	224	160	14,080	19,841
Total Ireland (Co. Kerry)	**3,216**	**1,457**	**2,685**	**525,212**	**122,240**	**585,206**

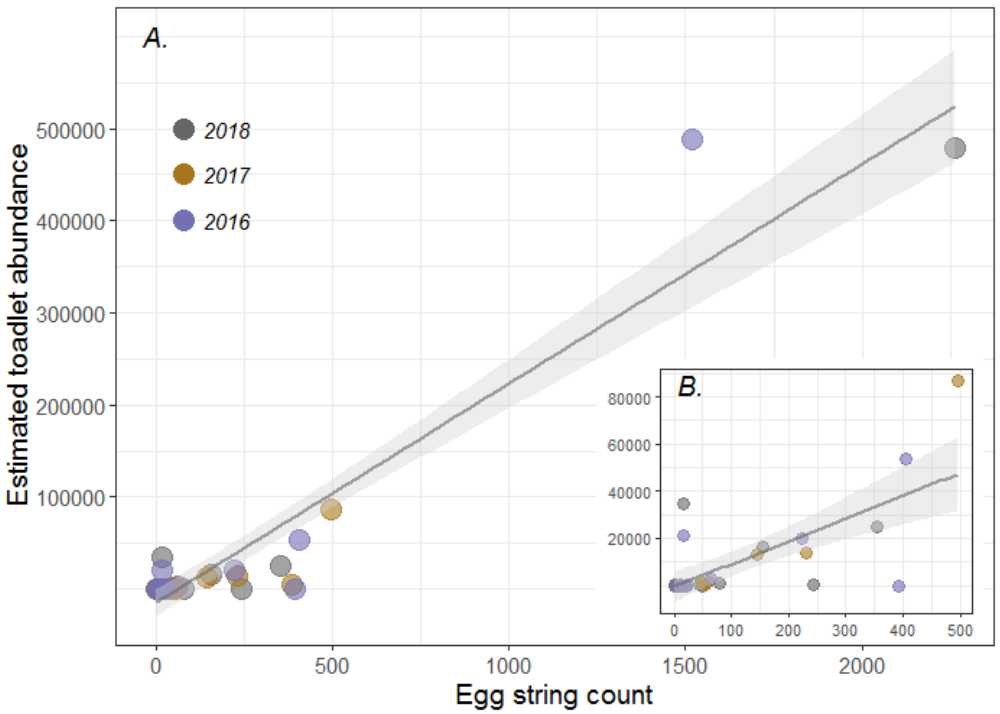


Figure A.1 Relationship between egg string count per metapopulation and toadlet abundance for (A) all Natterjack toad populations and (B) excluding the Magharees.

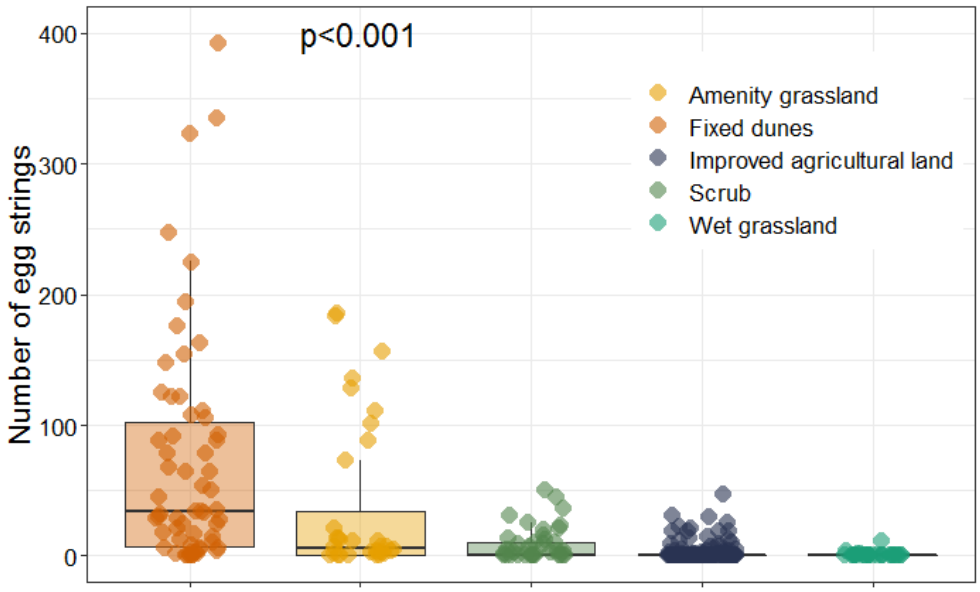


Figure A.2 Number of egg strings recorded at each habitat type during 2016-18 Natterjack toad survey.

10.2 Appendix B

Development and validation of a quantitative qPCR assay for detecting Natterjack toad (*Epidalea calamita*) eDNA samples.

A manuscript based on this study was published as:

Reyne M, Naaum A, Marnell F, Reid N, Helyar S. (2021) Development and validation of quantitative PCR assay for detecting Natterjack toad (*Epidalea calamita*) eDNA samples. *Conservation Genetics Resources.* DOI: 10.1007/s12686-021-01199-3

Abstract

The Natterjack toad (*Epidalea calamita*) is the rarest amphibian species in Ireland, regionally Red-Listed as Endangered. We applied an eDNA approach to detect species presence in breeding pond water samples. We developed a species-specific qPCR assay targeting the cytochrome c oxidase subunit I (COI). The assay was tested *in silico*, *in vitro* (DNA extracted from tissue) and *in vivo* (DNA extracted from water samples). Water samples were collected from five ponds with known Natterjack toad presence or absence to validate the sensitivity and specificity of the assay. The assay was shown to be highly specific to the Natterjack toad and tested positive only against toad tissue samples and eDNA samples from ponds with known species presence. We believe this method can be used for rapid assessment of species occurrence.

Species distribution is among the most basic and important data in ecology and conservation of wild populations, but often obtaining robust distribution records can be challenging (Mazerolle et al. 2007). Recent developments in molecular methods can offer a solution through non-invasive genetic monitoring, where DNA can be extracted from the environment (e.g. water, soil) to obtain targeted presence-absence data (Deiner *et al*. 2017). Environmental DNA (eDNA) analysis has already demonstrated that it is a powerful biodiversity monitoring tool with diverse applications in conservation management. This technique is especially useful for monitoring elusive species susceptible to disturbance (Laramie *et al.* 2015; Ma *et al.* 2016; Dougherty *et al.* 2016; Vörös *et al.* 2017), has been shown to be time and cost effective (Biggs *et al.* 2015; Boussarie *et al.* 2018) and can have higher detection rates compared to traditional survey methods (Hunter *et al.* 2015; Smart *et al.* 2015; Torresdal *et al*. 2017). However, there are challenges associated with using eDNA for biodiversity monitoring like optimisation of water collection and laboratory protocols, DNA behaviour in the environment, contamination (e.g. Buxton *et al*. 2017, 2018; Harper *et al*. 2019) that can influence DNA capture and detection.

In this study, we developed and optimized a quantitative PCR (qPCR) assay for detecting the presence of the Natterjack toad (*Epidalea calamita*) in water samples from breeding ponds. The species is the rarest amphibian in Ireland, regionally Red-listed as Endangered (King *et al*. 2011). The Natterjack toad is a subject to considerable conservation efforts including an agri-environment Pond Creation Scheme and a Head-Start and Translocation Programme by the National Parks & Wildlife Service (NPWS) to create artificial ponds on farmland and promote

colonisation (Reyne et al. 2019). The eDNA protocols developed here could be used for rapid assessment of species presence, especially for surveillance of colonisation rates of the newly created breeding sites, detecting toad presence before field signs of breeding are obvious and for monitoring post-release survival of translocated individuals (Rojahn *et al.* 2018).

Fieldwork was conducted in 2017 during the Natterjack toad breeding season (April-July) in Co. Kerry, Ireland (Figure B.1). We collected tadpoles and Natterjack toad tissue samples from dead individuals found *in situ*. DNA was preserved in 100% ethanol at ambient temperature. We collected water samples from ponds with well-known Natterjack toad presence and absence based on intensive field surveys (Bécart *et al.* 2007; Sweeney *et al.* 2013; Reyne *et al.* 2019). Water samples (30ml) were collected at ten sites around the pond margin, pooled and gently mixed in a sterile self-supporting plastic bag. From each of these pooled samples, 3x15ml were taken with a sterile pipette and added to a 50ml centrifuge tube containing 33 mL 100% ethanol and 1.5 ml 3M sodium acetate. A negative control of distilled water was used following the field protocol to test for cross-contamination between samples. All samples were stored at -20°C until extraction. Work was conducted in a UV sterilisable chamber with air ventilation. Genomic DNA was extracted using DNeasy Blood and Tissue extraction kit (Qiagen, Valencia CA, USA), while DNA extraction from water samples followed Williams *et al.* (2017). In summary, we used centrifugation to concentrate DNA from water, then DNA was purified using the CTAB (cetyltrimethyl ammonium bromide) protocol (Coyne *et al.* 2001; Turner *et al.* 2014),

followed by a post-extraction inhibitor removal step using a OneStep PCR Inhibitor Removal kit (Zymo Inc., Irvine, California, USA).

Development of the cytochrome c oxidase subunit I (COI) based qPCR assay was conducted on sequences of three individuals obtained from GenBank (accession numbers: HM901944-47). AlleleID software version 7.5 (Premier Biosoft, USA) was used to align the COI regions, identify consensus regions and design primers. We developed an assay consisting of forward (*Ecal_COI_F* 5'-CCGTCAATAACTCAATACC-3') and reverse (*Ecal_COI_R* 5'-GCAAGAACTGGTAGAGAA-3') primers and a FAM-labelled MGB non-fluorescent quencher probe (*Ecal_COI_probe* 6FAM-5'-AATCACTGCCGTCTTGCTTCT-3') that amplifies an 89 base pair (bp) region. After the primer design, specificity was assessed via an NCBI BLAST search (Ye *et al.* 2012). The assay was tested *in silico* against COI sequences of three European toad species (*Bufo bufo*, *B. spinosus* and *Bufotes viridis*) and *in vitro* against a panel of tissue samples of the target organism and closely related non-target species present in Ireland (the common frog *Rana temporaria* and smooth newt *Lissotriton vulgaris*) to empirically demonstrate the specificity of the developed assay. Amplification was validated via Sanger sequencing and a subsequent BLAST search on GenBank. The assay was also tested *in situ* on samples collected from ponds with known Natterjack toad presence and absence. We performed assay optimisation using different primer/probe concentrations and thermocycling conditions including two and three step protocols. qPCR was performed using a Magnetic Induction Cycler (MIC) platform (Bio Molecular Systems) in a final reaction volume of 20µL, which included 4µL of template DNA, 10µL SensiFAST™ Probe No-ROX (Bioline Meridian BioScience, Cincinnati, Ohio, USA),

4µL ddH2O, 0.8µL of each primer and 0.2µL probe. This mix was then placed into dedicated reaction tubes manufactured for MIC platform and prefilled with high viscosity silicon oil (Bio Molecular Systems) to prevent evaporation and contamination of amplicon. PCR reactions had the following thermal cycling conditions: activation step 95°C for 5min, followed by 35 cycles of 95°C for 10 sec and one step for annealing and extension of 60°C for 35 sec. Tissues samples of the Natterjack toad, common frog and smooth newt were used for positive and specificity controls respectively. The results obtained from qPCR and Sanger sequencing demonstrate that the developed COI assay tested positive against only Natterjack toad tissue samples and when the species was known to be present in breeding ponds (Table B.1). No amplification occurred at sites where the species was absent, or in negative controls or blanks.

The assay presented is highly specific to the Natterjack toad. We believe this method has potential to be used for species detection during monitoring and surveillance across its distribution range in Europe and for evaluating species conservation strategies including post-release survival of translocated individuals.

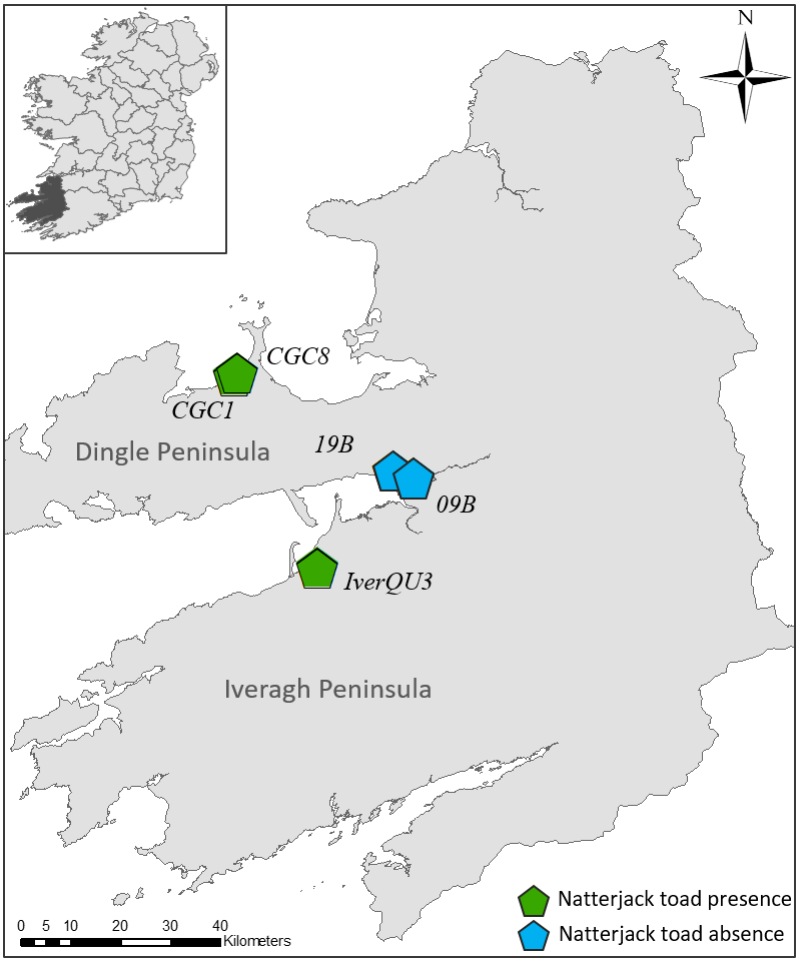


Figure B.1 Map of the study area (insert shows Co. Kerry within Ireland) and sampling locations of collected water samples used for qPCR assay validation.

Table B.1 Summary of the results of the developed qPCR assay for detection presence of the Natterjack toad in environmental DNA samples. Efficiency was calculated from the gradient of a standard curve using tissue DNA and the linearity measured as R^2.

Location	Pond ID	Natterjack toad presence*	Amplification	Efficiency	R^2	Cq
Castlegregory Golf Course	CGC1	Yes	Yes	0.87	0.999	27.42
Castlegregory Golf Course	CGC8	Yes	Yes	0.84	0.998	27.83
Roscullen Island	09B	No	No	-	-	-
Keel	19B	No	No	-	-	-
Glenbeigh	IverQU3	Yes	Yes	0.79	0.996	23.64

*Presence was confirmed via field surveys

10.3 Appendix C

Chapter 3. Artificial agri-environment scheme ponds do not replicate natural environments despite higher aquatic and terrestrial invertebrate richness and abundance

Methods

Invertebrate sampling

Aquatic macroinvertebrates were collected using sweep netting (effective in collecting slow moving species from the water column or sedentary invertebrates from amongst aquatic plants) covered an area of $2m^2$ along the pond edge swept for 20 seconds. Contents of three separate sweeps were pooled together into one sample per pond. Bottle traps (effective in collecting fast moving, highly mobile predatory invertebrates, principally water beetles i.e. *Dytiscid* spp.) were made of 2 litre plastic bottles with a one-way entrance funnel and were baited with cat food. Traps were submerged vertically (entrance lowermost in the water column capturing an air bubble within the upturned bottle to provide a breathing chamber for any trapped invertebrates) close to areas of dense aquatic vegetation. Bottle traps were fixed to a one metre bamboo pole and were checked after 24 hours. Aggregated sweep nets and bottle traps were pooled per pond.

Terrestrial spiders were collected using pitfall trapping using plastic containers (9 x 8.5cm) placed at the south side of each pond <2m from the water's edge and in open ground avoiding areas of scrub and overhanging vegetation. The top of the container was level with the soil surface. The opening was covered with mesh (to

prevent the unintentional capture of protected pygmy shrews (*Sorex minutus*) and capped with a raised cover (to prevent flooding by rainwater). Containers were one third filled with Propanediol (a non-toxic antifreeze) to preserve samples. Traps were left *in-situ* for a period of four weeks (between late April and late May 2017).

Taxonomic resolution

All aquatic macroinvertebrates were identified following Croft (1986), Friday (1988), Cham (2012) and Pawley *et al.* (2012). Species lists were checked by NPWS Invertebrate Ecologist, Brian Nelson (see Acknowledgements), who provided quality assurance by flagging any likely misidentifications for checking. Aquatic invertebrates that could not be identified to species-level (larvae) or due to poor sample preservation were excluded from the analysis (Table C.1). Terrestrial spiders (taken as bioindicators of the surrounding terrestrial invertebrate community) were identified by Myles Nolan to species-level following Roberts (1993) and Merrett *et al.* (2015).

Statistical analysis

Environmental parameters were standardised to have a $\bar{x} = 0$ and $\sigma = 1$ prior analyses. All 17 environmental variables (Table 3.1) were tested for multicollinearity and one of each highly correlated set of bivariates (correlation coefficients >0.7) were removed from further analysis.

Multivariate ordination analyses were used to examine variation in aquatic and terrestrial invertebrate community composition using PRIMER6 with PERMANOVA^{+}

software. Separate analyses were conducted for aquatic and spider communities. In each case, species were pooled together in a single species matrix (pond x species) and were related to an environmental matrix (pond x environmental variables). Species abundance data was transformed using the square-root function. Resemblance tables were calculated using Euclidean distances for environmental parameters and Bray-Curtis similarity for species data.

Results

Given the coastal nature of the study areas, conductivity and salinity were highly correlated (r=0.721, p<0.001) as were habitat type and management activity (r=0.900, p<0.001). We, therefore, excluded salinity and management activity from analyses. It should be noted that there was no significant difference in salinity between natural and artificial ponds (Mann Whitney U=472.5, Z=-1.709, p=0.087).

There was no significant difference in the number of natural and artificial ponds classified as permanent or ephemeral (χ^2=1.75, d.f.=1, p=0.186, Table S4). In contrast, a significantly larger proportion of the surface area of natural ponds dried up as the season progressed (Table 3.2). Single variable analysis suggested that species richness and abundance were unrelated to the proportion of the surface area of either natural or artificial ponds that dried up (Figure C.3 and C.4).

Pond age was also not retained in the top set of GLMs for aquatic macroinvertebrate richness and abundance (Table 3.3). There was a suggestion of a weak trend for ponds <5 years old to have lowest species richness and abundance

with 9 year old ponds having highest species richness (Figure C.5). Nevertheless, pond species richness and abundance were not significantly affected by pond age.

Presence of vertebrate predators (fish and amphibians) was found in 83% of the sampled ponds but had no significant effect on aquatic invertebrate community, abundance and richness, thus was not retained in the top set of GLMs (Table 3.3). However, rarefaction suggested that species richness might be higher at ponds with vertebrate predator presence (Figure C.6).

It should be noted that the analyses and Results reported in the main paper were initially conducted with all taxa including those identified at species-level plus those at lower taxonomic levels e.g. Genus-level etc. Subsequently analyses were split into common taxa (>10% occurrence) and rare taxa (<10% occurrence) before the final analysis of all of taxa identified to species-level only was retained. The overall interpretation of the results re: differences in natural and artificial ponds remained identical for all analytical permutations as did most correlated environmental parameters. Thus, taxonomic resolution and the subset of data included did not change the Results appreciably. The approach reported in the main paper was that settled upon after two rounds of peer-review.

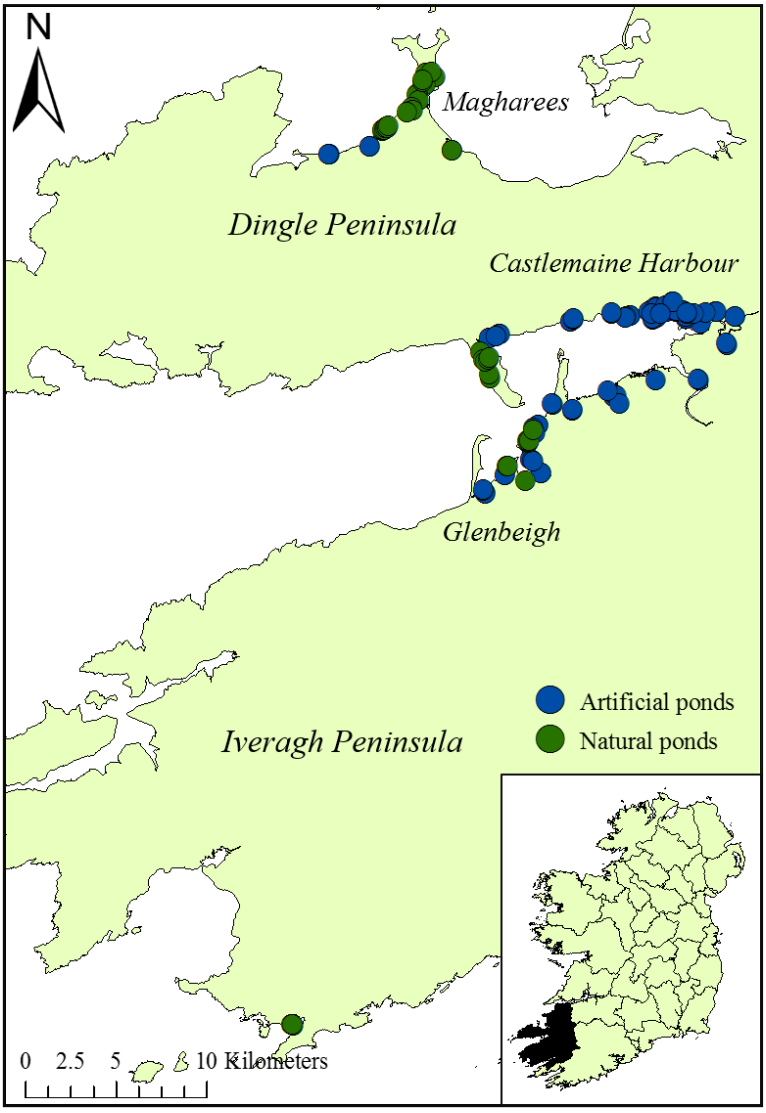
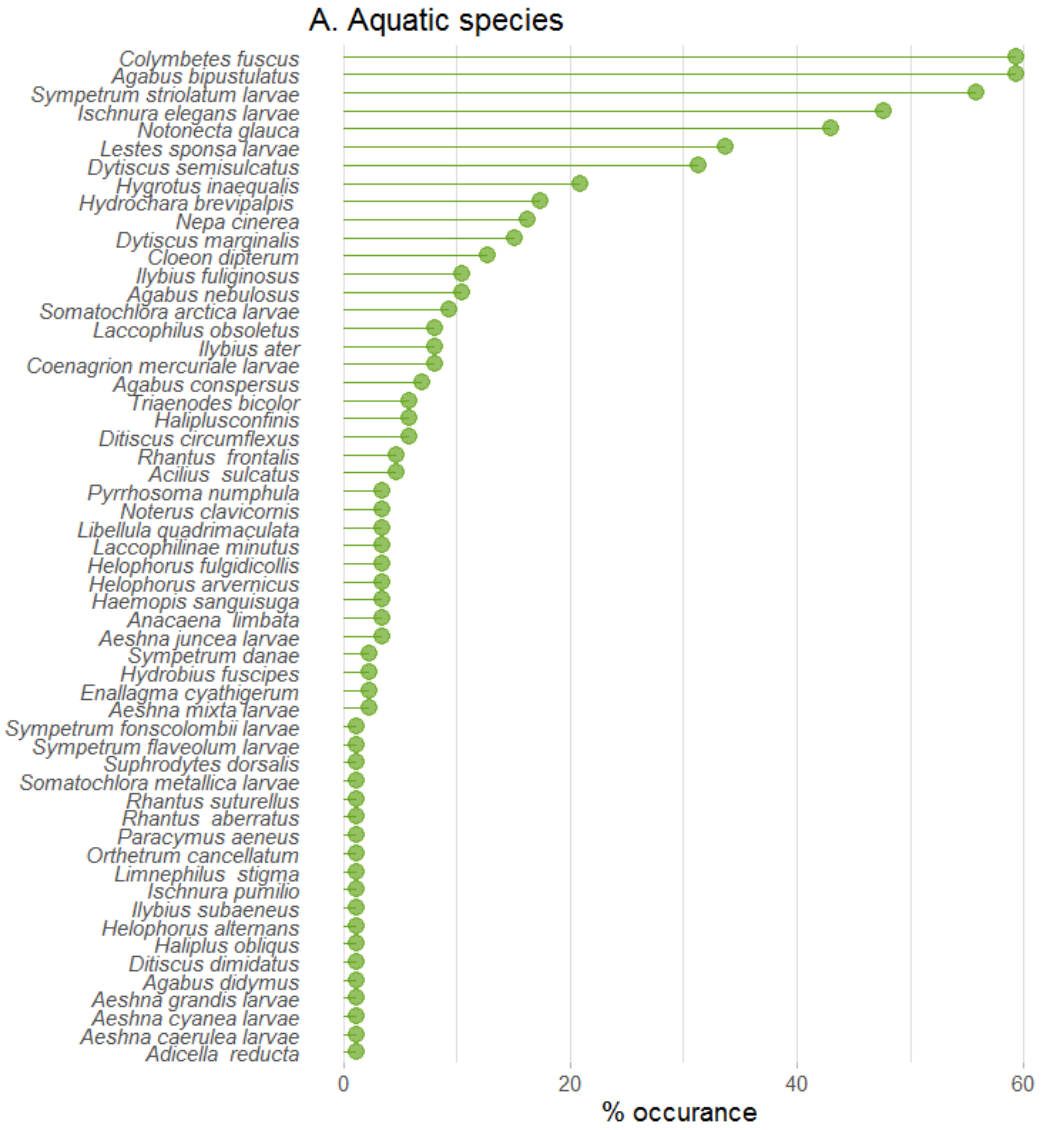


Figure C.1 Map of County Kerry, Ireland showing the locations of 139 ponds surveyed in the study. All ponds were restricted to coastal areas of the Dingle and Iveragh Peninsulas (some dots represent more than one pond).


A. Aquatic species
Colymbetes fuscus
Agabus bipustulatus
Sympetrum striolatum larvae
Ischnura elegans larvae
Notonecta glauca
Lestes sponsa larvae
Dytiscus semisulcatus
Hygrotus inaequalis
Hydrochara brevipalpis
Nepa cinerea
Dytiscus marginalis
Cloeon dipterum
Ilybius fuliginosus
Agabus nebulosus
Somatochlora arctica larvae
Laccophilus obsoletus
Ilybius ater
Coenagrion mercuriale larvae
Agabus conspersus
Triaenodes bicolor
Haliplusconfinis
Ditiscus circumflexus
Rhantus frontalis
Acilius sulcatus
Pyrrhosoma numphula
Noterus clavicornis
Libellula quadrimaculata
Laccophilinae minutus
Helophorus fulgidicollis
Helophorus arvernicus
Haemopis sanguisuga
Anacaena limbata
Aeshna juncea larvae
Sympetrum danae
Hydrobius fuscipes
Enallagma cyathigerum
Aeshna mixta larvae
Sympetrum fonscolombii larvae
Sympetrum flaveolum larvae
Suphrodytes dorsalis
Somatochlora metallica larvae
Rhantus suturellus
Rhantus aberratus
Paracymus aeneus
Orthetrum cancellatum
Limnephilus stigma
Ischnura pumilio
Ilybius subaeneus
Helophorus alternans
Haliplus obliqus
Ditiscus dimidatus
Agabus didymus
Aeshna grandis larvae
Aeshna cyanea larvae
Aeshna caerulea larvae
Adicella reducta
0
20
40
60
% occurance

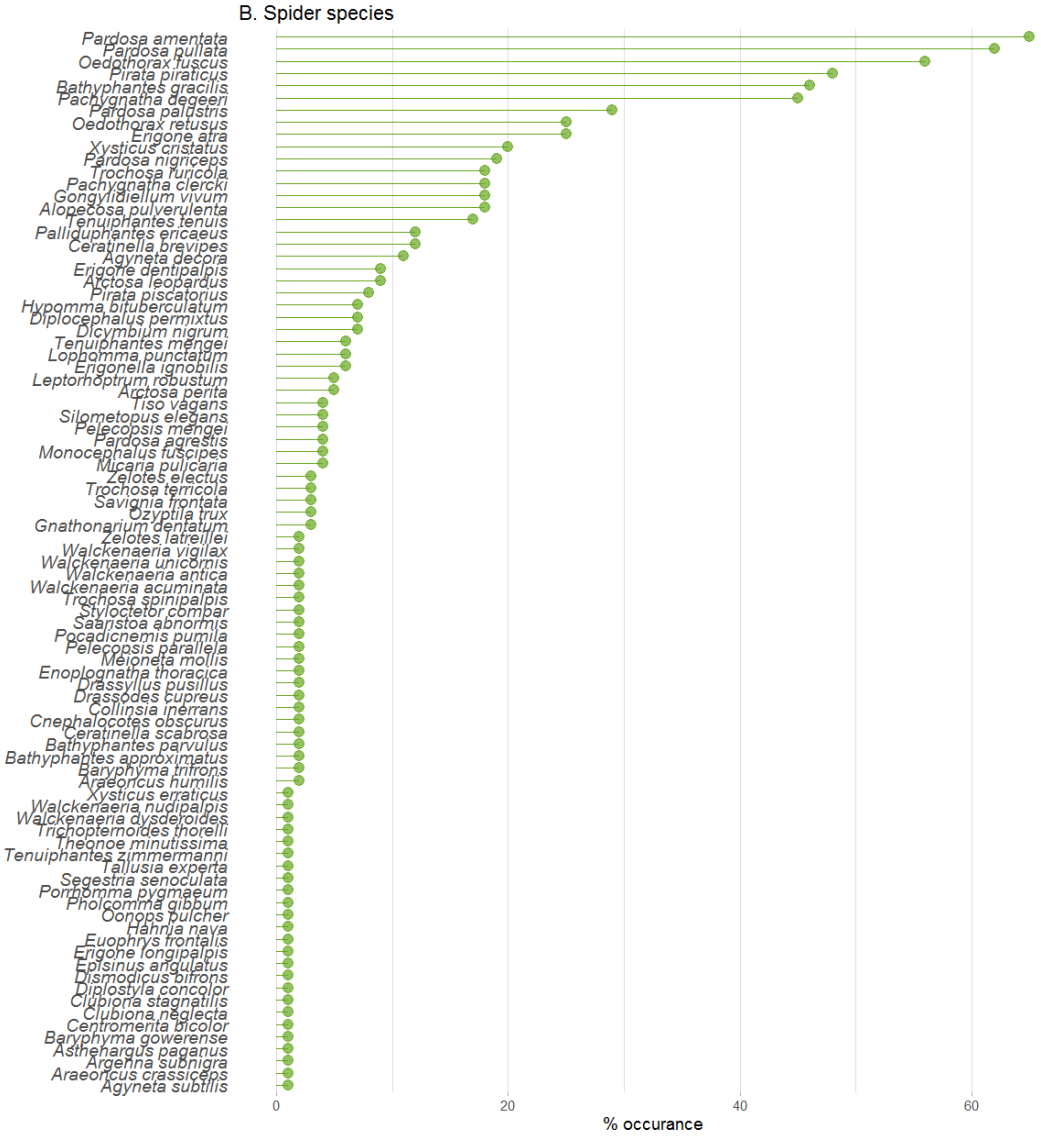


Figure C.2 Percentage occurrence of **(a)** aquatic species and **(b)** spider species.

Table C.1 Unidentified to species level taxa removed from statistical analysis and their occurrence in artificial and natural ponds.

Taxa	Artificial ponds		Natural ponds	
	Number of ponds	% occurrence	Number of ponds	% occurrence
Dytiscidae nymph	27	40	8	42
Agabus spp.	6	9	0	0
Suphrodytes sp.	1	1	0	0
Hydroporus spp.	15	22	4	21
Helophorus sp.	12	18	4	21
Haliplus sp.	3	4	4	21
Gyrinus sp.	6	9	1	5
Scirtidae	1	1	0	0
Notonecta sp.	56	84	12	63
Corixa spp.	48	72	11	58
Gerris sp.	40	60	5	26
Anisoptera larvae	14	21	3	16
Aeshna sp.	2	3	0	0
Coenagrion sp.	3	4	0	0
Zygoptera larvae	16	24	7	37
Limnephilus spp.	13	19	1	5
Leptoceridae	1	1	6	32
Hirudinae	3	4	4	21
Physidae	1	1	1	5
Planorbidae	6	9	4	21
Sphaeriidae	6	9	4	21
Crangonyx spp.	13	19	1	5
Hydracarina	10	15	3	16
Ephemeroptera	3	4	4	21

Table C.2 Mean values ± 1 standard deviation (SD) for environmental parameters associated with habitat types and a test of difference.

Environmental parameter	Habitat type				Kruskal-Wallis		
	Improved grassland	Wet grassland	Fixed dunes	Amenity grassland	χ^2	d.f.	*p*
Size							
Surface area (m^2)	82.7 ± 282	46.4 ± 20.9	372.0 ± 488.7	491 ± 520	20.214	3	<0.001
Area that dried (m^2)	44.5 ± 25.6	41.0 ± 22.6	65.3 ± 34.7	81.8 ± 107	5.577	3	0.134
Water							
pH	6.7 ± 0.7	6.4 ± 0.5	8.0 ± 0.5	7.4 ± 0.4	79.461	3	<0.001
Conductivity(µS/cm)	458.3 ± 717.5	1499.2 ± 1719	608.7 ± 142.7	460.5 ± 96.2	11.781	3	0.008
Oxygen (mg/l)	6.1 ± 2.3	5.08 ± 2.3	7.2 ± 1.9	5.6 ± 1.9	2.485	3	0.478
Vegetation (%)							
Emerged vegetation	26.7 ± 27.1	39.1 ± 37.7	41.6 ± 24.8	36.9 ± 28.6	3.182	3	0.364
Bare substrate	19.3 ± 27.1	19.1 ± 27.1	13.3 ± 17.5	6.3 ± 9.2	2.387	3	0.496
Aquatic plants surface	19.3 ± 25.3	25.0 ± 33.0	30.0 ± 39.5	26.3 ± 33.7	0.820	3	0.845
Plant litter	19.5 ± 28.9	69.1 ± 32.7	50.0 ± 26.8	26.3 ± 26.2	6.474	3	0.910
Aquatic plants substrate	49.2 ± 36.0	14.1 ± 29.6	75.0 ± 39.8	77.5 ± 35.7	12.444	3	0.006
Filamentous algae	37.6 ± 36.4	3.6 ± 9.2	8.3 ± 9.8	5.0 ± 14.1	4.430	3	0.219
Sward height (%)							
<5cm	24.3 ± 26.4	6.3 ± 10.1	45.2 ± 26.5	36.7 ± 20.7	29.507	3	<0.001
5-20cm	28.1 ± 23.3	18.4 ± 23.4	11.2 ± 20.3	16.7 ± 5.2	18.976	3	<0.001
>20cm	47.6 ± 26.7	76.3 ± 27.3	43.6 ± 28.9	46.7 ± 20.7	15.679	3	<0.001

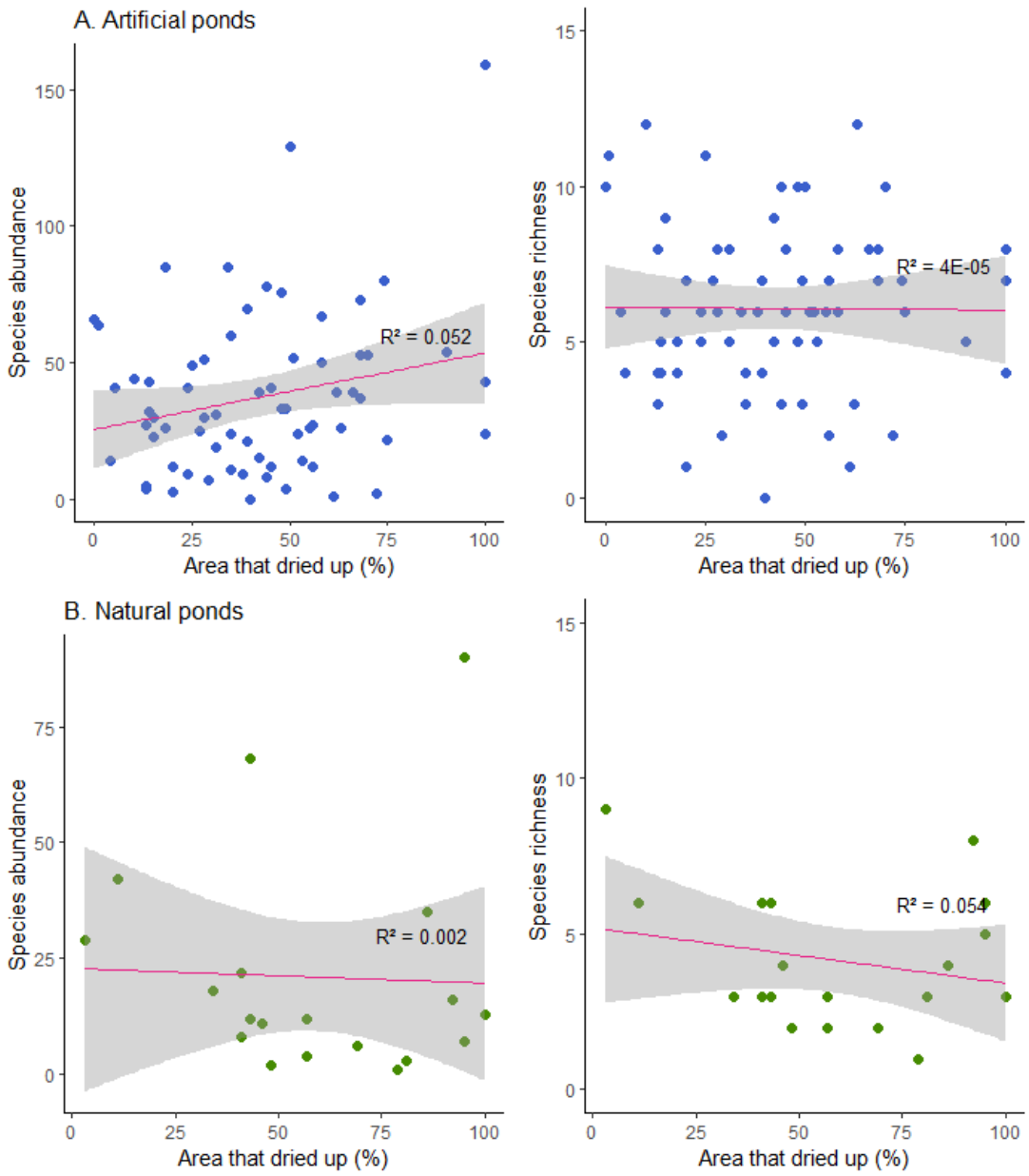


Figure C.3 Linear relationship ±95%CI between species richness/abundance and area of the pond that dried up (%) for **(a)** artificial and **(b)** natural ponds.

Table C.3 Number of permanent and ephemeral ponds per pond type and across habitats.

Habitat type	Natural ponds		Artificial ponds		Total
	Permanent	Ephemeral	Permanent	Ephemeral	
Improved agricultural grassland	0 (0.0%)	2 (2.3%)	36 (41.9%)	23 (26.7%)	61 (70.9%)
Wet grassland	0 (0.0%)	3 (3.5%)	7 (8.1%)	1 (1.2%)	11 (12.8%)
Amenity grassland	7 (8.1%)	1 (1.2%)	0 (0.0%)	0 (0.0%)	8 (9.3%)
Fixed dunes	2 (2.3%)	4 (4.7%)	0 (0.0%)	0 (0.0%)	6 (7.0%)
Total	9 (10.5%)	10 (11.6%)	43 (50%)	24 (27.9%)	86 (100%)

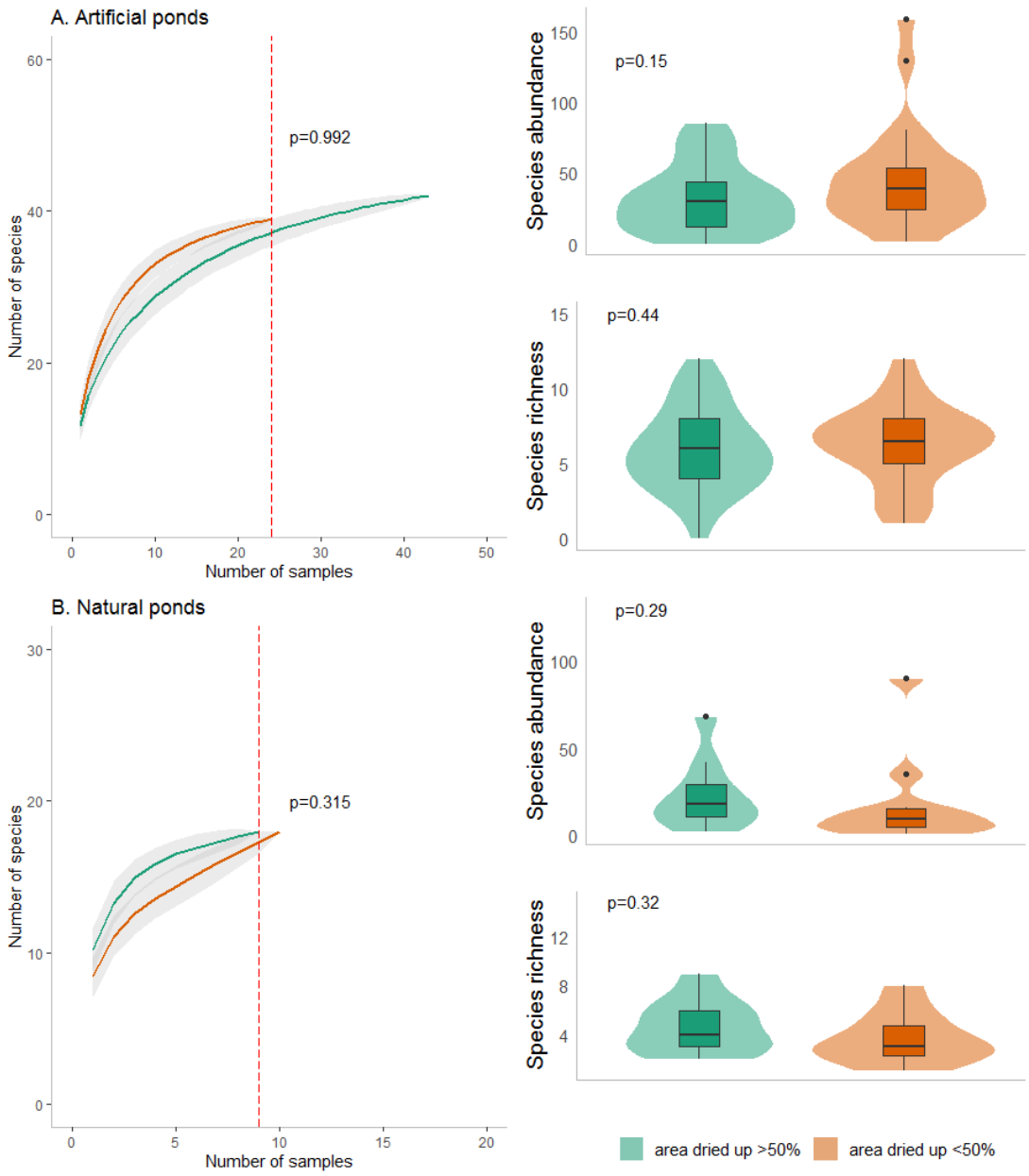


Figure C.4 Sample-based rarefaction curves and violin plots of aquatic species richness and abundance between **(a)** natural ponds that were largely ephemeral (>50% surface dried) or permanent (<50% surface dried) and **(b)** artificial ponds.

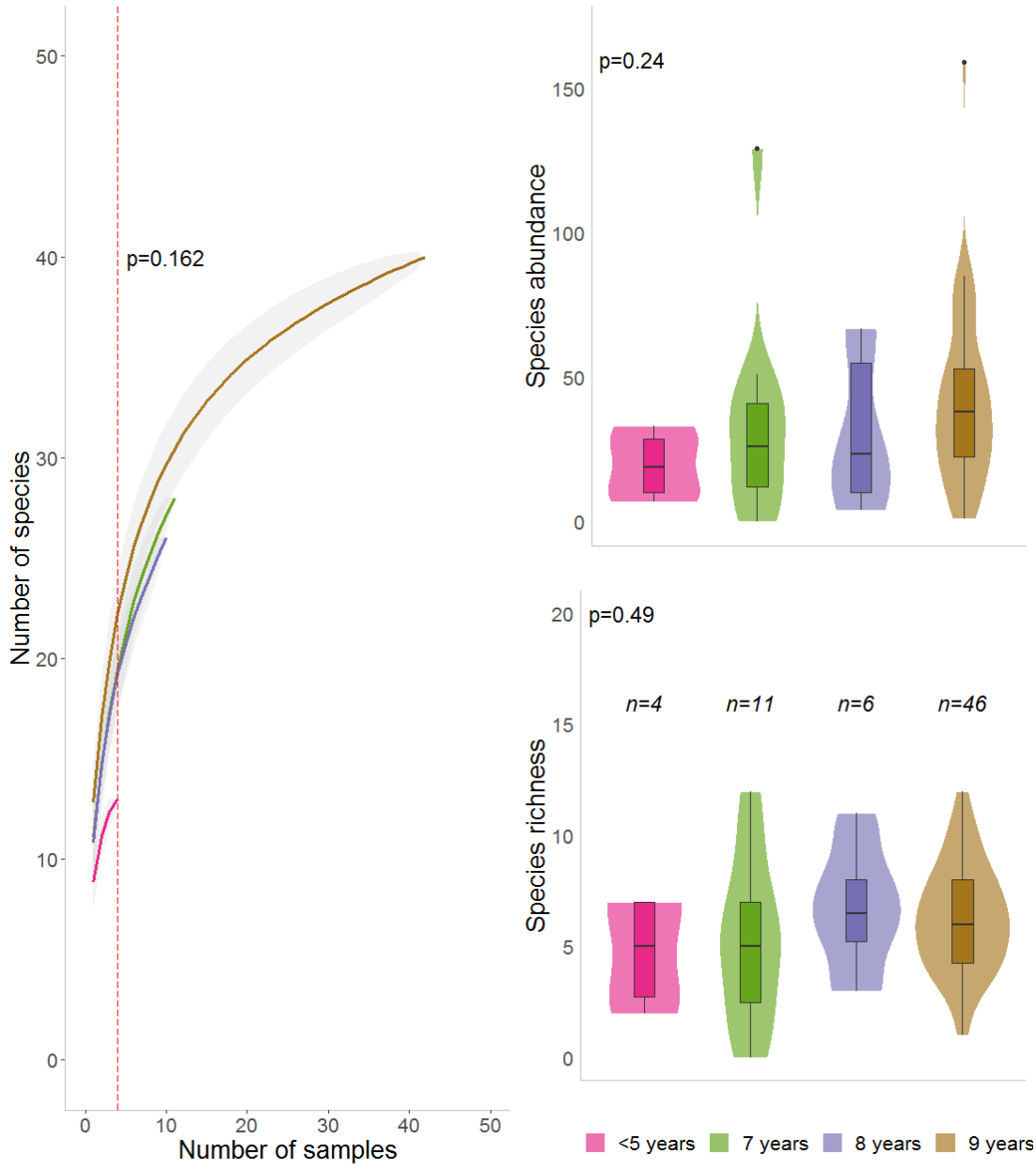


Figure C.5 Sample-based rarefaction curves and violin plots of aquatic species richness and abundance between ponds of different age.

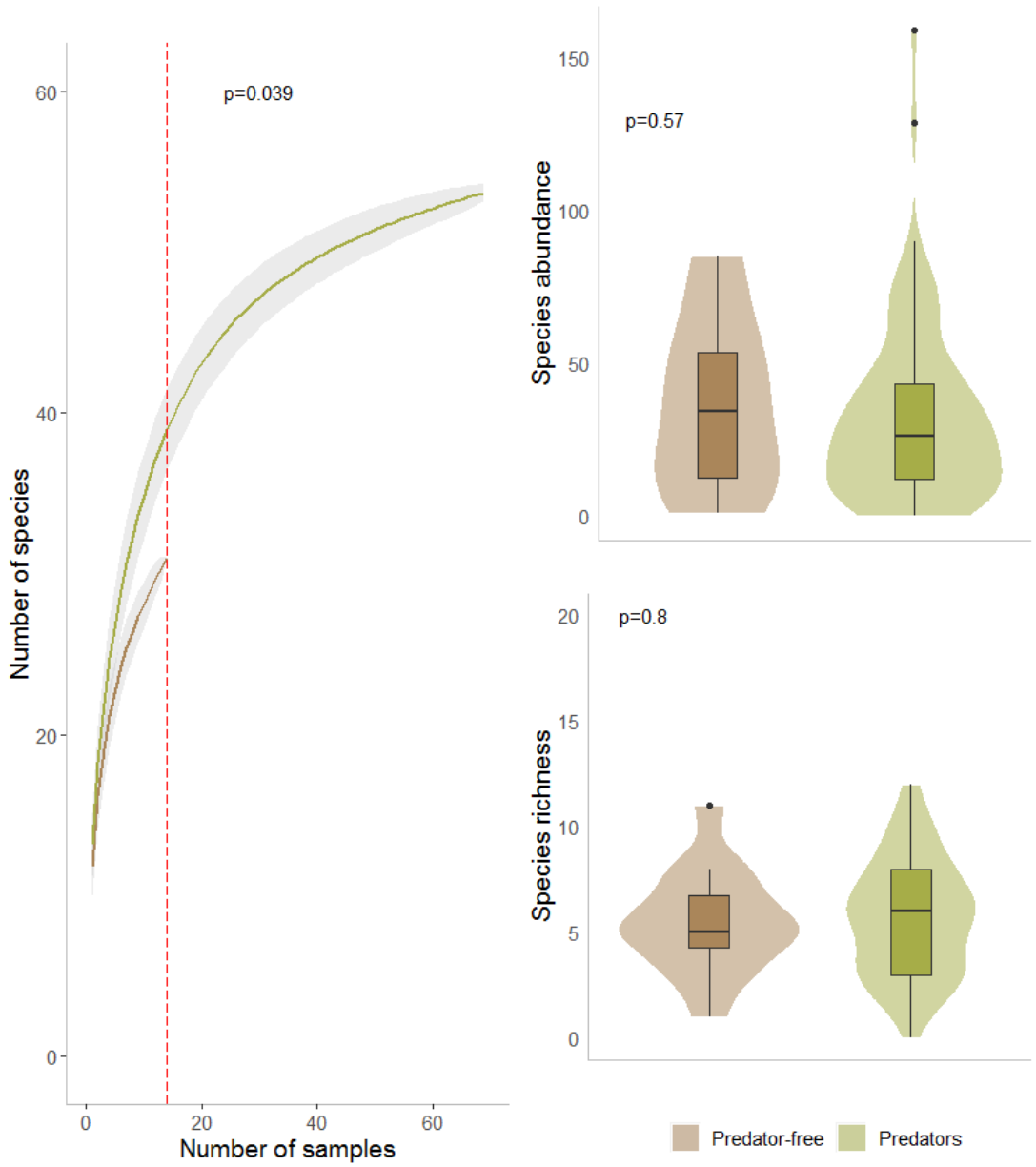


Figure C.6 Sample-based rarefaction curves and violin plots of aquatic species richness and abundance between ponds with and without predators.

Table C.4 PERMANOVA results of the pair-wise comparison tests of invertebrate communities between pond and habitat types.

Groups	t	Unique perms	P(perm)
a) Pond type			
Aquatic macroinvertebrates			
Natural vs artificial	1.907	999	0.001
Spider species			
Natural vs artificial	12.153	999	0.001
b) Habitats			
Aquatic macroinvertebrates			
Improved agricultural land vs Wet grassland	1.232	999	0.144
Improved agricultural land vs Fixed dunes	1.570	998	0.006
Improved agricultural land vs Amenity grassland	1.818	999	0.001
Wet grassland vs Fixed dunes	1.257	960	0.138
Wet grassland vs Amenity grassland	1.466	988	0.023
Fixed dunes vs Amenity grassland	1.696	829	0.002
Spider species			
Improved agricultural land vs Wet grassland	2.035	996	0.001
Improved agricultural land vs Fixed dunes	3.212	999	0.001
Improved agricultural land vs Amenity grassland	2.879	997	0.001
Wet grassland vs Fixed dunes	2.982	999	0.001
Wet grassland vs Amenity grassland	2.495	999	0.001
Fixed dunes vs Amenity grassland	1.815	999	0.001

Table C.5 Environmental variables that best explained variation in invertebrate communities chosen by Distance-based Linear Model (DistLM). Prop. is proportion of variance explained by each variable.

Variable	Pseudo -F	Prop.	*p*
Aquatic species			
pH	3.185	0.036	0.001
Conductivity	2.487	0.014	0.230
Aquatic plants substrate	2.886	0.033	0.002
Spider species			
<5cm	4.378	0.033	0.001
5-20 cm	2.303	0.017	0.007
>20cm	4.600	0.035	0.001

Discussion

Water permanency

Water permanency has been identified as an important factor affecting macroinvertebrate community structure with higher taxa richness and abundance in permanent ponds than those of ephemeral ponds (Legnouo *et al*. 2014). However, we did not find significant impact of pond permanency on species richness and abundance. Indeed, most of the recorded aquatic invertebrates (*Coleoptera, Odonata, Hemiptera*) are highly mobile species that disperse actively by flight as adults enabling them to easily recolonise ephemeral ponds scattered in a landscape together with permanent water bodies (Sanderson *et al.* 2005). Failure to detect an effect of pond permanency may also have been the result of the measure of ephemerality used: ephemeral ponds were defined as ponds having less than 50% of

their original surface area at the end of the sampling season. This definition does not necessarily mean that ponds classed as ephemeral in the current study completely dried up. Indeed, only four ponds completely dried up during the sampling season which is why a broader definition of ephemerality was used.

Vertebrate predation

It is well-known that vertebrate predators are important determinants of invertebrate community structure (e.g. Nyström *et al.* 2001; Batzer *et al.* 2000). Predators like fish have been shown to alter the composition of aquatic macroinvertebrate communities but the effect is not uniform across different taxonomic groups. For instance, fish presence can have a negative relationship with *Coleoptera* species richness and a positive relationship with *Odonata, Hemiptera* and *Mollusca* species richness (Hinden *et al*. 2005; Hassal *et al.* 2011). Yet in this study no significant effects were found. This may be attributed to the widespread (near ubiquitous) occurrence of vertebrate predators throughout studied ponds: predators (amphibians, fish or both) were recorded in 83% of the surveyed ponds. Besides, predator presence was determined via field observations during invertebrate surveys, but no active sampling have been conducted, hence this may result in underestimation of predator occurrence (Stefanoudis *et al.* 2017). Moreover, a formal quantification of predator density, abundance or relative activity may be needed to capture any effects on pond communities.

Pond age

Ponds are dynamic systems. Hence, heterogeneity of pond invertebrate communities occurs through time and is partly the result of historical events (Jeffries 2011). Invertebrate communities of older ponds are expected to be more diverse. A weak tendency supporting this was observed in the current study, however, this non-significant trend was probably attributable to low statistical power due to small sample size within each age category. Sampled ponds were small making them more susceptible to changes in the surrounding environment (e.g. land use changes, shading history, hydroperiod, management, direct interference) consequently impacting their invertebrate communities (Declerck *et al.* 2006; Mokany et al. 2008; Kneital & Lesson 2010). Thus, snap-shot studies may fail to explain some of the observed heterogeneity as a result of past management practises, natural succession and key historical events (Jeffries 2011, 2012).

Species diversity

Overall, we recorded lower aquatic macroinvertebrate diversity (56 species) in the landscape compared to other studies (e.g. Bloechl *et al.* 2010; Gioria *et al.* 2010). This could be because of exclusion of *Heteroptera* and unidentified larvae of *Odonata* and *Coleoptera* from analysis.

10.4 Appendix D

Chapter 4. Combining spawn egg counts, individual photo-ID and genetic fingerprinting to estimate the population size and sex ratio of an Endangered amphibian.

Protocol D.1 High salt DNA extraction protocol for skin swabs, modified from Miller *et al.* (1988).

1. Centrifuge the swab samples for 30 minutes at full speed (14,000 rpm).

2. Remove the ethanol. Dry the swabs and sampling tubes for 24 hours at room temperature.

3. Add 600 µl of TNES buffer[1] and 20 µl of Proteinase-K (>600 mAU/ml) to the swabs. Mix the samples by inverting the tube.

4. Incubate the samples for 12 hours at 54˚C.

5. Remove the swabs.

6. Add 167 µl of 6M NaCl and centrifuge the samples at full speed (14,000 rpm) for 5 minutes. 7. Remove supernatant to a new 1.5 ml micro centrifuge tube.

8. Add 800 µl ice cold 100% ethanol (C_2H_6O) and centrifuge at full speed (14,000 rpm) for 15 minutes.

9. Remove the supernatant and keep the DNA pellet.

10. Add 500 µl of 100% ethanol (C_2H_6O) at room temperature and centrifuge at 6,000 rpm for 1 minute.

[1] Solution recipes for 100 ml TNES buffer: 5 ml TRIS (1M, 7.5 pH), 8 ml NaCl (5M), 20 ml EDTA (0.5M) and 10 ml SDS (0.5%). Top up with dH_2O, vortex and autoclave the solution.

11. Repeat steps 9 and 10.

12. Leave the samples to dry for 2 hours at room temperature and re-suspend the DNA in 50 µl dH_2O.

Protocol D.2 Ethanol clean-up**.**

1. To 15 µl extracted DNA add 1.5 µl 3M Sodium acetate and 60 µl 100% ice-cold ethanol.

2. Vortex the samples and incubate at -20°C for 15min.

3. Centrifuge at full speed (14,000rpm) for 30min.

4. Remove the supernatant and add 200 µl ice-cold 75% ethanol.

5. Centrifuge at full speed (14,000rpm) for 10min.

6. Remove the supernatant, leave samples to dry for 2 hours at room temperature and re-suspend the DNA in 50 µl dH_2O.

Table D.1 Characterization of the seven microsatellite markers used in the study. *A* is the number of alleles and C_F is the final primer concentration in the PCR reaction.

Locus	Repeated structure	Primers (5'- 3')	Allelic size range (bp)	*A*	C_F	Dye	GenBank	Reference
BC02	$(GATA)_{14}$	F: TTGCTTGAGAAAAGTCCAACA	191 - 218	7	0.3µM	VIC	KX237585	Faucher *et al*. 2016
		R: ACTTGCCAACTCTCCCAGAA			0.3µM			
BC08	$(TAGA)_{11}$	F: CTCTTGTGCAAGATCTCTGGG	241 - 279	9	0.1µM	FAM	KX237574	Faucher *et al.* 2016
		R: TACTGACTGCTGCCCTCTCC			0.1µM			
BC22	$(ATCT)_9$	F: TGCAGATTGCCAGCAGTTTA	314 - 339	10	0.1µM	FAM	KX237578	Faucher *et al.* 2016
		R: CACTTCCTCAAGGTGGTGCT			0.1µM			
Bcalµ1	$(AT)4(GT)_{22}$	F: TGGGAATCCTTAGTGGTGAGCC	122 - 138	11	0.1µM	VIC	X99281	Rowe *et al.* 1997
		R: TGAACCCATCTTGTAAATGGCC			0.1µM			
Bcalµ3	$(TC)_{21}$	F: TGGGTGTCATGTTAGATTCCC	109 - 129	13	0.3µM	FAM	X99283	Rowe *et al.* 1997
		R: TGGACACTATTTGGGACTTGC			0.3µM			
Bcalµ8	$(CT)_6GT(CT)_4GT(CT)_{24}ATAC(AT)_7$	F: TGCTAGGGAATAACTGGAGAGC	153 - 179	12	0.3µM	NED	X99288	Rowe *et al.* 1997
		R: GTGAACAGAAATGGTTTAGGGC			0.3µM			
Bcalµ11	$(AG)_{14}$	F: TCATAGGTCAGTGGAAAGAGCA	165 - 193	12	0.1µM	FAM	AF267240	Rowe *et al.* 2000
		R: CGTCAACTTCAATTCGCTCA			0.1µM			

Table D.2 Genotyping error rate at 7 loci estimated with PEDANT version beta 1.3 with 10,000 search steps for enumerating each error rate. ε1 is the rate of allelic dropout (ADO), ε2 is the rate of false alleles (FA), GER is genetic error rate and GS is genotyping success.

Locus	Sample size	ε1	ε2	GER	GS
BC02	320	0.039	0.000	0.039	40.31
BC08	320	0.025	0.000	0.025	26.56
BC22	320	0.000	0.000	0.000	8.44
Bcalμ1	320	0.008	0.000	0.008	41.88
Bcalμ3	320	0.049	0.005	0.054	39.69
Bcalμ8	320	0.015	0.004	0.019	38.13
Bcalμ11	320	0.027	0.000	0.027	37.81

Table D.3 Program RELEASE goodness-of-fit test results for the fully time dependant Cormack-Jolly-Seber model tested on Mark-Recapture data of male Natterjack toads using open population POPAN parameterization in program MARK. $\hat{c}$ is a *post-hoc* variance inflation factor (VIF). TEST2 tested for equal detection, TEST3 for equal survival between sampling events, TEST3.SR tested if the survival depended on wherever an individual was caught previously, and TEST3.SM tested if an individual recapture depended on whether the individual was caught previously. The asterisk indicates significant deviation from the model's main assumption.

Test	**Chi-square**	***d.f.***	***p***	**ĉ**
TEST2	16.0276	10	0.099	1.603
TEST3	19.6078	12	0.075	1.634
TEST3.SR	17.1103	7	0.017	2.444
TEST3.SM	2.4974	5	0.777	0.499
TEST2 + TEST3	35.6354	22	0.033*	1.620

10.5 Appendix E

New records of Natterjack toad (*Epidalea calamita*) natural breeding sites in Ireland.

A manuscript based on this study is published as:

Reyne M, McFarlane C, Marnell F, Helyar SJ, Reid N (2020) New records of Natterjack toad (*Epidalea calamita*, Laurenti 1768) natural breeding sites in Ireland. *Herpetology Notes* **13**, 479-482.

The Natterjack toad (*Epidalea calamita*) has a wide distribution throughout Europe, ranging from the Iberian Peninsula to the Baltic coast with several isolated populations in Great Britain and Ireland (Gasc *et al.* 1997). Despite its widespread distribution, the conservation status for this species has been assessed as 'unfavourable' throughout most European populations (European Topic Centre 2012). In Ireland, the Natterjack toad is at the extreme western edge of its range and is regionally IUCN Red Listed as 'Endangered' (King *et al.* 2011). It is highly range restricted in Ireland confined to the south-west of the country in County Kerry and one small introduced population to the south-east of the country in County Wexford The latest conservation assessment suggests that the population is declining (Reyne *et al.* 2019) mostly likely due to the degradation of suitable breeding sites (Beebee 2002). Ireland lost over half its farmland ponds during the 20th century associated with agricultural intensification and large-scale land drainage schemes that destroyed

amphibian breeding habitat (Reid *et al.* 2014). Natterjack toads are presently restricted to seven discreet sites representing metapopulations (named: Magherees, Inch, Roscullen, Dooks, Yganavan, Glenbeigh and Caherdaniel) (Beebee 2002).

The Natterjack toad is listed under Annex IV of the EU Habitat and Species Directive (92/43/EEC) with EU member states required under Article 17 to report regularly to the European Commission on species' population size and trend. At intervals of roughly 6 years, Ireland's National Parks & Wildlife Service commissions monitoring and surveillance of all known breeding sites. Field surveys occur over 2-3 consecutive years where spawn is recorded every 2 weeks from April to July, to coincide with the breeding season, thereby enabling estimation of the breeding population size (Bécart *et al.* 2007; Sweeney *et al.* 2013; Reyne *et al.* 2019). As part of this program, we conducted extensive field searches for new natural breeding sites from 2016 to 2018 in order to update the species known range in Ireland. Surveys were conducted in County Kerry including the known species occurrence range as well as suitable areas (sand dunes, coastal grasslands and marshes) outside the distribution range. All newly discovered breeding sites were included in the annual survey and visited every 2 weeks after the initial discovery. The perimeter of each potentially suitable water body was surveyed for presence of egg strings by walking slowly along the shore and conducting zigzag transects across shallow water. Sweep netting was used to determine presence of tadpoles. We collected tissue samples from each site where Natterjack toad eggs and tadpoles were detected in order to confirm the species. Samples were stored in 100% ethanol until extraction, which was carried out following a high salt protocol (Miller *et al.* 1988). A 710bp fragment of the

mitochondrial cytochrome c oxidase subunit I gene (COI) was amplified using LCO1490 and HCO2198 primers (Folmer *et al.* 1994). The polymerase chain reaction and cycling program followed the original protocol (Folmer *et al.* 1994) but the annealing temperature was increased to 46°C to reduce nonspecific amplifications. All PCR products occurred at the correct fragment size and were sent to Eurofins Genomics Ltd. for Sanger sequencing. Sequence similarity searches were performed in GenBank BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) and BOLD International System (http://www.boldsystems.org).

All samples were successfully amplified and confirmed to be Natterjack toad. In total, 20 new natural breeding sites were discovered during the 2016-18 field survey (Table E.1, Figure E.1), expanding the known recorded range of the species (at a 2km grid cell resolution) by +19% (with an additional 3 cells occupied) since the last survey during 2011-12 (Sweeney *et al.* 2013). Half of the new locations were recorded from coastal sand dunes systems. Inch sand dunes (52.2806°N, -10.0299°E) has been historically recognised as important breeding area (Beebee 2002), however prior to the survey only three breeding sites were known. The discovery of five new sites with high numbers of egg strings (232 in 2018) and tadpoles (>10,000) highlights the ongoing importance of this location in an Irish context. We extensively searched two other sand dune systems in Co Kerry: Banna strand (52.3375°N, -9.8342°E) and Rossbeigh (52.0682°N, -9.9716°E) but no evidence of breeding was found, probably due to high water salinity (>20ppt) recorded at ponds within the dune slacks. Seven of the new locations were found along the shores of two large lakes: Yganavan Lake

(52.0954°N, -9.8891°E) and Lough Gill (52.2601°N, -10.0450°E), two unusual breeding sites for the species which typically uses shallow ephemeral ponds, avoiding permanent waterbodies where tadpoles may suffer from increased predation and competition (Griffiths *et al.* 1991; Stevens & Baguette, 2008). All lake sites were in small shallow (therefore warm) bays sheltered from wave activity avoiding deep cold water. Breeding activity was also recorded at small temporary puddles formed after heavy rains, where egg string and tadpole survival was likely to be low due to desiccation. A paucity of potentially suitable farmland ponds at the landscape-scale may be a reason for toads selecting habitats that might be otherwise perceived as unsuitable. Continued monitoring of all known breeding locations (including those new locations reported here) will be crucial in determining population trajectories. Maintaining the suitability of breeding sites (preventing ecological succession and controlling the impacts of agricultural intensification) will be necessary to stop further declines.

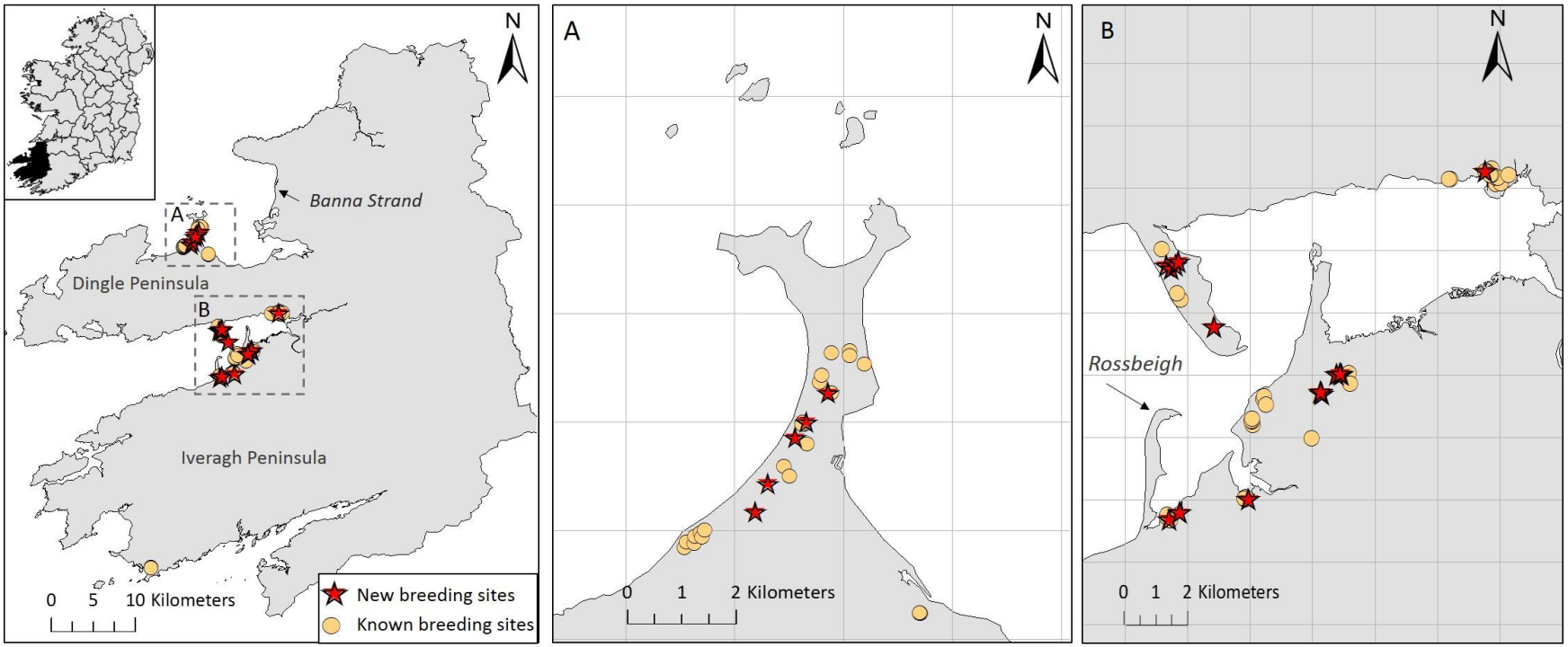


Figure E.1 Map of the known and newly discovered Natterjack toad breeding sites in Ireland (insert highlights County Kerry with a 2km grid). **A)** Magharees sand dune system. **B)** Castlemaine Harbour.

Table E.1 List of newly discovered breeding sites during the 2016-2018 Natterjack toad survey along with their coordinates, date of discovery and type of water body.

Area	Latitude	Longitude	Date	Water body type
Glenbeigh	52.0585	-9.9634	12th April 2016	Temporary puddle
	52.0605	-9.9588	4th June 2018	Temporary puddle
Inch	52.1315	-9.9680	27th May 2016	Temporary pond formed in dune slacks
	52.1306	-9.9659	27th May 2016	Temporary pond formed in dune slacks
	52.1318	-9.9639	27th May 2016	Temporary pond formed in dune slacks
	52.1329	-9.9624	27th May 2016	Temporary pond formed in dune slacks
	52.1142	-9.9449	7th May 2016	Temporary pond formed in dune slacks
Iveragh Quarry	52.9631	-9.9667	5th April 2016	Temporary puddle
Magharees	52.2635	-10.0463	14th April 2016	Lake
	52.2836	-10.0276	8th May 2018	Temporary pond formed in dune slacks
	52.2682	-10.0430	25th April 2016	Temporary pond formed in dune slacks
	52.2786	-10.0332	9th June 2016	Temporary pond formed in dune slacks
	52.2760	-10.0360	25th April 2016	Temporary pond formed in dune slacks
Roscullen Island	52.1611	-9.8201	25th May 2017	Drainage ditch
Yganavan Lake	52.0962	-9.8941	6th April 2016	Lake
	52.0957	-9.8939	27th April 2016	Lake
	52.1015	-9.8849	27th April 2016	Lake
	52.1014	-9.8871	27th April 2016	Lake
	52.1017	-9.8858	24th May 2016	Puddles formed in wheel tracks
	52.0962	-9.8944	5th May 2016	Lake

10.6 Appendix F

Chapter 5. Population genetic structure of the Natterjack toad (*Epidalea calamita*), in Ireland to inform conservation management.

Protocol F.1 High salt method for DNA extraction

All working surfaces, buffers and equipment were daily cleaned before starting laboratory work by using UV lamp, flame, autoclave and a high-level disinfectant (ChemGene HLD$_4$L).

Phase 1: DNA extraction

1. A small amount of tissue was placed in a sterile 1.5ml microcentrifuge tube with 600µl of TNES buffer[2] and 20µl Proteinase-K (Qiagen ™).
2. Samples were incubated at 56°C for 2 hours or until full digestion.
3. Samples were vortexed.
4. 167µl of 6M NaCl was added to the sample.
5. Samples were microfuge for 5min at 14,000 rpm speed.
6. Supernatant was moved to a new sterile 1.5ml microcentrifuge tube.

Phase 2: DNA precipitation

1. 800µl of 100% ice-cold ethanol was added to the sample and microfuge for 15min at 14,000 rpm speed.
2. Supernatant was removed and the formed DNA pellet kept.

[2] For 100ml TNES buffer: 5ml Tris (1M) pH 7.5, 8ml NaCl (5M), 20ml EDTA (0.5M), 10ml SDS (0.5%). Top up to 100ml with dH_2O, vortex and autoclave.

3. 500µl 100% ethanol (at room temperature) was added to the sample.
4. Samples were microfuge for 1min at 6,000 rpm speed.
5. DNA wash (step 3 and 4) was performed twice.
6. Samples were dried overnight.
7. DNA pellet was resuspended in 50 µl of ddH_2O

Table F.1 Genotyping error rate at 13 loci estimated with PEDANT version beta 1.3 with 10,000 search steps for enumerating each error rate. ε1 is the rate of allelic dropout (ADO) and ε2 is the rate of false alleles (FA). Samples were genotyped three times and results were compared between each set.

Locus name	Sample size	1&2		1&3		2&3		Mean	
		ε1	ε2	ε1	ε2	ε1	ε2	ε1	ε2
BC01	28	0.056	0.000	0.064	0.000	0.032	0.000	0.050	0.000
BC09	28	0.072	0.000	0.089	0.000	0.020	0.000	0.060	0.000
BC11	28	0.139	0.000	0.071	0.000	0.097	0.000	0.102	0.000
BC37	28	0.017	0.000	0.033	0.000	0.017	0.000	0.022	0.000
BC39	28	0.013	0.000	0.000	0.000	0.014	0.000	0.009	0.000
BC45	28	0.128	0.023	0.074	0.025	0.105	0.026	0.102	0.025
BC02	28	0.000	0.000	0.046	0.000	0.000	0.000	0.015	0.000
BC08	28	0.000	0.029	0.000	0.029	0.000	0.019	0.000	0.026
BC22	28	0.000	0.031	0.000	0.033	0.000	0.022	0.000	0.029
Bcalμ1	28	0.000	0.036	0.000	0.033	0.000	0.018	0.000	0.029
Bcalμ3	28	0.039	0.034	0.039	0.034	0.000	0.018	0.026	0.029
Bcalμ8	28	0.061	0.000	0.061	0.000	0.000	0.000	0.041	0.000
Bcalμ11	28	0.022	0.000	0.022	0.000	0.000	0.000	0.015	0.000

Table F.2 Genetic characterization of the Natterjack toads in Ireland at locus level. Sample size (*N)*, total number of alleles (A_T), allelic richness (A_R), private allelic richness (A_P), observed (H_O) and expected *(*H_E) heterozygosity and inbreeding coefficient (F_{IS}) are given for each locus and population. Asterisk indicates significant deviation from the Hardy-Weinberg equilibrium after Bonferroni correction (adjusted α threshold= 0.007).

Locus	***N***	A_T	A_R	A_P	H_O	H_E	F_{IS}
BC01	316	8	6.17	0.17	0.70	0.74	0.04*
BC09	316	6	3.69	0.13	0.46	0.47	0.02
BC11	316	11	8.05	0.35	0.54	0.7	0.24*
BC37	316	9	5.72	0.20	0.48	0.50	0.04*
BC39	316	11	5.29	0.49	0.52	0.54	0.03
BC45	316	8	5.87	0.37	0.41	0.57	0.29*
BC02	316	7	5.16	0.16	0.61	0.64	0.05*
BC08	316	9	3.64	0.30	0.41	0.37	-0.08
BC22	316	10	6.31	0.35	0.56	0.63	0.11*
Bcalμ1	316	11	7.05	0.40	0.82	0.70	-0.17
Bcalμ3	316	13	6.69	0.50	0.46	0.58	0.21*
Bcalμ8	316	12	6.88	0.40	0.61	0.64	0.05*
Bcalμ11	316	12	5.80	0.47	0.47	0.52	0.10*
Mean	**-**	**10**	**5.87**	**0.33**	**0.54**	**0.59**	**0.07***

Table F.3 Results of the linkage-disequilibrium (LD) analysis. 28 out of 78 comparisons had *p* values less than 0.05. Only significant results after Bonferroni are shown in the table.

Locus 1		**Locus 2**	***p* value**
BC01	x	BC11	0.004
BC01	x	BC39	0.003
BC01	x	BC45	0.001
BC01	x	Bcalμ1	0.018
BC01	x	Bcalμ11	0.020
BC01	x	Bcalμ8	0.028
BC02	x	Bcalμ1	0.007
BC02	x	Bcalμ3	0.021
BC02	x	Bcalμ8	0.023
BC08	x	BC22	0.001
BC08	x	Bcalμ1	0.031
BC08	x	Bcalμ11	0.039
BC09	x	BC37	0.030
BC11	x	BC02	0.029
BC11	x	BC37	0.044
BC11	x	BC39	0.011
BC11	x	BC45	0.011
BC11	x	Bcalμ1	0.003
BC11	x	Bcalμ3	0.047
BC22	x	Bcalμ1	0.009
BC22	x	Bcalμ3	0.015
BC39	x	BC08	0.032
BC39	x	BC22	0.039
BC39	x	BC45	0.008
BC45	x	Bcalμ1	0.008
Bcalμ1	x	Bcalμ3	0.048
Bcalμ3	x	Bcalμ11	0.001
Bcalμ8	x	Bcalμ1	0.001

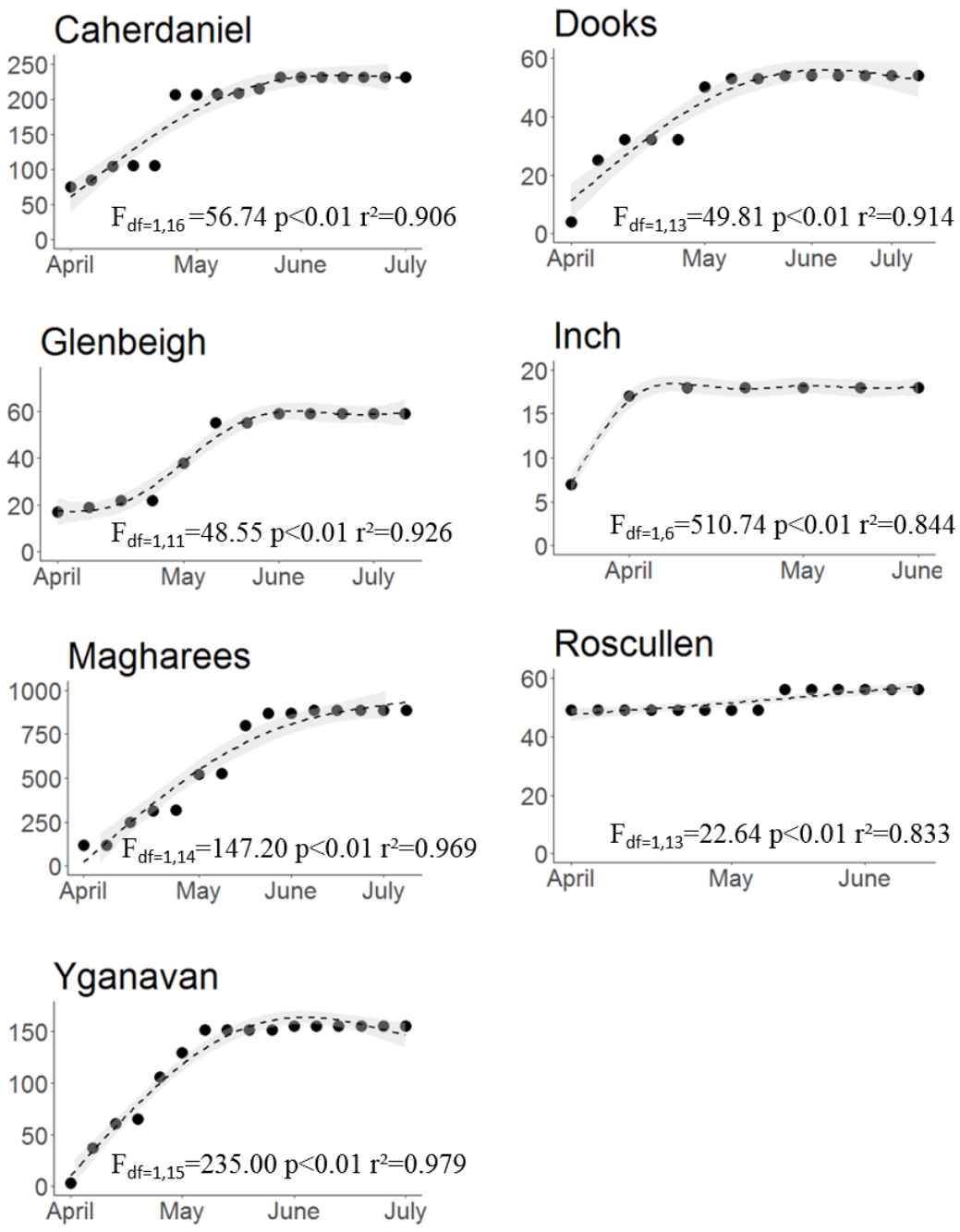


Figure F.1 Cumulative egg string counts / minimum number of females from weekly surveys from April-July for seven Natterjack toad populations in Ireland. Dashed lines represent a *Generalized Additive Model* (GAM) and shading its 95% Confidence Intervals. GAM results are shown for each graph.

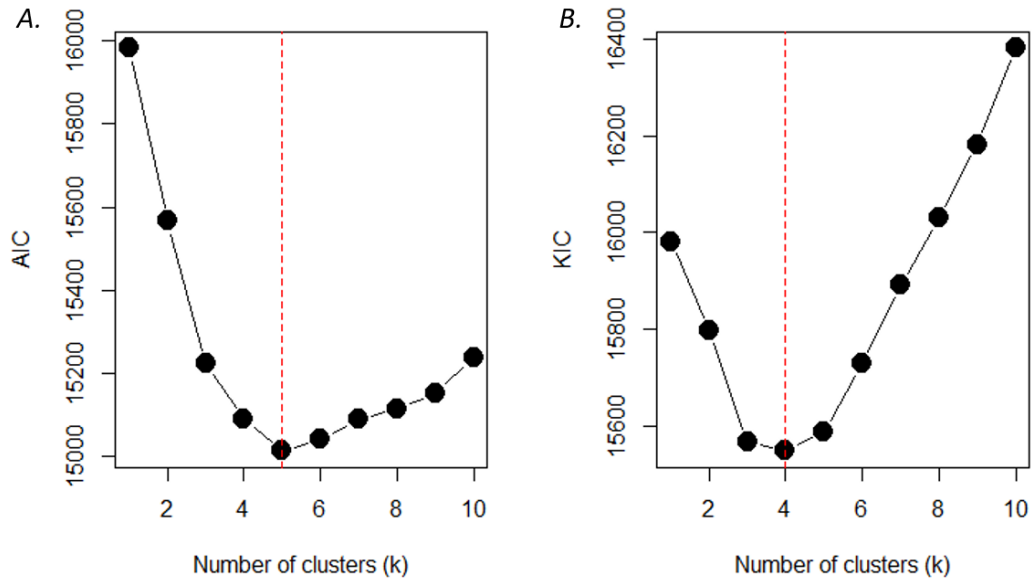


Figure F.2 Selection of the optimal number of genetic clusters (K) on the basis of a) AIC and b) KIC criteria for the Natterjack toad population in Ireland. The optimal K is indicated by the dashed red lines.

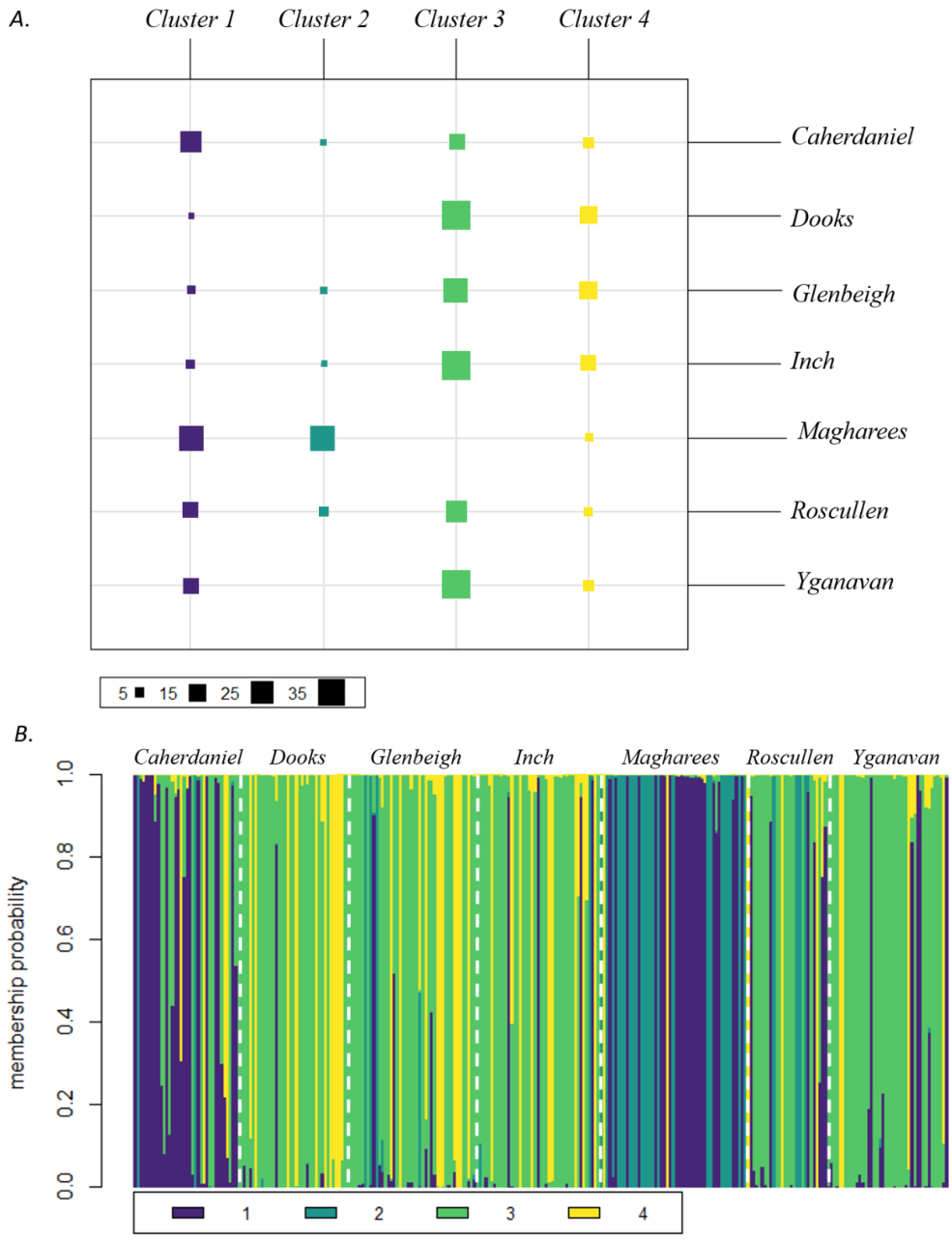


Figure F.3 Population genetic structure based on the analysis of 316 Natterjack toads from seven population in Co. Kerry, Ireland using *snapclust* analysis. a) Contingency table with population assignment to four genetic clusters identified in the analysis (K=4); b) diagram with assignment of individuals from different populations to clusters shown as different colours. Each bar represents an individual with the height of the column segments showing the probability of being assigned to one of the four clusters. Individuals are grouped according to their population of origin.

Figure F.4 Summary of the population genetic studies on the Natterjack toad using microsatellite markers described by Rowe et al. (1997, 2000), Rogell et al. (2005) and Faucher et al. (2016). Sample size (*N*), average number of alleles per locus (*A*), allelic richness (A_R) independent of sample size, observed (H_O) and expected (H_E) heterozygosity, - data was not reported in the study.

Country	Population	*N*	*A*	A_R	H_O	H_E	Microsatellite markers	Reference
Belgium	Frasnes-lez-Couvin	43	3.67	-	0.65	-	Bcalμ1, Bcalμ2, Bcalμ5-7, Bcalμ10	Stevens *et al.* 2006
	Mariembourg	33	3.83	-	0.45	-		
	Merlemont	33	3.67	-	0.48	-		
	Romedenne	28	3.83	-	0.57	-		
Denmark	Avernakø	40	-	2.11	-	0.35	Bcalμ1 - 8, Bcalμ10, Bcalμ11, Buca5, Buca6	Allentoft *et al.* 2009
	Fyns Hoved East	39	-	2.43	-	0.41		
	Fyns Hoved West	40	-	2.47	-	0.43		
	Espe	40	-	2.52	-	0.38		
	Munke Bjergby	38	-	2.6	-	0.18		
	Dybsø	31	-	2.06	-	0.31		
	Honum	41	-	2.16	-	0.32		
	Hedensted	39	-	2.77	-	0.36		
	Råbjerg Mile	40	-	2.06	-	0.30		
	Grærup	35	-	1.81	-	0.30		
	Harboøre	39	-	2.24	-	0.27		
	Bygholm Vejle	20	-	1.89		0.23		
Switzerland	PopD	68	-	2.51	0.59	0.59	Bcalμ1 - 8, Bcalμ10, Buca1, Buca2, Buca5, Buca6	Gomez-Mestre & Tejedo 2004
	PopE	24	-	2.37	0.55	0.55		
	PopF	36	-	2.45	0.56	0.57		
	PopG	16	-	2.46	0.56	0.58		
	PopH	41	-	2.40	0.55	0.55		
	PopI	9	-	2.39	0.56	0.54		
	PopJ	21	-	2.43	0.61	0.55		
	PopK	48	-	2.45	0.58	0.56		
Sweden	Bohuslän islands	180	-		0.11	-	Bcalμ1-4, Bcalμ6-11, Buca1-3, Buca5	Rogell *et al.* 2010

Table F.4 cont.

Netherlands	Ooy-Polder	40	5.13	-	0.47	0.52	Bcalμ1 - 8	Beebee & Rowe 2000
	Texel	40	2.63	-	0.43	0.37		
France	Brittany	32	4.38	-	0.36	0.49		
	Boulogne	15	3.88	-	0.46	0.46		
UK	Merseyside	40	2.63	-	0.30	0.29		
Poland	-	40	2.00	-	0.28	0.25		
Spain	Velez	11	4.88	-	0.61	0.69		
Sweden	Continental	40	1.63	-	0.14	0.12		
UK	Cumbria	40	3.75	-	0.34	0.39		Rowe & Beebee 2004
	Easr/south-east	40	2.50	-	0.29	0.35		
	sampling year 1994							
	Ainsdale	40	-	3.00	0.30	0.31		
	Saltfleetby	40	-	2.33	0.22	0.19		
	sampling year 2000							
	Ainsdale	80	-	3.02	0.31	0.29		
	Saltfleetby	80	-	2.33	0.19	0.18		
	Gibraltar point	80	-	2.33	0.22	0.18		
Spain	South-west/North	53	-	7.05	-	0.807	Bcalμ1 - 8	Rowe *et al.* 2006
Ireland	Yganavan	39	-	2.64	0.42	0.44	Bcalμ1-5, Bcalμ8, Bcalμ11, Buca1, Buca2	May & Beebee 2010
	Glenbeigh	36	-	2.89	0.43	0.43		
	Roscullen	40	-	2.22	0.39	0.39		
	Inch	40	-	2.42	0.3	0.31		
	Castlegregory	40	-	1.86	0.34	0.35		
	Tullaree	40	-	1.89	0.29	0.29		
France	North Coastline	273	3.55	3.11	0.48	0.48	Bcalμ1 - 11, Buca1 -6, BC01-46	Faucher *et al.* 2017
	Northern Coalfield	686	3.52	3.22	0.52	0.51		
	Eastern France	59	3.66	3.34	0.44	0.50		
	Lorraine	10	3.09	3.45	0.50	0.49		
	Alps	21	4.34	3.17	0.50	0.58		
	Lot	20	3.91	2.23	0.53	0.56		
	Western France	68	3.02	2.68	0.40	0.41		
Switzerland	-	17	3.11	1.92	0.44	0.49		
Sweden	-	10	1.49	3.32	0.14	0.16		

10.7 Appendix G

Three primer method

Tail primer method was selected to label DNA fragments with fluorescent dye as a cost-effective alternative to directly labelling locus-specific primers for microsatellite analysis (Margulies *et al.* 2005). This method uses modified two-part primers (tailed-forward primer) in which universal tail is added to the 5'-end of the forward locus-specific primer sequence. Then, the PCR reaction is performed with three primers: forward tailed primer containing a universal tail primer sequence at the 5'-end, reverse locus-specific primer and universal tail primer labelled with a fluorescent dye (Steffens *et al.* 1993; Oetting *et al.* 1995; Neilan *et al.* 1997; Schuelke 2000; Missiaggia & Grattapaglia 2006). This method has been widely used on a variety of taxonomic groups (for instance amphibians *Hale et al.* 2011; plants James *et al.* 2011; molluscs Miller *et al.* 2011) especially in small molecular projects where budget is a limiting factor and has been shown to have similar PCR efficiency to using directly labelled primers (Blacket *et al.* 2012). Four universal primers: Tail A, Tail B, M13 and T7 were added to 25 forward primers specifically developed for the Natterjack toad (Table E.1) and descried by Rowe *et al.* (1997, 2000), Rogell *et al.* (2005) and Faucher *et al.* (2016). Tails were labelled with 6-FAM™, NED™, VIC™ and PET™ fluorescent dye (Applied Biosystems). During the initial cycles the newly synthesis DNA was a result of the forward tailed primer and reverse primer and contained the tail sequence incorporated into the PCR product by the tailed-forward primer (Step 1 and 2, Figure G.1). During the amplification after the depletion of the modified forward tailed

primer as a result of a lower starting concentration, the labelled universal tail primer took over the amplification creating PCR product bearing the fluorescent dye that can be detecting by ABI 3730xl Genetic Analyzer (Step 3 and 4, Figure G.1).

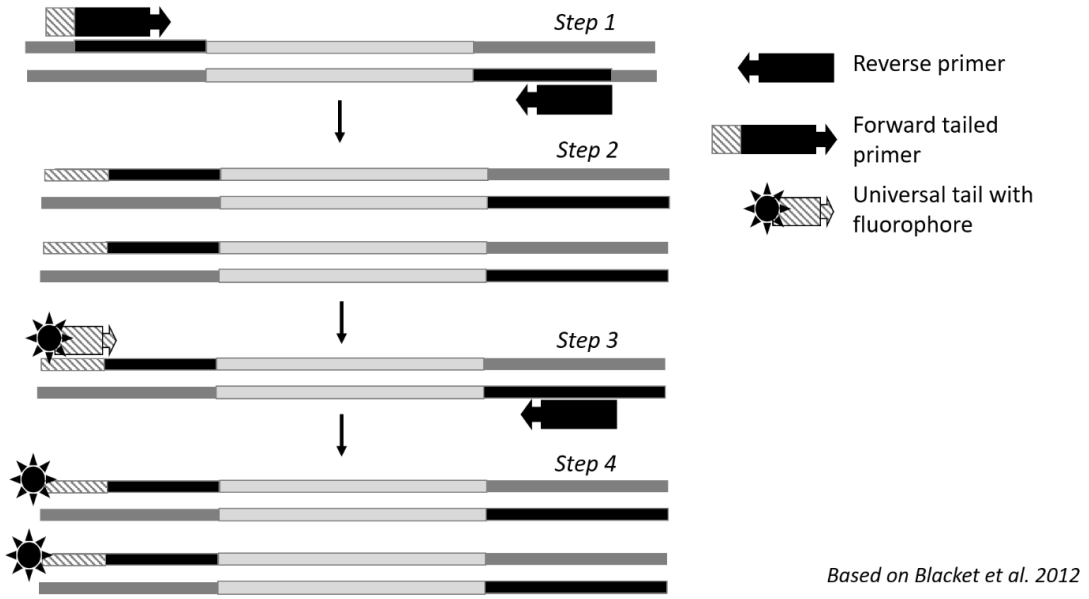


Figure G.1 Amplification of DNA fragment with forward tailed primer and fluorescently labelled universal primer.

All primers were initially tested in single plex PCR reactions before running multiplexes. Assay was optimised via temperature gradient PCR and visualization on 1% agarose gel stained with ethidium bromide and under UV light. PCR reactions were performed at four multiplexes with multiple tail primers labelled with fluorescent dye and size overlapping markers. Concentrations of the primers in the final reaction were optimized. We run 10µl reactions containing 1µl of genomic DNA, 5µl Type-it Multiplex PCR Master Mix (Qiagen), 1µl primer mix of labelled tail, tailed-forward and reverse primers (final concentrations at 0.2µM, 0.05 µM and 0.2µM, respectively) and 3µl ddH_2O.

Primer performance was evaluated under three different PCR cycling programs. The first PCR program had two steps: first step for incorporating the tail into the PCR product and second step for the labelled universal tail to take over the amplification and incorporates the fluorescent dye into the final PCR product. The program started with initial denaturation at 95°C for 15min, step one consisted of 13 cycles of 94°C for 30sec, Ta (55°C or 58°C) for 90sec, 72°C for 60sec, step two consisted of 31 cycles of 94°C for 30sec, 50°C for 90sec and 72°C for 60sec, and final extension at 72°C for 30min. The second PCR cycling program followed the manufactures recommendations (Type-it Qiagen multiplex kit) and had an initial denaturation of 95°C for 15min; 35 cycles of 94 °C for 30sec, annealing temperature of 55°C or 58°C for 90sec, and 72°C for 60sec, and final extension at 60°C for 30min. Finally, for the third option we performed two separate PCRs. First PCR reaction was run with the tailed-forward primer and reverse locus-specific primer. Then, we added the tail primer and run a second PCR reaction in order to increase the primer specificity. The cycling program followed that of a two-step PCR protocol described above.

Table G.1 Characterization of thetwenty-five microsatellite markers used in the three primer method

Locus	Tail	Tail (5' - 3')	Tail size	Dye	Modified forward primer and reverse primer (5' - 3')	Multiplex	Ta (C°)
BC01	T7	TAATACGACTCACTATAG	18	PET	F: TAATACGACTCACTATAGTCCATAATCAGGCGCTCATA R: TCTATTCTCTTAAACCGGAGAGG	1	55
BC02	T7	TAATACGACTCACTATAG	18	PET	F: TAATACGACTCACTATAGTTGCTTGAGAAAAGTCCAACA R: ACTTGCCAACTCTCCCAGAA	3	58
BC04	M13	TGTAAAACGACGGCCAGT	18	NED	F: TGTAAAACGACGGCCAGTTGCTCCTGACAATTAACTTTGG R: ATCTGTGTCAGGGCATCTCC	2	55
BC05	M13	TGTAAAACGACGGCCAGT	18	NED	F: TGTAAAACGACGGCCAGTCATTGATATGGCTGCCAACTT R: CATGGGGATCAATGGCTACT	1	55
BC08	Tail B	GCCTTGCCAGCCCGC	15	VIC	F: GCCTTGCCAGCCCGCCTCTTGTGCAAGATCTCTGGG R: TACTGACTGCTGCCCTCTCC	1	55
BC09	Tail A	GCCTCCCTCGCGCCA	15	6-FAM	F: GCCTCCCTCGCGCCAGGTGGTGGCACATTTCTTTT R: GTAGTTTGCCAGCAATGCCT	2	55
BC11	Tail B	GCCTTGCCAGCCCGC	15	VIC	F: GCCTTGCCAGCCCGCAGCCTTCTTTGCATCACTGC R: TAGCGGGAAGAGATGTACGC	1	55
BC15	T7	TAATACGACTCACTATAG	18	PET	F: TAATACGACTCACTATAGTGCTCCTCAAGTGTTGTTGG R: TGGGACGACAGGAACGTACT	2	55
BC18	T7	TAATACGACTCACTATAG	18	PET	F: TAATACGACTCACTATAGCCTTAATGGCCCAAGCCTAT R: AGACAGGGATGGATAGATGGA	2	55
BC22	Tail A	GCCTCCCTCGCGCCA	15	6-FAM	F: GCCTCCCTCGCGCCATGCAGATTGCCAGCAGTTTA R: CACTTCCTCAAGGTGGTGCT	1	55
BC24	T7	TAATACGACTCACTATAG	18	PET	F: TAATACGACTCACTATAGACGGTTTTCTGAAGCAATGG R: GCATGTGCAGAAGACTTCAAA	1	55
BC29	M13	TGTAAAACGACGGCCAGT	18	NED	F: TGTAAAACGACGGCCAGTGTTGCGACTGGGGAAATAAC R: GCTTCACAAGACATGCAGGA	1	55
BC37	Tail B	GCCTTGCCAGCCCGC	15	VIC	F: GCCTTGCCAGCCCGCTCACCTGTACCCCTCTGGG R: CCATCCATGACACAGACCAG	2	55
BC39	Tail B	GCCTTGCCAGCCCGC	15	VIC	F: GCCTTGCCAGCCCGCTCTGTCCTTCTGTCCAATCTG R: GCACCTTTGTTCAGGATGGT	2	55
BC45	Tail A	GCCTCCCTCGCGCCA	15	6-FAM	F: GCCTCCCTCGCGCCACCCTTGCAGCCAAAATAAAA R: TAACAGGAAACGGATTTGGG	2	55
BC46	Tail A	GCCTCCCTCGCGCCA	15	6-FAM	F: GCCTCCCTCGCGCCATGAATAGACAGACATTTGTCCAAGA R: TTCTACCGGTCAACCTATCCA	1	55
Bcalμ1	Tail B	GCCTTGCCAGCCCGC	15	VIC	F: GCCTTGCCAGCCCGCTGGGAATCCTTAGTGGTGAGCC	4	58

Table G.1 cont.

					R: TGAACCCATCTTGTAAATGGCC		
Bcalμ11	Tail A	GCCTCCCTCGCGCCA	15	6-FAM	F: GCCTCCCTCGCGCCATCATAGGTCAGTGGAAAGAGCA	4	58
					R: CGTCAACTTCAATTCGCTCA		
Bcalμ2	M13	TGTAAAACGACGGCCAGT	18	NED	F: TGTAAAACGACGGCCAGTTTCGGGGCCTGAGAAGAGG	3	58
					R: AGGGTGAGTGGAGTGACAACCC		
Bcalμ3	Tail A	GCCTCCCTCGCGCCA	15	6-FAM	F: GCCTCCCTCGCGCCATGGGTGTCATGTTAGATTCCC	3	58
					R: TGGACACTATTTGGGACTTGC		
Bcalμ4	M13	TGTAAAACGACGGCCAGT	18	NED	F: TGTAAAACGACGGCCAGTTGTTGGGGCTGATGTCACTA	4	58
					R: CTTTTATAGCCTTTCCCAGGC		
Bcalμ5	Tail A	GCCTCCCTCGCGCCA	15	6-FAM	F: GCCTCCCTCGCGCCAACGTGACACGGAGTAATAGCTG	3	58
					R: TGGAGCCTTTGGAAATGAAC		
Bcalμ8	Tail B	GCCTTGCCAGCCCGC	15	VIC	F: GCCTTGCCAGCCCGCTGCTAGGGAATAACTGGAGAGC	3	58
					R: GTGAACAGAAATGGTTTAGGGC		
Buca1	M13	TGTAAAACGACGGCCAGT	18	NED	F: TGTAAAACGACGGCCAGTATGGATTGTAAGACGCATCTC	2	55
					R: TTTCCTCTGCCGATATGATT		
Buca2	M13	TGTAAAACGACGGCCAGT	18	NED	F: TGTAAAACGACGGCCAGTATCAGAATCTCAGCACATCTACT	1	55
					R: GAGGGCACAGAGGTAGTTC		

Three primer method did not provide reliable data due to presence of non-specific amplification. Examples of PCR products amplified by different primers and cycling programs are illustrated on Figure F.2. A, B and C show presence of additional bands (non-specific amplification) in comparisons to D where the reaction was run with only two primers: directly fluorescently labelled forward and locus-specific reverse primers. Each reaction was locus-specific confirmed by presence of one DNA fragment per reaction at the expected size. Even though, the one step PCR cycling program recommended by Qiagen reduced the amount of non-specific amplifications seen on 1% agarose gel (Fig. 1B), we were still not able to distinguish true allele peaks in GeneMarker V1.8. Hence, the reason why we decided not to use the three-primer method and to directly fluorescently label 13 of the most polymorphic primers with high performance.

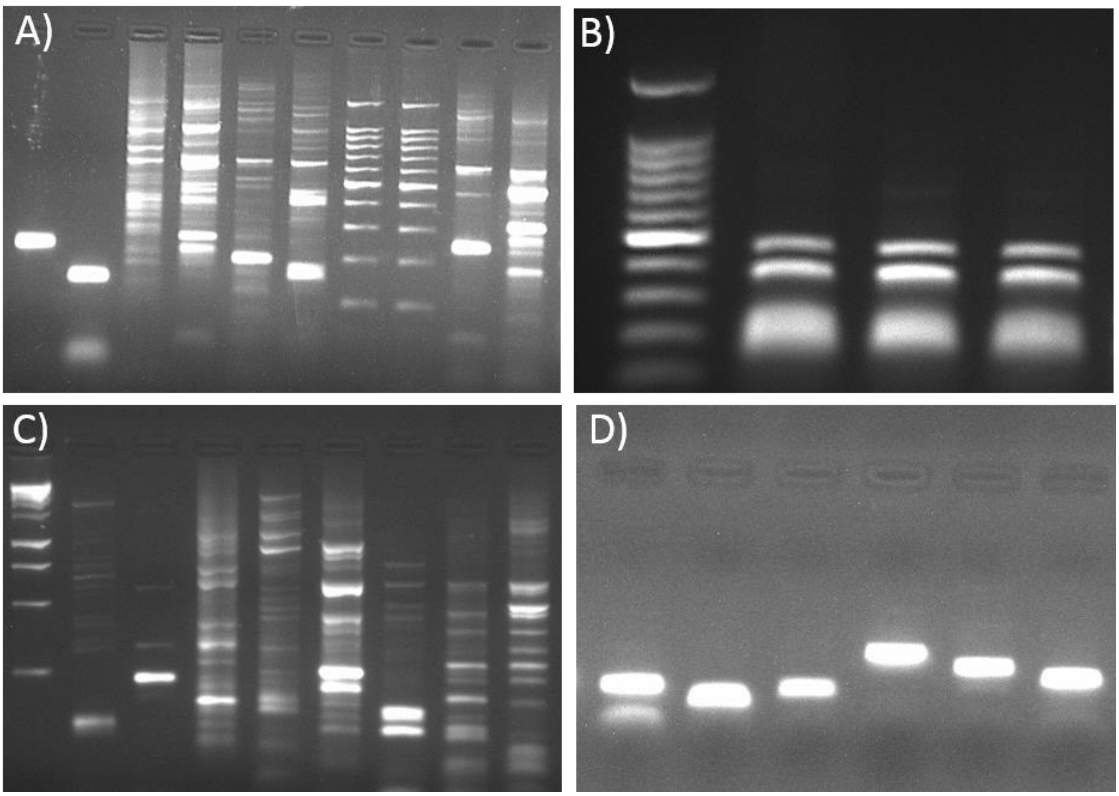


Figure G.2 Comparison of several methods for phuorescently labelling DNA fragments visualised on 1% agarose gel stained with ethidium bromide and under UV light. A) Results of the two-step PCR cycling program for three-primer method (left to right: BC01, BC02, BC08, BC09 BC29, BC45, Lader,Lader,Buca1, Buca2). B) Results of the Quigen recommended cycling PCR program for three-primer method (left to right: lader, BC04, BC22, BC37). C) Results of a PCR run with only universal tails with fluorophor and reverse locus-specific primer for two different tissue samples (left to right: T7 ind1, T7 ind2, Tail A ind1, Tail A ind2, Tail B ind 1, Tail B ind2, M13 ind1, M13 ind2). D) Example of PCR products by directly fluorescently labelled forward and reverse primers (left to right: Bcalμ8, Bcalμ3, Bcalμ1, BC22, BC8 and Bc2).

10.8 Appendix H

Chapter 6. Landscape genetics identifies barriers to Natterjack toad metapopulation dispersal.

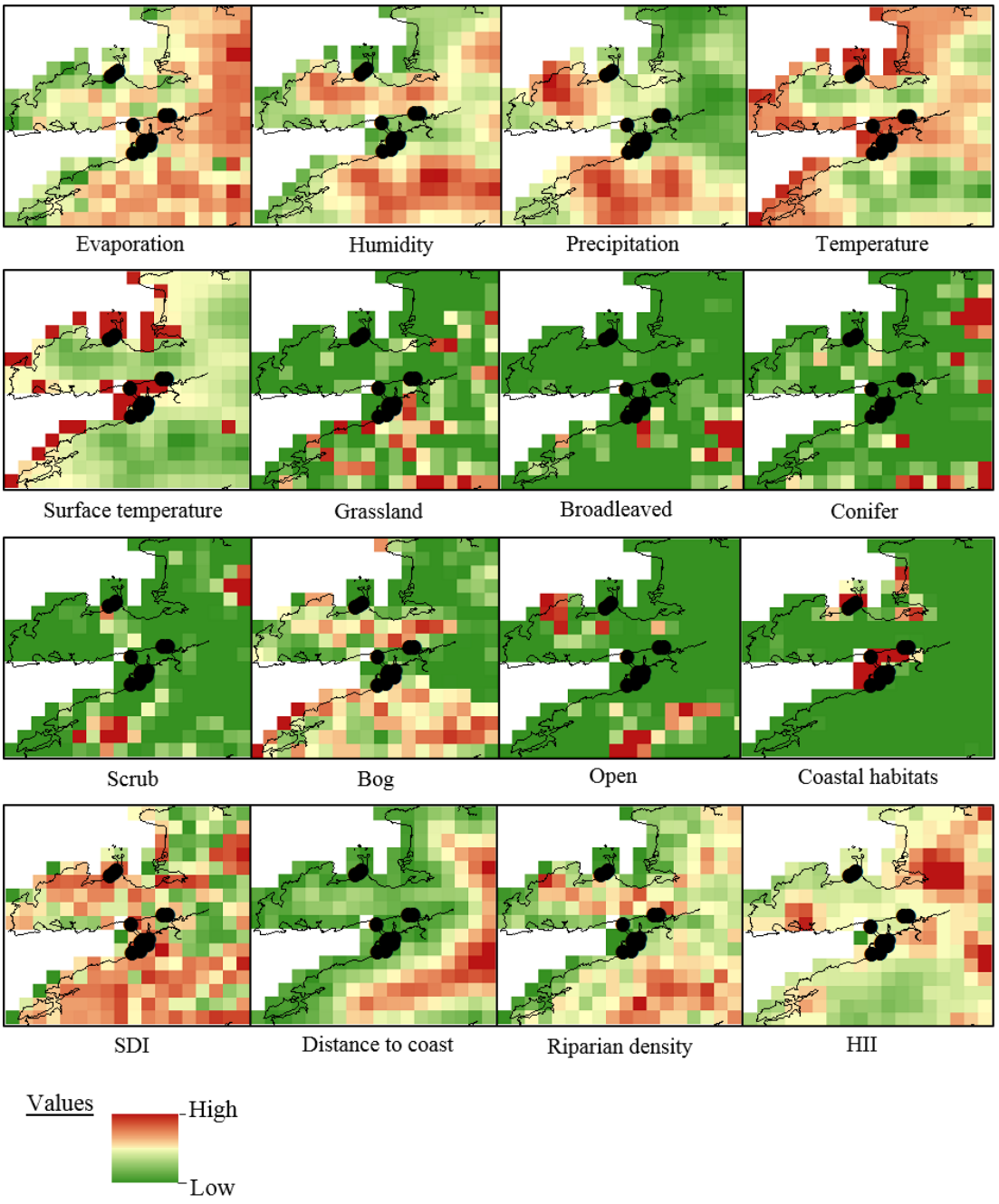


Figure H.1 Sixteen environmental predictors used to create resistance surfaces for the Natterjack toad dispersal and gene flow in Ireland. Black circles indicate breeding sites.

Table H.1 Null allele frequency and genotyping error rate at 13 loci estimated with PEDANT version beta 1.3 with 10,000 search steps for enumerating each error rate. ADO is the rate of allelic dropout and FA is the rate of false alleles. Samples were genotyped three times and results were averaged between each set.

Locus name	ADO	FA	Null allele
BC01	0.020	0.000	0.058
BC09	0.066	0.000	0.013
BC11	0.096	0.000	0.128
BC37	0.139	0.000	0.047
BC39	0.010	0.000	0.021
BC45	0.109	0.017	0.094
BC02	0.018	0.000	0.040
BC08	0.031	0.000	0.020
BC22	0.011	0.000	0.094
Bcalμ1	0.041	0.012	0.000
Bcalμ3	0.046	0.000	0.104
Bcalμ8	0.047	0.000	0.063
Bcalμ11	0.014	0.000	0.066

Table H.2 Pairwise genetic differentiation of Natterjack toad population at 12 metapopulations. F_{ST} genetic distance values are above the diagonal and G''_{ST} genetic distance values are below the diagonal. Significance (*) is shown after Bonferroni correction (α=0.05) and 10,000 test permutations.

	Glenbeigh	Quarry	DGC	Dooks	Nambrackdarrig	Yganavan	Inch	Roscullen	Killeen	CGC	Lough Gill	Magharees
Glenbeigh	-	0.031	0.038*	0.023*	0.020	0.038*	0.040*	0.065*	0.068*	0.082*	0.102*	0.110*
Quarry	0.068*	-	0.037*	0.037*	0.033	0.029*	0.025*	0.051*	0.041	0.053*	0.074*	0.071*
DGC	0.112*	0.147*	-	0.026	0.024	0.037*	0.034*	0.066*	0.076*	0.084*	0.097*	0.121*
Dooks farm	0.158*	0.118*	0.058*	-	0.027	0.023	0.023*	0.039*	0.050*	0.073*	0.090*	0.109*
Nambrackdarrig	0.013	0.075*	0.045*	0.051*	-	0.020	0.029	0.048*	0.055*	0.067*	0.090*	0.105*
Yganavan	0.111*	0.077*	0.128*	0.058*	0.031	-	0.022*	0.039*	0.039*	0.048*	0.074*	0.081*
Inch	0.125*	0.069*	0.103*	0.066*	0.061*	0.049*	-	0.029*	0.043*	0.067*	0.086*	0.093*
Roscullen	0.202*	0.169*	0.223*	0.127*	0.126*	0.101*	0.091*	-	0.034	0.067*	0.087*	0.099*
Killeen	0.205*	0.111*	0.264*	0.154*	0.149*	0.096*	0.127*	0.053*	-	0.038*	0.048	0.054*
CGC	0.272*	0.191*	0.322*	0.281*	0.230*	0.177*	0.243*	0.219*	0.085*	-	0.022	0.024
Lough Gill	0.301*	0.228*	0.320*	0.303*	0.281*	0.251*	0.273*	0.265*	0.099*	-0.001	-	0.020
Magharees	0.371*	0.259*	0.454*	0.420*	0.384*	0.318*	0.343*	0.342*	0.163*	0.057*	-0.010	-

Table H.3 Pairwise matrices of **(a)** Euclidean and **(b)** geographical distances. Current geographical distances values are below the diagonal and historical distances are above the diagonal. Distances are in kilometres.

	Glenbeigh	Quarry	DGC	Dooks	Nambrackdarrig	Yganavan	Inch	Roscullen	Killeen	CGC	Lough Gill	Magharees
(a) Euclidean distances												
Glenbeigh	-	-	-	-	-	-	-	-	-	-	-	-
Quarry	2.52	-	-	-	-	-	-	-	-	-	-	-
DGC	4.05	2.53	-	-	-	-	-	-	-	-	-	-
Dooks	4.82	3.26	0.79	-	-	-	-	-	-	-	-	-
Nambrackdarrig	5.23	2.90	2.02	2.00	-	-	-	-	-	-	-	-
Yganavan	6.31	4.23	2.42	1.86	1.54	-	-	-	-	-	-	-
Inch	8.02	7.92	5.69	5.32	7.27	6.41	-	-	-	-	-	-
Roscullen	15.08	13.08	11.04	10.28	10.24	8.85	10.91	-	-	-	-	-
Killeen	14.04	12.17	10.00	9.22	9.41	7.95	9.46	1.50	-	-	-	-
CGC	23.05	23.37	21.15	20.71	22.55	21.39	15.47	20.28	19.09	-	-	-
Lough Gill	23.39	23.56	21.26	20.77	22.56	21.34	15.64	19.63	18.50	1.46	-	-
Magharees	23.89	23.97	21.64	21.13	22.88	21.62	16.06	19.53	18.46	2.33	0.90	-
(b) Geographical distances												
Glenbeigh	-	2.52	6.00	6.56	5.42	6.96	10.74	28.06	29.53	28.26	28.14	28.57
Quarry	2.52	-	2.97	3.57	2.90	4.44	8.22	22.26	20.92	25.65	25.62	26.19
DGC	6.00	2.97	-	0.79	2.02	2.65	5.69	18.91	17.45	22.14	21.70	22.15
Dooks	6.56	3.57	0.79	-	2.00	1.86	5.32	18.55	16.82	20.79	20.96	21.45
Nambrackdarrig	5.42	2.90	2.02	2.00	-	1.54	7.32	20.72	19.25	23.49	23.49	24.06
Yganavan	6.96	4.44	2.65	1.86	1.54	-	6.41	20.32	18.88	23.67	21.82	24.35
Inch	39.68	37.61	34.69	33.90	33.43	31.86	-	13.42	11.94	15.47	15.64	16.06
Roscullen	25.46	3.57	24.50	21.08	23.52	20.09	13.42	-	1.50	20.56	20.24	20.53
Killeen	23.96	26.44	25.90	22.14	25.12	21.43	11.94	1.50	-	19.50	19.07	19.34
CGC	48.90	49.69	45.06	44.64	44.58	40.87	15.47	20.56	19.50	-	1.46	2.35
Lough Gill	48.28	49.99	44.92	44.41	44.08	40.91	15.64	20.24	19.07	1.46	-	0.90
Magharees	48.56	50.70	45.01	44.68	44.46	40.85	16.06	20.53	19.34	2.35	0.90	-

Table H.4 Mantel tests and single-predictor *least-cost path* ResistanceGA analysis using two measures of genetic distance **(a-b)** and 16 environmental predictors. Surface optimisation was performed twice to check for convergence (Run 1 and Run 2). Environmental predictors are ranked in ascending order of AIC_c values. K is the number of parameters, ΔAIC_c is the difference in the AIC_c values between the best supported model (marked in bold) and each subsequent model; R^2m and R^2c are the marginal and conditional R^2 values of the fitted MLPE model; LL is log likelihood.

	Run 1								*Run 2*							
	Mantel test		**MLPE**						**Mantel test**		**MLPE**					
	R	**p**	**K**	**AICc**	**ΔAIC_c**	**R2m**	**R2c**	**LL**	**R**	**p**	**K**	**AICc**	**ΔAIC_c**	**R2m**	**R2c**	**LL**
(a) F_{ST} genetic distance																
Null	-	-	1	-173.640	104.725	0.000	0.187	88.020	-	-	1	-173.640	104.748	0.000	0.187	88.020
Isolation-by-distance																
Euclidean dist.	0.864	0.000	2	-265.200	13.164	0.782	0.819	135.267	0.864	0.000	2	-265.2	13.187	0.782433	0.818522	135.2669
Isolation-by-resistance																
Riparian dens.	**0.834**	**0.001**	**4**	**-278.365**	**0.000**	**0.847**	**0.895**	**146.039**	**0.834**	**0.001**	**4**	**-278.388**	**0.000**	**0.847**	**0.895**	**146.051**
Dist. to coast	0.877	0.001	4	-269.545	8.819	0.823	0.877	141.630	0.878	0.001	4	-269.640	8.747	0.824	0.878	141.677
HII	0.841	0.001	4	-267.464	10.900	0.824	0.838	140.589	0.841	0.001	4	-267.024	11.363	0.823	0.838	140.369
Coastal habitats	0.862	0.001	4	-262.393	15.972	0.811	0.853	138.053	0.880	0.001	4	-260.627	17.761	0.803	0.826	137.171
SDI	0.787	0.001	4	-260.617	17.748	0.801	0.821	137.166	0.787	0.001	4	-260.700	17.688	0.801	0.821	137.207
Temperature	0.793	0.001	4	-260.159	18.206	0.795	0.830	136.937	0.793	0.001	4	-260.119	18.269	0.794	0.829	136.917
Humidity	0.835	0.002	4	-260.154	18.211	0.791	0.834	136.934	0.836	0.001	4	-260.130	18.257	0.791	0.835	136.922
Evaporation	0.840	0.001	4	-258.896	19.469	0.795	0.827	136.305	0.840	0.001	4	-258.896	19.492	0.795	0.827	136.305
Precipitation	0.778	0.001	4	-258.752	19.613	0.794	0.828	136.233	0.778	0.001	4	-258.749	19.638	0.794	0.828	136.232
Conifer	0.847	0.001	4	-258.648	19.717	0.799	0.816	136.181	0.859	0.001	4	-258.682	19.706	0.800	0.816	136.198
Grassland	0.879	0.001	4	-257.190	21.174	0.787	0.817	135.452	0.879	0.001	4	-257.192	21.196	0.787	0.817	135.453
Bog	0.870	0.001	4	-257.134	21.230	0.783	0.819	135.424	0.885	0.001	4	-257.312	21.075	0.759	0.845	135.513
Open	0.815	0.001	4	-256.822	21.542	0.782	0.819	135.268	0.815	0.001	4	-256.820	21.568	0.782	0.819	135.267
Broadleaved	0.867	0.001	4	-256.820	21.545	0.782	0.819	135.267	0.867	0.001	4	-256.820	21.568	0.782	0.819	135.267
Surface temperature	0.785	0.002	4	-256.820	21.545	0.782	0.819	135.267	0.785	0.001	4	-256.820	21.568	0.782	0.819	135.267
Scrub	0.800	0.001	4	-256.820	21.545	0.782	0.819	135.267	0.802	0.001	4	-256.876	21.512	0.783	0.821	135.295

Table H.4 cont.

	Run 1								*Run 2*							
	Mantel test		**MLPE**						**Mantel test**		**MLPE**					
	R	**p**	**K**	**AICc**	**ΔAIC$_c$**	**R2m**	**R2c**	**LL**	**R**	**p**	**K**	**AICc**	**ΔAIC$_c$**	**R2m**	**R2c**	**LL**
(b) G"$_{ST}$ genetic distance																
Null	-	-	1	7.449	111.510	0.000	0.208	-2.524	-	-	1	7.449	111.509	0.000	0.208	-2.524
Isolation-by-distance																
Euclidean dist.	0.859	0.000	2	-84.039	20.022	0.771	0.832	44.686	0.859	0.000	2	-84.039	20.022	0.771	0.832	44.686
Isolation-by-resistance																
Riparian dens.	**0.892**	**0.001**	**4**	**-104.061**	**0.000**	**0.850**	**0.915**	**58.888**	**0.892**	**0.001**	**4**	**-104.061**	**0.000**	**0.850**	**0.915**	**58.887**
Dist. to coast	0.918	0.001	4	-94.282	9.779	0.826	0.899	53.998	0.917	0.001	4	-94.728	9.332	0.825	0.898	54.221
HII	0.885	0.002	4	-84.317	19.744	0.816	0.849	49.016	0.878	0.001	4	-80.771	23.290	0.800	0.841	47.243
Scrub	0.862	0.001	4	-83.123	20.938	0.791	0.875	48.418	0.864	0.001	4	-83.875	20.185	0.793	0.873	48.795
Humidity	0.878	0.001	4	-81.951	22.109	0.788	0.854	47.833	0.878	0.001	4	-81.896	22.165	0.788	0.854	47.805
Coastal habitats	0.902	0.001	4	-81.293	22.768	0.799	0.852	47.504	0.925	0.001	4	-89.404	14.657	0.827	0.879	51.559
Temperature	0.848	0.001	4	-81.057	23.004	0.794	0.855	47.386	0.844	0.001	4	-80.909	23.151	0.791	0.849	47.312
SDI	0.835	0.001	4	-80.187	23.873	0.796	0.837	46.951	0.835	0.001	4	-80.187	23.874	0.796	0.837	46.951
Surface temperature	0.839	0.001	4	-78.855	25.206	0.786	0.848	46.285	0.839	0.001	4	-78.574	25.486	0.784	0.846	46.144
Precipitation	0.830	0.001	4	-78.651	25.410	0.790	0.849	46.182	0.830	0.001	4	-78.651	25.410	0.790	0.849	46.182
Evaporation	0.878	0.001	4	-78.453	25.608	0.790	0.844	46.084	0.878	0.001	4	-78.453	25.608	0.790	0.844	46.083
Open	0.865	0.001	4	-76.908	27.153	0.776	0.844	45.311	0.866	0.001	4	-76.909	27.152	0.776	0.843	45.311
Grassland	0.900	0.001	4	-76.451	-27.610	0.782	0.833	45.083	0.900	0.001	4	-76.443	-27.617	0.782	0.832	45.079
Conifer	0.875	0.001	4	-75.658	-28.403	0.771	0.832	44.686	0.889	0.001	4	-78.002	-26.058	0.796	0.835	45.858
Broadleaved	0.883	0.001	4	-75.658	-28.403	0.771	0.832	44.686	0.883	0.001	4	-75.658	-28.403	0.771	0.832	44.686
Bog	0.885	0.001	4	-75.658	-28.403	0.771	0.832	44.686	0.906	0.001	4	-75.658	-28.403	0.769	0.871	48.844

Table H.5 Mantel tests and single-predictor *circuit theory* ResistanceGA analysis using two measures of genetic distance **(a-b)** and 16 environmental predictors. Surface optimisation was performed twice to check for convergence (Run 1 and Run 2). Environmental predictors are ranked in ascending order of AIC_c values. K is the number of parameters, ΔAIC_c is the difference in the AIC_c values between the best supported model (marked in bold) and each subsequent model; R^2m and R^2c are the marginal and conditional R^2 values of the fitted MLPE model; LL is log likelihood.

	Run 1								*Run 2*							
	Mantel test		**MLPE**						**Mantel test**		**MLPE**					
	R	**p**	**K**	**AICc**	**ΔAIC_c**	**R2m**	**R2c**	**LL**	**R**	**p**	**K**	**AICc**	**ΔAIC_c**	**R2m**	**R2c**	**LL**
(a) FST genetic distance																
Null	-	-	1	-173.640	85.331	0.000	0.187	88.020	-	-	1	-173.640	85.331	0.000	0.187	88.020
Isolation-by-distance																
Euclidean dist.	0.864	0.000	2	-258.971	0.000	0.773	0.776	132.152	0.864	0.000	2	-258.971	0.000	0.773	0.776	132.152
Isolation-by-resistance																
Conifer	**0.931**	**0.001**	**4**	**-257.933**	**1.038**	**0.796**	**0.797**	**135.824**	**0.927**	**0.001**	**4**	**-256.477**	**2.494**	**0.794**	**0.797**	**135.096**
Dist. to coast	0.932	0.002	4	-255.474	3.497	0.798	0.840	134.594	0.932	0.001	4	-255.471	3.500	0.799	0.842	134.593
Grassland	0.918	0.001	4	-252.196	6.775	0.779	0.781	132.955	0.918	0.001	4	-252.197	6.775	0.779	0.781	132.955
Bog	0.907	0.001	4	-252.190	6.781	0.779	0.780	132.952	0.907	0.001	4	-252.190	6.781	0.779	0.780	132.952
Broadleaved	0.917	0.001	4	-252.186	6.785	0.778	0.780	132.950	0.916	0.001	4	-252.186	6.785	0.778	0.780	132.950
Surface temperature	0.848	0.002	4	-251.854	7.118	0.776	0.776	132.784	0.848	0.001	4	-251.854	7.118	0.776	0.776	132.784
Humidity	0.878	0.001	4	-251.543	7.428	0.775	0.775	132.629	0.878	0.001	4	-251.543	7.428	0.775	0.775	132.629
Temperature	0.850	0.001	4	-251.323	7.648	0.774	0.774	132.519	0.851	0.001	4	-251.322	7.649	0.774	0.774	132.518
Open	0.873	0.001	4	-250.954	8.017	0.773	0.773	132.334	0.873	0.001	4	-250.954	8.017	0.773	0.773	132.334
Evaporation	0.893	0.001	4	-250.827	8.144	0.773	0.773	132.271	0.893	0.001	4	-250.827	8.144	0.773	0.773	132.271
Precipitation	0.873	0.001	4	-250.822	8.150	0.773	0.773	132.268	0.873	0.001	4	-250.821	8.150	0.773	0.773	132.268
HII	0.894	0.001	4	-250.694	8.278	0.774	0.778	132.204	0.907	0.001	4	-251.905	7.066	0.786	0.803	132.810
Riparian dens.	0.895	0.001	4	-250.639	8.333	0.773	0.776	132.177	0.895	0.001	4	-250.639	8.332	0.773	0.776	132.177
Scrub	0.873	0.001	4	-250.596	8.375	0.773	0.776	132.155	0.873	0.001	4	-250.596	8.375	0.773	0.776	132.155
Coastal habitats	0.908	0.001	4	-250.590	8.381	0.773	0.776	132.152	0.908	0.001	4	-250.590	8.381	0.773	0.776	132.152
SDI	0.869	0.001	4	-250.590	8.381	0.773	0.776	132.152	0.869	0.001	4	-250.590	8.381	0.773	0.776	132.152

Table H.5 cont.

	Run 1								*Run 2*							
	Mantel test		**MLPE**						**Mantel test**		**MLPE**					
	R	**p**	**K**	**AICc**	**ΔAIC$_c$**	**R2m**	**R2c**	**LL**	**R**	**p**	**K**	**AICc**	**ΔAIC$_c$**	**R2m**	**R2c**	**LL**
(b) G"st genetic distance																
Null	-	-	1	7.449	85.156	0.000	0.208	-2.524	-	-	1	7.449	85.155	0.000	0.208	-2.524
Isolation-by-distance																
Euclidean dist.	0.859	0.000	2	-75.216	2.491	0.768	0.791	40.275	0.859	0.000	2	-75.215	2.492	0.768	0.791	40.274
Isolation-by-resistance																
Dist. to coast	**0.937**	**0.001**	**4**	**-77.707**	**0.000**	**0.802**	**0.866**	**45.711**	**0.937**	**0.001**	**4**	**-77.707**	**0.000**	**0.803**	**0.867**	**45.710**
Conifer	0.928	0.001	4	-75.127	2.580	0.800	0.820	44.421	0.930	0.001	4	-75.134	2.572	0.800	0.820	44.424
HII	0.910	0.001	4	-69.593	8.114	0.785	0.821	41.654	0.910	0.002	4	-69.533	8.174	0.785	0.821	41.624
Coastal habitats	0.919	0.001	4	-69.577	8.130	0.776	0.824	41.646	0.918	0.001	4	-69.572	8.135	0.776	0.827	41.643
Scrub	0.893	0.001	4	-68.225	9.482	0.777	0.805	40.970	0.906	0.001	4	-69.361	8.346	0.789	0.835	41.538
Broadleaved	0.910	0.002	4	-68.189	9.519	0.777	0.798	40.951	0.909	0.001	4	-68.233	9.474	0.777	0.797	40.974
Grassland	0.917	0.001	4	-68.025	9.683	0.778	0.800	40.869	0.917	0.001	4	-68.025	9.682	0.778	0.800	40.869
Bog	0.901	0.002	4	-67.903	9.804	0.776	0.795	40.809	0.900	0.001	4	-67.811	9.896	0.776	0.796	40.763
Open	0.898	0.001	4	-67.365	10.343	0.771	0.800	40.539	0.898	0.001	4	-66.835	10.871	0.768	0.791	40.275
SDI	0.881	0.001	4	-67.196	10.511	0.770	0.791	40.455	0.880	0.001	4	-68.162	9.545	0.747	0.811	40.938
Precipitation	0.887	0.001	4	-67.044	10.663	0.773	0.798	40.379	0.887	0.002	4	-67.044	10.662	0.773	0.798	40.379
Surface temperature	0.874	0.001	4	-67.033	10.674	0.769	0.790	40.374	0.874	0.001	4	-67.033	10.674	0.769	0.790	40.373
Humidity	0.884	0.001	4	-67.020	10.687	0.770	0.790	40.367	0.884	0.002	4	-67.020	10.687	0.770	0.790	40.367
Temperature	0.875	0.002	4	-66.987	10.720	0.769	0.790	40.351	0.875	0.001	4	-66.987	10.720	0.769	0.790	40.350
Riparian dens.	0.890	0.001	4	-66.899	10.808	0.769	0.791	40.307	0.890	0.002	4	-66.899	10.807	0.769	0.791	40.307
Evaporation	0.893	0.001	4	-66.892	10.815	0.770	0.793	40.303	0.893	0.001	4	-66.889	10.818	0.770	0.792	40.302

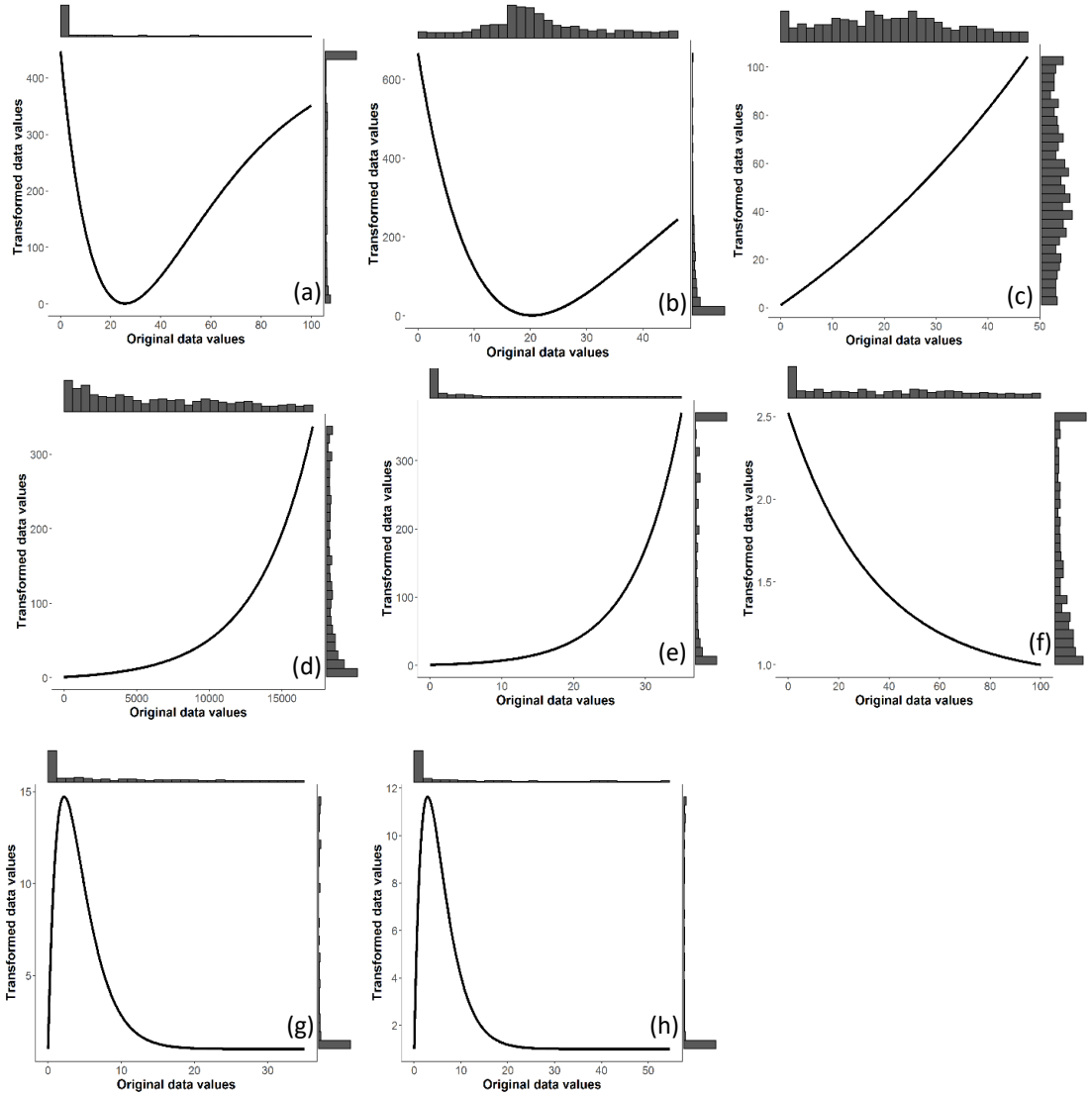


Figure H.2 Transformations applied to each variable to generate resistance surfaces for multiple resistance surface optimisations: **(a)** coastal, **(b)** Human Influence Index, **(c)** riparian density, **(d)** distance to coast, **(f)** bog, **(e)** conifer, **(g)** grassland and **(h)** scrub. Original values are represented in the x-axis while transformed (resistance) values are shown in the y-axis. Four different transformations were used: **(a-b)** inverse Ricker transformation, **(c-e)** inverse-reverse monomolecular transformation; **(f)** inverse monomolecular transformation and **(g-h)** Ricker transformation

Table H.6 Multiple surface optimisation for **(a)** least-cost path and **(b)** circuit theory for two measures of genetic distance. Each surface optimisation was performed twice to check for convergence (Run 1 and Run 2). Model evaluation metrics were produced using 1,000 bootstrap iterations: Avg.AIC_C is an averaged AICc value; avg.weight is the averaged AIC_C weights; avg.mR^2 is the averaged marginal R^2.

Model	*Run 1*						*Run 2*					
	k	**avg.AICc**	**ΔAIC_c**	**avg.weight**	**avg.R^2m**	**avg.LL**	**k**	**avg.AICc**	**ΔAIC_c**	**avg.weight**	**avg.R^2m**	**avg.LL**
(a) Least-cost path												
F_{ST} genetic distance												
Dist. to coast + Riparian dens. + Coastal habitats	10	-263.459	0.000	0.274	0.837	80.729	10	-263.459	0.000	0.274	0.837	80.729
Dist. to coast + HII+ Riparian dens.	10	-263.196	0.263	0.229	0.835	80.598	10	-263.196	0.263	0.229	0.835	80.598
HII + Riparian dens. + Coastal habitats	10	-263.390	0.069	0.268	0.836	80.695	10	-263.390	0.069	0.268	0.836	80.695
Dist. to coast + HII + Coastal habitats	10	-259.187	4.272	0.229	0.819	78.593	10	-259.187	4.272	0.229	0.819	78.593
Dist. to coast + HII + Riparian dens. + Coastal habitats	13	-225.408	38.051	0.000	0.833	80.304	13	-225.408	38.051	0.000	0.833	80.304
HII	4	-147.261	116.198	0.000	0.858	82.630	4	-147.261	116.198	0.000	0.858	82.630
Riparian dens.	4	-143.473	119.986	0.000	0.837	80.737	4	-143.473	119.986	0.000	0.837	80.737
Euclidean dist.	2	-139.928	123.530	0.000	0.773	74.964	2	-139.928	123.530	0.000	0.773	74.964
Coastal habitats	4	-139.527	123.931	0.000	0.820	78.764	4	-139.527	123.931	0.000	0.820	78.764
Dist. to coast	4	-138.706	124.752	0.000	0.818	78.353	4	-138.706	124.752	0.000	0.818	78.353
Dist. to coast + HII	7	-43.166	220.292	0.000	0.849	81.583	7	-43.166	220.292	0.000	0.849	81.583
Dist. to coast + Riparian dens.	7	-42.103	221.356	0.000	0.839	81.051	7	-42.103	221.356	0.000	0.839	81.051
Riparian dens. + Coastal habitats	7	-41.530	221.929	0.000	0.837	80.765	7	-41.530	221.929	0.000	0.837	80.765
HII + Riparian dens.	7	-41.280	222.179	0.000	0.836	80.640	7	-41.280	222.179	0.000	0.836	80.640
HII + Coastal habitats	7	-38.825	224.634	0.000	0.829	79.412	7	-38.825	224.634	0.000	0.829	79.412
Dist. to coast + Coastal habitats	7	-36.686	226.773	0.000	0.819	78.343	7	-36.686	226.773	0.000	0.819	78.343

Table H.6 cont.

Model	Run 1						Run 2					
	k	**avg.AICc**	**ΔAIC_c**	**avg.weight**	**avg.R^2m**	**avg.LL**	**k**	**avg.AICc**	**ΔAIC_c**	**avg.weight**	**avg.R^2m**	**avg.LL**
G''_{ST} genetic distance												
HII + Riparian dens. +Scrub	10	-166.716	0.000	0.319	0.833	32.358	10	-166.716	0.000	0.319	0.833	32.358
Dist. to coast + Riparian dens. + HII	10	-167.340	0.624	0.354	0.843	32.670	10	-167.340	0.624	0.354	0.843	32.670
Dist. to coast + Riparian + Scrub	10	-166.867	0.152	0.267	0.837	32.434	10	-166.867	0.152	0.267	0.837	32.434
Dist. to coast + HII + Scrub	10	-161.473	5.242	0.059	0.818	29.737	10	-161.473	5.242	0.059	0.818	29.737
Dist. to coast + HII + Riparian dens. + Scrub	13	-127.845	38.871	0.000	0.830	31.522	13	-127.845	38.871	0.000	0.830	31.522
Riparian dens.	4	-47.185	119.531	0.000	0.843	32.592	4	-47.185	119.531	0.000	0.843	32.592
HII	4	-43.546	123.170	0.000	0.842	30.773	4	-43.546	123.170	0.000	0.842	30.773
Dist. to coast	4	-42.384	124.332	0.000	0.822	30.192	4	-42.384	124.332	0.000	0.822	30.192
Euclidean dist.	2	-40.595	126.121	0.000	0.756	25.298	2	-40.595	126.121	0.000	0.756	25.298
Scrub	4	-36.306	130.410	0.000	0.777	27.153	4	-36.306	130.410	0.000	0.777	27.153
Riparian dens. + Scrub	7	54.180	220.895	0.000	0.842	32.910	7	54.180	220.895	0.000	0.842	32.910
Dist. to coast + Riparian dens.	7	54.519	221.234	0.000	0.844	32.741	7	54.519	221.234	0.000	0.844	32.741
Riparian dens. + HII	7	54.841	221.557	0.000	0.843	32.580	7	54.841	221.557	0.000	0.843	32.580
Dist. to coast + HII	7	58.924	225.640	0.000	0.826	30.538	7	58.924	225.640	0.000	0.826	30.538
Dist. to coast + Scrub	7	59.759	226.475	0.000	0.821	30.120	7	59.759	226.475	0.000	0.821	30.120
HII + Scrub	7	64.356	231.071	0.000	0.784	27.822	7	64.356	231.071	0.000	0.784	27.822

Table H.6 cont.

Model	Run 1						Run 2					
	k	**avg.AICc**	**ΔAIC$_c$**	**avg.weight**	**avg.R^{2}m**	**avg.LL**	**k**	**avg.AICc**	**ΔAIC$_c$**	**avg.weight**	**avg.R^{2}m**	**avg.LL**
(b) Circuit theory distance												
F_{ST} genetic distance												
Grassland + Conifer + Bog	**10**	**-258.845**	**0.000**	**0.420**	**0.817**	**78.422**	**10**	**-258.845**	**0.000**	**0.420**	**0.817**	**78.422**
Dist. to coast + Grassland + Bog	10	-257.404	1.441	0.295	0.816	77.702	10	-257.404	1.441	0.295	0.816	77.702
Dist. to coast + Conifer + Bog	10	-257.606	1.239	0.236	0.814	77.803	10	-257.606	1.239	0.236	0.814	77.803
Dist. to coast + Conifer + Grassland	10	-253.342	5.503	0.049	0.787	75.671	10	-253.342	5.503	0.049	0.787	75.671
Dist. to coast + Grassland + Conifer + Bog	13	-222.351	36.494	0.000	0.820	78.775	13	-222.351	36.494	0.000	0.820	78.775
Euclidean dist.	2	-137.780	121.065	0.000	0.762	73.890	2	-137.780	121.065	0.000	0.762	73.890
Conifer	4	-134.113	124.732	0.000	0.790	76.057	4	-134.113	124.732	0.000	0.790	76.057
Grassland	4	-132.758	126.087	0.000	0.784	75.379	4	-132.758	126.087	0.000	0.784	75.379
Dist. to coast	4	-132.684	126.161	0.000	0.787	75.342	4	-132.684	126.161	0.000	0.787	75.342
Bog	4	-130.722	128.123	0.000	0.767	74.361	4	-130.722	128.123	0.000	0.767	74.361
Grassland + Bog	7	-35.090	223.755	0.000	0.811	77.545	7	-35.090	223.755	0.000	0.811	77.545
Conifer + Bog	7	-34.280	224.565	0.000	0.803	77.140	7	-34.280	224.565	0.000	0.803	77.140
Dist. to coast + Bog	7	-33.337	225.508	0.000	0.809	76.669	7	-33.337	225.508	0.000	0.809	76.669
Dist. to coast + Grassland	7	-33.541	225.304	0.000	0.799	76.771	7	-33.541	225.304	0.000	0.799	76.771
Grassland + Conifer	7	-33.187	225.658	0.000	0.796	76.594	7	-33.187	225.658	0.000	0.796	76.594
Dist. to coast + Conifer	7	-32.048	226.797	0.000	0.790	76.024	7	-32.048	226.797	0.000	0.790	76.024

Table H.6 cont.

Model	*Run 1*						*Run 2*					
	k	**avg.AICc**	**ΔAIC_c**	**avg.weight**	**avg.R^2m**	**avg.LL**	**k**	**avg.AICc**	**ΔAIC_c**	**avg.weight**	**avg.R^2m**	**avg.LL**
G''_{ST} genetic distance												
Dist. to coast + HII + Conifer	**10**	**-156.989**	**0.000**	**0.291**	**0.804**	**27.494**	**10**	**-156.989**	**0.000**	**0.291**	**0.804**	**27.494**
Coastal + HII + Conifer	10	-155.932	1.057	0.285	0.805	26.966	10	-155.932	1.057	0.285	0.805	26.966
Dist. to coast + Coastal habitats + Conifer	10	-155.636	1.353	0.220	0.795	26.818	10	-155.636	1.353	0.220	0.795	26.818
Dist. to coast + HII + Coastal habitats	10	-155.301	1.687	0.204	0.794	26.651	10	-155.301	1.687	0.204	0.794	26.651
Dist. to coast + HII + Coastal habitats + Conifer	13	-117.522	39.466	0.000	0.792	26.361	13	-117.522	39.466	0.000	0.792	26.361
Euclidean dist.	2	-37.168	119.821	0.000	0.752	23.584	2	-37.168	119.821	0.000	0.752	23.584
Dist. to coast	4	-34.340	122.649	0.000	0.786	26.170	4	-34.340	122.649	0.000	0.786	26.170
HII	4	-34.218	122.771	0.000	0.801	26.109	4	-34.218	122.771	0.000	0.801	26.109
Conifer	4	-33.968	123.021	0.000	0.789	25.984	4	-33.968	123.021	0.000	0.789	25.984
Coastal habitats	4	-31.676	125.313	0.000	0.756	24.838	4	-31.676	125.313	0.000	0.756	24.838
HII + Conifer	7	65.501	222.489	0.000	0.807	27.250	7	65.501	222.489	0.000	0.807	27.250
Dist. to coast + HII	7	65.809	222.798	0.000	0.799	27.095	7	65.809	222.798	0.000	0.799	27.095
Dist. to coast + Conifer	7	66.138	223.127	0.000	0.796	26.931	7	66.138	223.127	0.000	0.796	26.931
Conifer + Coastal habitats	7	67.061	224.050	0.000	0.792	26.470	7	67.061	224.050	0.000	0.792	26.470
Dist. to coast + Coastal habitats	7	67.979	224.968	0.000	0.785	26.011	7	67.979	224.968	0.000	0.785	26.011
HII + Coastal habitats	7	68.170	225.159	0.000	0.781	25.915	7	68.170	225.159	0.000	0.781	25.915

Table H.7 The percent contribution of environmental predictors in isolation-by-resistance (IBR) multiple surface optimisation models for **(a)** least-cost path and **(b)** circuit theory distance.

Model	Percent contribution			
pred1 + pred2 + pred3 + pred4	**pred1**	**pred2**	**pred3**	**pred4**
(a) Least-cost path				
F_{ST} genetic distance				
Dist. to coast + Riparian dens. + Coastal habitats	13.1	83.8	3.1	-
Dist. to coast + HII + Riparian dens.	20.6	2.5	76.8	-
HII + Riparian dens. + Coastal habitats	3.5	93.1	3.5	-
Dist. to coast + HII + Coastal habitats	22.2	1.4	76.4	-
Dist. to coast + HII + Riparian dens. + Coastal habitats	8.1	15.4	74.4	2.2
G''_{ST} genetic distance				
HII + Riparian dens. +Scrub	17.6	68.6	13.8	-
Dist. to coast + Riparian dens. + HII	32.3	2.4	65.3	-
Dist. to coast + Riparian dens. + Scrub	4.6	83.9	11.5	-
Dist. to coast + HII + Scrub	75.0	8.2	16.8	-
Dist. to coast + HII + Riparian dens. + Scrub	16.0	1.0	73.0	10.0
(b) Circuit theory distance				
F_{ST} genetic distance				
Grassland + Conifer + Bog	25.2	29.4	45.3	-
Dist. to coast + Conifer + Bog	47.2	25.2	27.6	-
Dist. to coast + Grassland + Bog	54.2	18.8	27.0	-
Dist. to coast + Conifer + Grassland	4.0	25.6	70.4	-
Dist. to coast + Grassland + Conifer + Bog	4.7	26.9	26.1	42.2
G''_{ST} genetic distance				
Dist. to coast + HII + Conifer	52.5	34.4	13.1	-
Coastal habitats + HII + Conifer	9.0	64.7	26.3	-
Dist. to coast + Coastal habitats+ Conifer	74.4	6.3	19.4	-
Dist. to coast + HII + Coastal habitats	60.5	35.4	4.0	-
Dist. to coast + HII + Coastal habitats + Conifer	29.8	1.7	17.2	51.3

10.9 Appendix I

Chapter 7. Will predicted positive effects of climate change be enough to reverse declines of the regionally Endangered Natterjack toad in Ireland?

Table I.1 Climatic and habitat variables used as explanatory environmental variables.

	Variable code	Description
a)	**Worldclim bioclimatic variables – Europe-wide extent (2.5° ~ 4km grid cells)**	
	bio1	Mean annual temperature (°C)
	bio2	Diurnal temperature range (°C)
	bio10	Mean temperature of warmest quarter (°C)
	bio11	Mean temperature of coldest quarter (°C)
	bio12	Annual total precipitation (mm)
	bio16	Precipitation of wettest quarter (mm)
	bio17	Precipitation of driest quarter (mm)
b)	**COSMO-CLM5 ensemble variables (ICHEC) – Ireland only (4km grid cells)**	
	T_S	Surface temperature at ground level (°C)
	T_SO_00540mm	Soil temperature at 54cm belowground (°C)
	TOT_PREC	Precipitation (kg/m^2)
	RUNOFF_G	Subsurface runoff (kg/m^2)
	WDSPD_10m	Wind speed at 10m (m/s)
c)	**CORINE2018 (EEA) – Europe (2.5° ~5km grid cells) and Ireland (4km grid cells)**	
	dist_to_coast	Nearest perpendicular distance to the coast (km)
	coastal_habs	Beaches, dunes, sand (331), salt marshes (421) and intertidal flats (423)
	freshwater	Water courses (511) and Water bodies (512)
	grassland	Pastures (231) and Natural grasslands (321)
	scrub	Transitional woodland-shrub (324) and Fruit trees and berry plantations (222)
	sparse_veg	Bare rocks (332) and Sparsely vegetated areas (333)

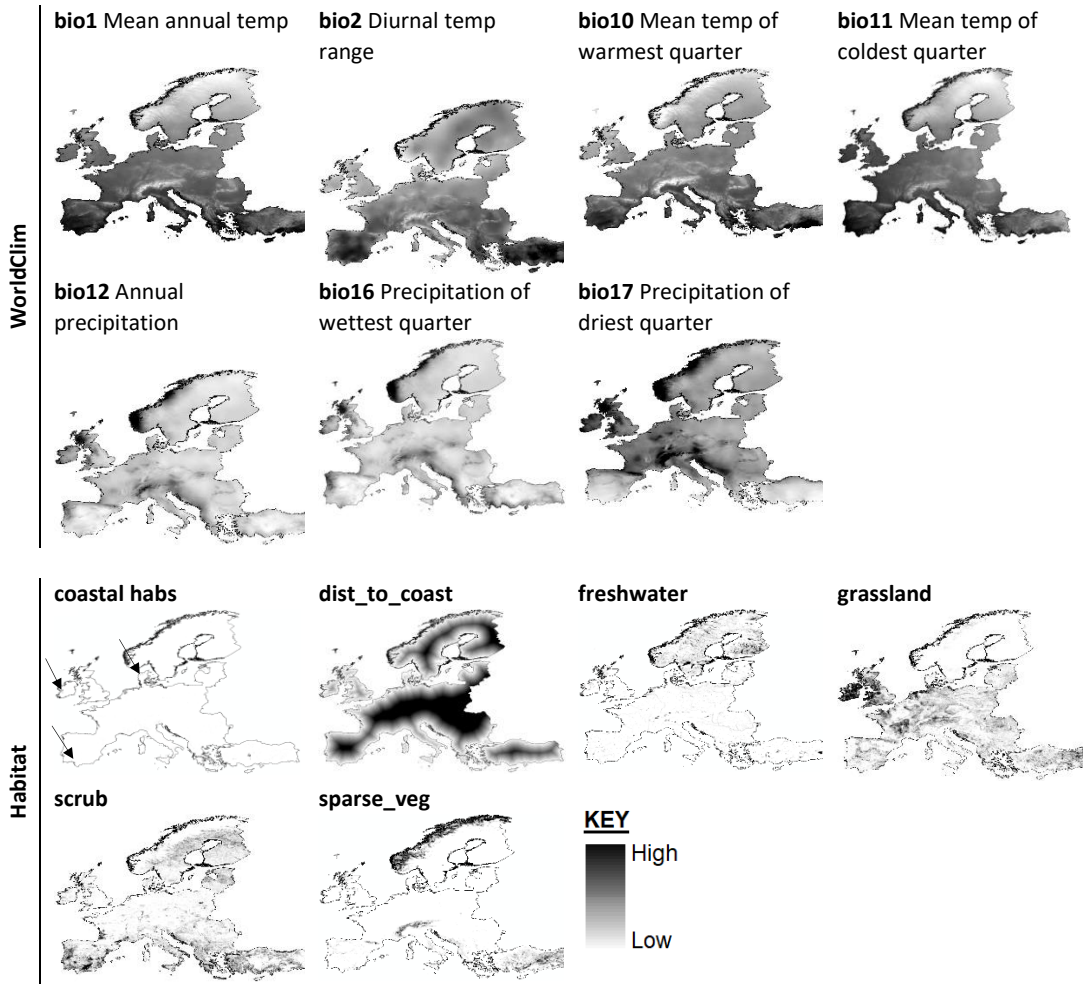


Figure I.1 Spatial variation of selected Worldclim climate variables for the current period (averaged from 1970-2000) and CORINE2018 habitat variables (using the classification in Table H.1) at a 2.5° ~4km grid cell resolution throughout Europe. The same climate variables were available projected for the future periods of the 2050s and 2070s under both RCP4.5 and RCP8.5 emissions scenarios (not shown).

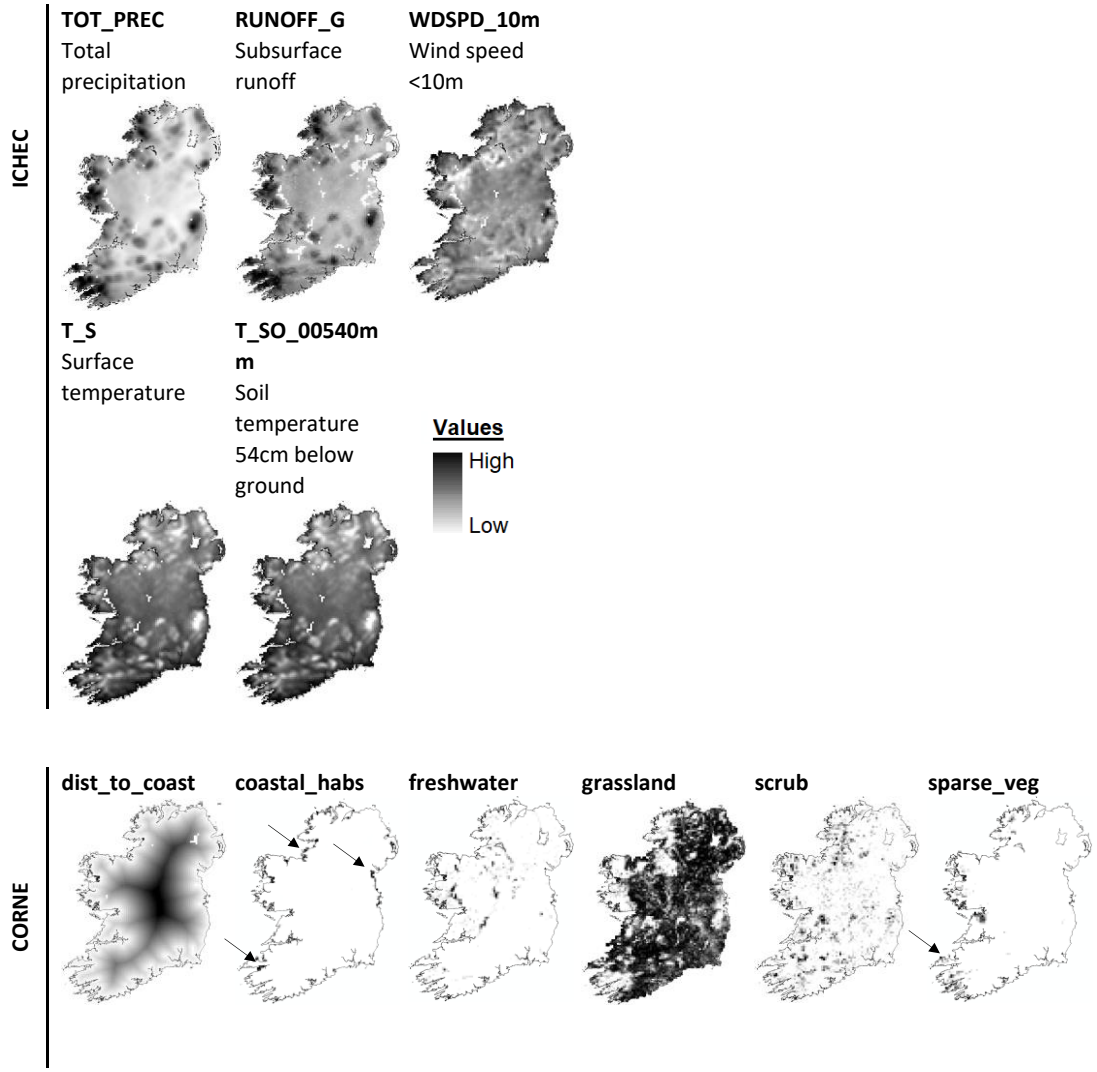


Figure I.2 Spatial variation of selected ICHEC climate variables (derived from the COSMO-CLM5 ensemble) for the current period (averaged from 1976-2005) and selected CORINE2018 habitat variables (using the classification in Table S1) at a 4km grid cell resolution throughout Ireland. The same climate variables were available projected for the future periods: 2050s and 2080s under both RCP4.5 and RCP8.5 emissions scenarios (not shown).

Table I.2 Validation of Natterjack toad Species Distribution Models for Europe and Ireland.

		Europe Worldclim	Ireland ICHEC	Ireland Worldclim
No threshold	AUC	0.832 ± 0.006	0.984 ± 0.009	0.965 ± 0.021
10^{th} Percentile training presence	AUC	0.762 ± 0.003	0.892 ± 0.075	0.835 ± 0.044
	Threshold	0.630 ± 0.001	0.477 ± 0.344	0.135 ± 0.062
	Sensitivity	0.900 ± 0.005	0.833 ± 0.192	0.708 ± 0.083
	Specificity	0.624 ± 0.003	0.950 ± 0.054	0.962 ± 0.008
	Omission rate	0.100 ± 0.005	0.167 ± 0.192	0.292 ± 0.083
	Proportion correct	0.661 ± 0.002	0.950 ± 0.054	0.962 ± 0.008
	Fractional predicted area	0.376 ± 0.003	0.050 ± 0.054	0.038 ± 0.008
	Kappa	0.263 ± 0.003	0.046 ± 0.049	0.030 ± 0.009
	TSS	0.524 ± 0.006	0.783 ± 0.150	0.670 ± 0.088
Maximum test sensitivity plus specificity	AUC	0.767 ± 0.005	0.900 ± 0.092	0.873 ± 0.043
	Threshold	0.638 ± 0.007	0.457 ± 0.235	0.111 ± 0.086
	Sensitivity	0.883 ± 0.018	0.833 ± 0.192	0.792 ± 0.083
	Specificity	0.651 ± 0.023	0.966 ± 0.017	0.954 ± 0.007
	Omission rate	0.117 ± 0.018	0.167 ± 0.192	0.208 ± 0.083
	Proportion correct	0.682 ± 0.018	0.966 ± 0.017	0.954 ± 0.008
	Fractional predicted area	0.349 ± 0.023	0.034 ± 0.017	0.046 ± 0.007
	Kappa	0.280 ± 0.014	0.037 ± 0.032	0.028 ± 0.007
	TSS	0.534 ± 0.010	0.800 ± 0.184	0.746 ± 0.086

Table I.3 Paired t-tests (paired at the level of the grid cell) for change in predicted suitability between current conditions and future climate scenarios for **a)** Europe using WorldClim (separated into regions), **b)** Ireland using WorldClim **c)** Ireland using ICHEC climate data, **d)** Ireland using ICHEC climate data and only those cells occupied by toads.

Region	Current	SD	Scenario	Prob	SD	%	Paired	df	p
a) Europe$_{Worldclim}$									
Ireland	0.653	0.017	2050	0.664	0.017	1.7	175.0	7,036	<0.001
			2050	0.655	0.017	0.3	18.1	7,036	<0.001
			2070	0.659	0.017	0.9	70.6	7,036	<0.001
			2070	0.664	0.017	1.6	83.8	7,036	<0.001
Great	0.636	0.042	2050	0.660	0.043	3.9	431.8	19,807	<0.001
			2050	0.658	0.048	3.5	248.9	19,807	<0.001
			2070	0.659	0.046	3.6	308.4	19,807	<0.001
			2070	0.665	0.054	4.6	241.8	19,807	<0.001
Europe	0.622	0.084	2050	0.678	0.054	8.9	755.7	277,597	<0.001
			2050	0.683	0.048	9.7	684.0	277,597	<0.001
			2070	0.684	0.047	9.9	684.7	277,597	<0.001
			2070	0.700	0.046	12.5	742.9	277,597	<0.001
Scandinavia	0.330	0.120	2050	0.460	0.120	38.0	1083.1	122,845	<0.001
			2050	0.500	0.120	50.8	1078.4	122,845	<0.001
			2070	0.480	0.120	45.3	1095.7	122,845	<0.001
			2070	0.510	0.120	54.1	953.2	122,845	<0.001
Baltic	0.452	0.035	2050	0.589	0.020	30.2	885.6	15,455	<0.001
			2050	0.613	0.017	35.6	934.3	15,455	<0.001
			2070	0.601	0.020	33.0	870.0	15,455	<0.001
			2070	0.629	0.019	39.1	878.1	15,455	<0.001
b) Ireland$_{Worldclim}$									
Ireland	0.020	0.090	2050	0.140	0.300	534.8	41.6	7,044	<0.001
			2050	0.180	0.300	721.8	49.9	7,044	<0.001
			2070	0.190	0.300	734.8	48.2	7,044	<0.001
			2070	0.260	0.400	1049.6	53.3	7,044	<0.001
c) Ireland$_{ICHEC}$ - All Ireland									
Ireland	0.045	0.138	2050	0.133	0.272	195.6	37.7	5,490	<0.001
			2050	0.142	0.283	215.6	38.2	5,490	<0.001
			2070	0.154	0.301	242.2	38.4	5,490	<0.001
			2070	0.167	0.310	271.1	40.8	5,490	<0.001
d) Ireland$_{ICHEC}$ - Natterjack occupied cells only									
Ireland	0.787	0.215	2050	0.992	0.014	26.0	3.4	11	0.005
			2050	0.998	0.003	26.8	3.4	11	0.006
			2070	0.995	0.007	26.4	3.4	11	0.006
			2070	0.999	0.002	26.9	3.4	11	0.006

Table I.4 2x2 χ^2 contingency tests of association for change in bioclimatic envelope (number of suitable cells above the MaxTSS threshold) for **a)** Europe using Worldclim (separated into regions), **b)** Ireland using Worldclim and **c)** Ireland using ICHEC climate data.

Region	*n* (cells)	Baseline	% suitable	% unsuitable	Future scenario	% suitable	χ^2	df	*p*
a) Europe$_{Worldclim}$									
Ireland	7,037	Current	100	0	2050 (4.5)	100	*na*	1	1
					2050 (8.5)	100	*na*	1	1
					2070 (4.5)	100	*na*	1	1
					2070 (8.5)	100	*na*	1	1
Great Britain	19,808	Current	99.4	0.6	2050 (4.5)	100	104	1	<0.001
					2050 (8.5)	99.6	9	1	0.003
					2070 (4.5)	100	85	1	<0.001
					2070 (8.5)	99.7	17	1	<0.001
Europe	277,598	Current	90.9	9.1	2050 (4.5)	98.5	16,038	1	<0.001
					2050 (8.5)	99.4	21,366	1	<0.001
					2070 (4.5)	99.3	20,967	1	<0.001
					2070 (8.5)	99.4	21,978	1	<0.001
Scandinavia	122,846	Current	13.2	86.8	2050 (4.5)	37.1	18,666	1	<0.001
					2050 (8.5)	51.8	41,792	1	<0.001
					2070 (4.5)	45.9	31,561	1	<0.001
					2070 (8.5)	55.6	48,952	1	<0.001
Baltic	15,456	Current	7.3	92.7	2050 (4.5)	100	*Inf*	1	<0.001
					2050 (8.5)	100	*Inf*	1	<0.001
					2070 (4.5)	100	*Inf*	1	<0.001
					2070 (8.5)	100	*Inf*	1	<0.001
b) Ireland$_{Worldclim}$	7,045	Current	16.3	83.7	2050 (4.5)	31.4	1750	1	<0.001
					2050 (8.5)	37.5	1670	1	<0.001
					2070 (4.5)	37.7	1690	1	<0.001
					2070 (8.5)	41.8	2070	1	<0.001
c) Ireland$_{ICHEC}$	5,491	Current	3	97	2050 (4.5)	11.1	232	1	<0.001
					2050 (8.5)	12	280	1	<0.001
					2070 (4.5)	13.6	359	1	<0.001
					2070 (8.5)	14.9	429	1	<0.001

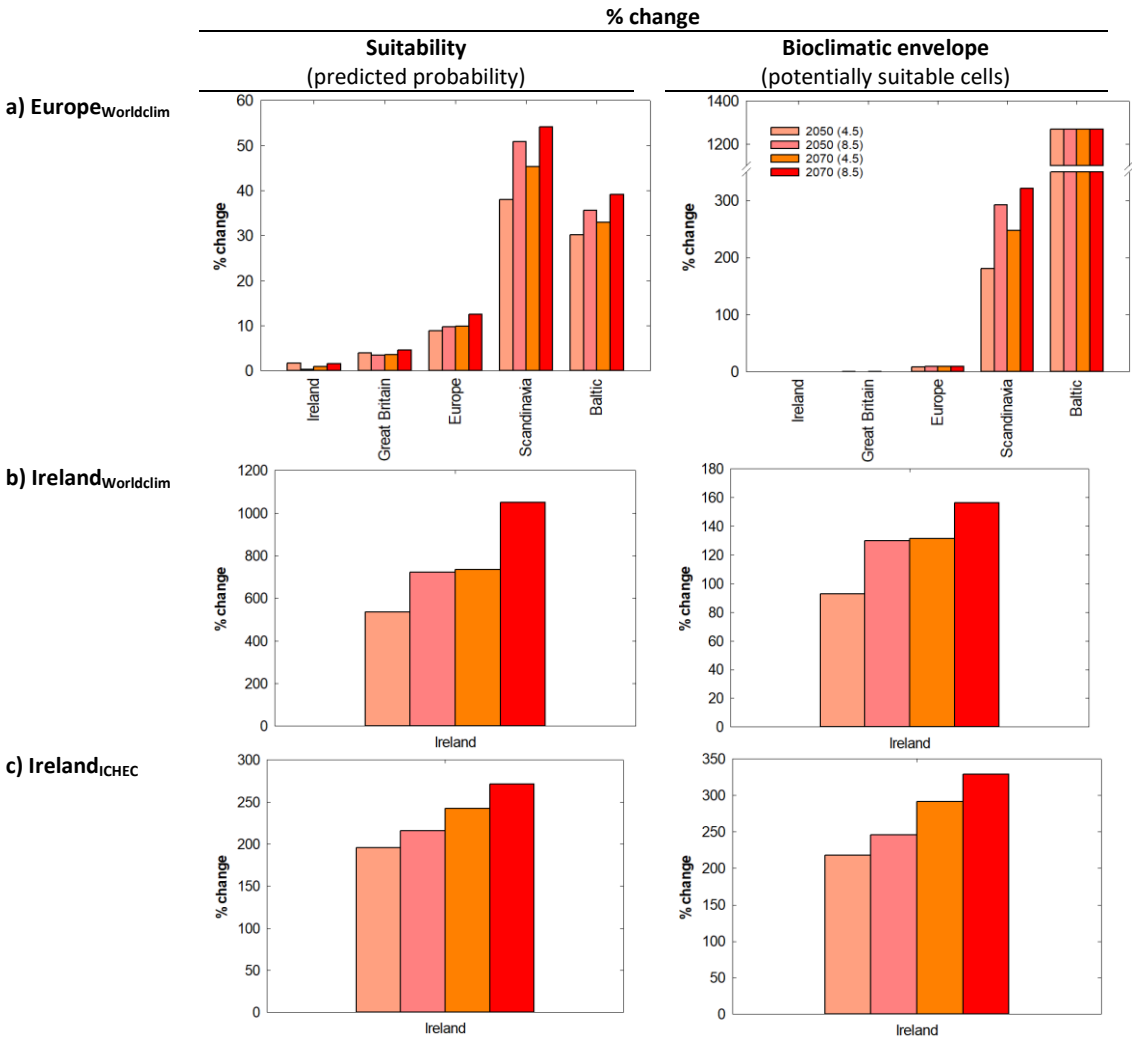


Figure I.3 Predicted percentage (%) change in suitability (left column) and potential suitable cells (right column) for Natterjack toads in **a)** Europe using Worldclim (separated into regions), **b)** Ireland using Worldclim and **c)** Ireland using ICHEC climate data between current conditions and future climate change scenarios (bars from left-to-right). Note *y*-axes values are not standardized due to the large range between regions and models.