Population genetics of the common frog (*Rana temporaria*) in relation to climate

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MSci (Hons)

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy.

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General abstract

Ecological responses to a changing climate have been well documented in a broad range of species, predominantly in terms of range movements and phenological changes. When faced with a changing environment, species survival will depend on the ability to: 1) evade changes in climate, via dispersal; 2) evolve, via natural selection; and/or 3) plastically change their phenotype, without underlying genetic changes. The potential for an organism to evade, evolve or plastically respond to a changing environment can be predicted by inferring relationships with current climatic conditions. Altitudinal gradients have been proposed as being particularly suitable for environmental change studies due to the rapid variation in climate even over short geographical distances. Species that inhabit altitudinal gradients experience a range of climatic conditions across their range and are thus subject to varying selection pressures. Changes in temperature are predicted to particularly influence ectotherms due to the direct effect on physiological processes. The common frog (*Rana temporaria*) occurs from zero to over a thousand metres along altitudinal gradients in Scotland, offering the opportunity to assess the influence of temperature on organism responses. The overall aim of this thesis was to assess population-level relationships with climate, in order to make predictions regarding susceptibility to a changing climate, focussing on *R. temporaria* in Scotland. In Chapter 2, I inferred colonisation patterns within Europe following the last glacial maximum by combining new and previously compiled mitochondrial cytochrome b DNA sequences. I found that the mitochondrial DNA sequences from my Scottish samples were identical to, or clustered with, the common haplotype previously identified from Western Europe. This clade showed very low mitochondrial genetic variation, consistent with a leptokurtic model of range expansion, where low numbers of long-distance dispersers cause multiple founder events. Second, I assessed fine-scale genetic variation in relation to current temperature gradients using microsatellites. No population structure was found within or between altitudinal gradients at any scale (3-50km; average F_{ST}= 0.02), despite a mean annual temperature difference of 4.5°C between low- and high-altitude sites. Levels of genetic diversity and

heterozygosity were considerable but did not vary by site, altitude or temperature. In Chapter 3, common temperature treatments were used to assess phenotypic differentiation and phenotypic plasticity variation in relation to altitude in terms of larval fitness traits. Local adaptation to altitude was assessed using Q_{ST}-F_{ST} analyses and adaptive phenotypic divergence was then related to environmental parameters using Mantel tests, to look for drivers of selection. I found that *R. temporaria* showed evidence of local adaptation in all larval fitness traits measured. However, only variation in larval period and growth rate was consistent with adaptation to altitude. Moreover, this was only evident in the three mountains with the highest high-altitude sites (at least 900m). Adaptive divergence in traits that were locally adapted to altitude was correlated with spring temperature, suggesting that temperature acts as a strong environmental selection pressure influencing local adaptation even in the face of high gene flow. In Chapter 4, the physiological and behavioural responses that facilitate survival in high-altitude environments were evaluated, in terms of routine metabolic rate and freeze tolerance in tadpoles, and breeding temperature in adults. I found that routine metabolic rate was lower for individuals sampled from high- than low-altitude sites but only from the three mountains with the highest high-altitude sites (at least 900m). Glucose accumulation during freezing was not significantly different based on altitude. However, individuals from low-altitude survived freezing significantly better than those from high-altitude, across all mountains. Breeding did not occur below 5˚C at any site and there was no significant difference in breeding temperature between high- and low-altitude sites, leading to high-altitude individuals spawning 30 days later than those at low-altitude. My results suggest that tadpoles are adapted physiologically to surviving at high-altitude via reduced routine metabolic rate, but only at the highest breeding sites. Finally, in Chapter 5, I assessed the spatial variation in species presence and composition of parasitic water moulds in the genus *Saprolegnia* found on *R. temporaria* eggs. Thirteen samples isolated from four sites were identified as members of the *Saprolegniaceae*. Four putative species of *Saprolegnia* were isolated overall, multiple *Saprolegnia* water moulds were isolated from within sites, and species composition varied between sites. Acidity was significantly lower at sites where

Saprolegniaceae were present, but genetic distance between samples was not correlated with environmental or geographic distance. These findings question the previous focus on *S. ferax* as the primary agent of *Saprolegnia* infection in amphibians and suggest that future studies of virulence need to consider the synergistic effect of multiple *Saprolegnia* species. In conclusion, *R. temporaria* show the potential for evasion, evolution and plastic responses to a changing climate and my results suggest that the outlook is positive for survival of the common frog in Scotland.

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Acknowledgements

First and foremost I'd like to thank my primary supervisor, Barbara Mable. Throughout my PhD she has given me the freedom to take the project and run with it, whilst always being available for questions, support and a nudge in the right direction when I've needed it. A good supervisor is rare, and one that you can share a hill walk and a drink with is even rarer, and I am very appreciative of all her support. I'd also like to thank my other two supervisors, Rob Thomas and Roman Biek, who have been very generous with their time and encouragement and who have always made me feel welcome when I've asked for help. This project was funded by the Biotechnology and Biological Sciences Research Council in partnership with the Royal Zoological Society of Scotland and I am very grateful for the opportunity to undertake this study. I would also like to thank the Glasgow Natural History Society, Royal Geographic Society and Scottish Mountaineering Trust for supporting my fieldwork.

A huge thanks to all the people who helped me with many rainy (and some sunny) hours of fieldwork: Rose Hanley-Nickolls, David Fettes, Romaine Furmston-Evans, Rachel Donnachie and Neil James. I'd also like to thank everyone at SCENE, particularly David Fettes and Rona Brennan, for all their help with housing 3000 tadpoles! Thanks to Aileen Adam and Elizabeth Kilbride for teaching me all I know in the lab, and to Aileen for all the chocolate and caffeine too! The Mable lab has been a very happy place to work over the last three years so I'd like to thank everyone who has passed through the offices for their support and banter, especially Clare Marsden, Andy Tedder, Jimena Guerrero-Flores, Jared Bakuza and Alan Reynolds in my office. Many more people from the Graham Kerr Building have helped me along the way and I'd particularly like thank Roger Downie, Pat Mclaughlin, Alan Law, Graham Law, Florence McGarrity, Lorna Kennedy, John Laurie and George the Janitor, plus many others.

Through the fun times and the tough Vic Paterson-Mackie, John Hume and Ashley Le Vin have been my main partners in crime in the GK and to them I am very

thankful. Thanks also to my lovely family for their endless support. Finally, but most importantly, thanks to Martin, who contributed so much of his time and energy to help me with, and support me though, this PhD and without whom it would not have been possible.

Author’s declaration

I declare that the work recorded in this thesis is entirely my own and is of my own composition. No part of this thesis has been submitted for another degree.

Romaine Furmston-Evans carried out the lab work in 2010 for Appendix D.

Chapter 1 – General Introduction

1.1 Global climate change

The earth's climate has warmed by 0.6°C over the past century (Berry *et al.* 2003; IPCC 2007). The scientific community are in agreement that the rate and scale of the changing climate is outwith that experienced through natural climatic cycles, and is instead the result of human activities (Oreskes 2004). Anthropogenic climate change has been linked to carbon emissions associated primarily with carbon-base fuel consumption (Karl & Trenberth 2003). The Intergovernmental Panel on Climate Change (IPCC) have predicted that as carbon emissions continue to rise, climate perturbations will continue, not only influencing temperature at a global level but also the hydrological cycle. Predicted consequences include contraction of snow cover, glaciers and ice sheets; increases in frequency of temperature and precipitation extreme events; and precipitation increases at high latitudes and decreases at low latitudes (IPCC 2007). The rate and scale of this change is dependent on the carbon emissions scenario (Tabor & Williams 2010), but whatever the level of change, species extinctions and global biodiversity reductions appear inevitable (Thomas *et al.* 2004). The greatest challenge currently facing researchers and conservationists is predicting responses to, and effectively conserving species in, a changing climate (Hannah *et al.* 2002; Hulme 2005; Reusch & Wood 2007; Williams *et al.* 2008; Early & Sax 2011).

1.2 Ecological responses to a changing climate

Worldwide ecological responses to recent climate change have been well documented in a broad range of terrestrial and marine taxa (Parmesan & Yohe 2003; Perry *et al.* 2005; Parmesan 2006). Records of species-level responses to a

changing climate predominantly fall into two categories: range movements and phenological changes (Visser 2008). Range movements, where species ranges' change to remain within climatic tolerance limits (i.e. their climatic envelope) (Walther *et al*. 2002), correlate with pace and direction of climatic warming (Parmesan & Yohe 2003). Resulting patterns observed include leading-edge range movements northwards and upwards within altitudinal gradients as temperatures rise (Parmesan & Yohe 2003; Perry *et al*. 2005). Often, although not always, this is accompanied by a contraction at the tail end of the range as tolerance levels are exceeded (Walther *et al*. 2002; Davis *et al*. 2005; Jump & Penuelas 2005). Range movement responses are dependent on the dispersal ability of the species in question and the availability of suitable habitat (Perry *et al*. 2005; Parmesan 2006; Gienapp *et al*. 2008).

The second observed response, phenological changes, have mainly been recorded as advancement of spring events such as spawning, emergence and flowering (Parmesan & Yohe 2003; Visser 2008). The underlying mechanisms of phenological changes are difficult to determine from temporal phenotypic records alone (but see Loehr et al. 2010; Phillimore et al. 2010). Observed changes could be due to phenotypic plasticity (where environmental cues act on the phenotype without genotypic changes) (Merilä *et al*. 2000b) and/or through natural selection (Gienapp *et al*. 2008; Loehr *et al*. 2010; Haye *et al*. 2010). Evolutionary responses though natural selection to climate are the most difficult to measure (Gienapp *et al*. 2008) and much debate exists as to the role evolutionary-scale changes will play in species survival as the climate continues to warm (Davis & Shaw 2001; Davis *et al*. 2005; Jump & Penuelas 2005; Gienapp *et al*. 2008). Evolutionary responses to a changing climate will depend on the level of adaptation to current environmental conditions (i.e. local adaptation), evolutionary potential to adapt to new conditions (Allendorf & Luikart 2007; Richter-Boix *et al*. 2011), and whether evolution can keep pace with climatic changes (Araújo *et al*. 2006; Pertoldi *et al*. 2007; North *et al*. 2010).

1.3 Inferring population-level relationships with climate

1.3.1 Local adaptation

Natural selection can act to adapt populations to the local environment, here defined as a fitness advantage of local genotypes over genotypes originating in other environments (Miller *et al*. 2011). In a heterogeneous environment this can lead to differentially adapted neighbour populations, even over short geographical distances (Palo *et al*. 2003). However, high levels of gene flow, via dispersal between populations, can act to reduce local adaptation by introducing alleles that are maladapted to the new environment and swamping the locally beneficial alleles (Allendorf & Luikart 2007; Bridle & Vines 2007). The levels of gene flow required to inhibit local adaptation is dependent on the strength of the selective force acting on a trait (Savolainen *et al*. 2007). Indeed, it has been suggested that directional selection acting on important life-history traits can act to maintain divergence between populations in terms of adaptive loci, whilst allowing homogenisation in other parts of the genome (Richter-Boix *et al*. 2011). The potential mechanism for adaptation to local conditions despite lack of neutral genetic isolation is via linkage disequilibrium among allele frequencies at the loci underlying the quantitative trait subject to divergent selection (Lind *et al*. 2011). Thus, adaptive trait divergence can be accompanied by limited allele frequency divergence (Latta 2003). There have been an increasing number of reports of local adaptation taking place in the face of gene flow, in response to strong natural selection (Atlantic salmon: Dionne *et al*. 2008; common frog: Richter-Boix *et al*. 2010; trees: Savolainen *et al*. 2007). The presence of local adaptation, levels of gene flow and strength of natural selection in current environmental conditions will affect population-level responses and ultimately species survival potential in changing environmental conditions.

Current levels of local adaptation are typically measured through reciprocal transplant experiments, to assess fitness of locals in comparison to transplanted

individuals; or through common garden experiments, to assess relative fitness of different populations in a neutral environment (Merilä *et al*. 2000a; Leinonen *et al*. 2011). To fully understand the adaptive basis of genetic variation, neutral genetic processes (such as genetic drift) must also be accounted for in the observed phenotypic differentiation (Loman 2003; Hangartner *et al*. 2011b). However, not all species can be experimentally manipulated due to their conservation status, large size, or complex environmental requirements (Kawecki & Ebert 2004; Antoniazza *et al*. 2010). Therefore, phenotypic variation is sometimes recorded in the field (e.g. feather colouration in European barn owls: Antoniazza *et al*. 2010), but this approach does not take into account genotype-by-environment interactions and conclusions can be misleading (Gienapp *et al*. 2008). This is particularly true if countergradient variation is present within the study system, where genetic influences act in opposition to environmental influences, resulting in cryptic phenotypic divergence (Conover & Schultz 1995). Accurately assessing the presence of local adaptation is important when forming conservation strategies for a changing climate (Savolainen *et al*. 2007).

Translocation ("artificial gene flow"; Fischer & Matthies 1997) has been suggested as a conservation measure to augment declining populations, move populations from areas under threat, and to allow dispersal-limited species to stay within their climatic tolerance limits during climate change (Walther *et al*. 2002; Hulme 2005; Petit *et al*. 2008; Rogell *et al*. 2011). However, movement of an individual away from an area they are locally adapted to may result in maladaptation and reduced survival in a new location (Rogell *et al*. 2011). Furthermore, if differentially adapted individuals are introduced to sites that are already inhabited by a locally adapted population, outbreeding depression can lead to reduced offspring survival due to breakdown of co-adapted gene complexes or reduced hybrid fitness (Sagvik *et al*. 2005). Evidence of outbreeding depression has indeed been found in reintroduced Arabian oryx (Marshall & Spalton 2000); immigrant song sparrows (Marr *et al*. 2002); and artificially crossed ambrosia beetles and pink salmon (Peer & Taborsky 2005;

Gilk *et al*. 2004, respectively), among others, but studies that have addressed the risks of outbreeding depression in animals are still limited (Edmands 2007). Finally, local adaptation may have occurred in response to a broad range of environmental selection pressures (Hangartner *et al*. 2011b). Therefore, artificial movement of populations in line with changes in climate, without knowledge of the environmental drivers of local adaptation to current conditions, may result in adaptive mismatches in the new location (Hulme 2005; Gienapp *et al*. 2008; Rogell *et al*. 2011). Mismatches with local conditions can also occur with regards to disease exposure and resistance (Hogg *et al*. 2006). Understanding local adaptation within a species is critical for forming knowledge-based action plans in light of a changing climate; however, this step is often lacking in studies of climate change susceptibility (Hulme 2005; Sork *et al*. 2010).

1.3.2 Phenotypic plasticity

Phenotypic plasticity, here defined as the predictable changes in phenotype of a single genotype as a result of environmental cues (Via 1993), can confer a fitness advantage in temporally and spatially heterogeneous environments (Merilä *et al*. 2000b; Lind *et al*. 2011) by affording a single genotype a broader tolerance to environmental conditions (Ghalambor *et al*. 2007). Although linked to fitness in variable environments, there has been considerable debate as to whether plasticity itself is an adaptive trait, or merely a by-product of selection on different trait means in different environments (Via *et al*. 1995). Via (1993) argues that differences in plasticity between species have evolved due to historical differences in range of environmental variation experienced and thus different patterns of selection on trait values within those environments. However, Scheiner (1993) believes that plasticity of a trait is determined by separate loci from those determining the mean of a character; a consensus view has still not been reached (Ghalambor *et al*. 2007; Hallsson & Björklund 2012). Further disagreement surrounds the role of phenotypic plasticity in adaptive evolution, which has been viewed as both impeding evolution, by shielding

genotypes from selection, and promoting evolution, by generating novel phenotypes for selection to act on (Thompson 1991; Ghalambor *et al*. 2007). Regardless of the ongoing controversy surrounding phenotypic plasticity theory, the presence and scale of phenotypic plasticity is likely to be important in predicting organism survival in a changing climate (Charmantier *et al*. 2008).

The greater environmental tolerance range of organisms as a result of phenotypic plasticity has the potential to prevent local extinctions resulting from changing environmental conditions (Gienapp *et al*. 2008). Organisms that show relatively higher levels of phenotypic plasticity are thus predicted to be less susceptible to a changing climate (Hallsson & Björklund 2012). Furthermore, plasticity can act rapidly, within a lifetime or generation, depending on the trait being measured (Nussey *et al*. 2005; Charmantier *et al*. 2008); an important consideration given the unprecedented rate of recent and predicted climate change (Davis & Shaw 2001). Therefore, in order to make predictions about species-specific susceptibility to a changing climate, it is necessary to know the level and variation of phenotypic plasticity within the species (Crozier *et al*. 2008). Phenotypic plasticity is generally measured using reaction norms: plots of phenotypic trait means per genotype, across multiple environments (Via *et al*. 1995; Nussey *et al*. 2005). The slope of the reaction norm is an estimation of the level of phenotypic plasticity and can be compared between genotypes and between populations (genotype-by-environment interactions; (Via & Lande 1985; Pigliucci 2005). Some recently observed phenotypic changes that have been linked to a changing climate, including changes in breeding phenology (red squirrel: Réale *et al*. 2003; great tits: Nussey *et al*. 2005; Charmantier *et al*. 2008), migration (avian meta-analysis: Gienapp *et al*. 2007) and morphology (thin horn sheep: Loehr *et al*. 2010) have been shown to be the result of phenotypic plasticity. Crozier *et al*. (2008) used knowledge of phenotypic plasticity and local adaptation of life-history traits to make predictions about susceptibility of salmon to a changing climate. They found that some traits were likely to plastically respond to changes in climate (e.g. emergence timing, smolt migration timing), while other traits would be subject to selection (e.g.

upstream migration date, spawning date), and concluded that trait plasticity should reduce mortality in the species compared to selection acting on the same traits (Crozier *et al.* 2008). Assessing phenotypic plasticity in conjunction with local adaptation is key to understanding the range of possible evolutionary responses to environmental change and an essential step in forming effective conservation strategies (Crozier *et al.* 2008; Shama *et al.* 2011).

1.3.3 Dispersal

A dispersal, or evasion, response to a changing climate allows organisms to avoid changes in climate by moving geographically to stay within their climatic envelope (Araújo *et al.* 2004; Sork *et al.* 2010). The climatic envelope, or bioclimatic niche, of a species is the range of climatic variables that do not exceed organism tolerance limits, the location of which can be geographically mapped using knowledge of local climatic conditions (Walther *et al.* 2002; Araújo *et al.* 2006; Early & Sax 2011). Range movements of organisms in line with their climatic envelope is the easiest to measure, and most commonly recorded, response to recent climate change, with a wide range of species having spread to higher latitude and altitude areas (Parmesan 1996, 2006). However, the ability of an organism to evade changes in climate is dependent on their dispersal ability (Hulme 2005), and thus differences in dispersal ability between species is likely to be one of the predictors of winners and losers in a changing climate (Baskin 1998).

Historical species' range expansions and movements, during the period of rapid warming following the last glacial maximum, can be used to make predictions about the dispersal potential of species as a result of current global warming (MacDonald *et al.* 2008; Normand *et al.* 2011). Knowledge of post-glacial colonisation routes, rates and modes has been widely gathered within the northern hemisphere using both fossil records and molecular methods (Hewitt 1999; MacDonald *et al.* 2008; Scoble & Lowe 2010). However, the current rate of

climate change is predicted to be ten times faster than that observed during the last glacial maximum (Davis *et al*. 2005). Dispersal ability in relation to current environmental conditions, i.e. recent demography and gene flow, can be estimated using rapidly evolving DNA markers such as microsatellites and SNPs (Safner *et al*. 2010; Bryja *et al*. 2010; Hansen *et al*. 2012). Using knowledge of dispersal ability in relation to current and historical climatic conditions, combined with mathematical and geographical modelling of predicted species envelope movement, can allow predictions of species-level dispersal potential (McCarty 2001; Scoble & Lowe 2010). Such integration of approaches is key to predicting range shifts, as recent predictions using bioclimatic models alone are limited by lack of knowledge of species-level dispersal ability and thus contribute little to conservation strategies (e.g. Araújo *et al*. 2006; Mitikka *et al*. 2007; Sheldon *et al*. 2011).

Dispersal responses to a changing climate mean that current conservation measures and existing protected areas may no longer ensure survival of the species they aim to protect (Hannah *et al*. 2002). New ways of designating protected areas are now needed to reflect the dynamic nature of environment, species range and community predicted in the coming years (Araújo *et al*. 2004; Sheldon *et al*. 2011). Reserve networks, landscape connectivity and climate paths are part of the new language of conservation in a changing climate (Hannah *et al*. 2002; Early & Sax 2011). Facilitating dispersal and range movement, as well as conserving the tail-edge of species range, will be important management goals to avoid extinctions as local environmental suitability changes (Hampe & Petit 2005; Early & Sax 2011). Planning locations of dispersal corridors, predicting rate and scale of climate change, and assessing species-specific dispersal abilities is the challenge for conservationists and researchers to ensure long-term species persistence in a changing climate (Hannah *et al*. 2002; Araújo *et al*. 2004; Early & Sax 2011).

1.4 Climate change and amphibian declines

Amphibian populations are declining across the globe at an unprecedented rate (Lips *et al*. 2008), with anurans (toads and frogs), urodeles (newts and salamanders) and apodans (caecilians) collectively more threatened than any other vertebrate taxa (Stuart *et al*. 2004). Nearly a third of all known species are threatened with extinction and the number of species classed as critically endangered has doubled since the 1980s (Stuart *et al*. 2004). The sudden and widespread declines have been well documented (Corn 2005) but the causes behind the losses are complex; the result of interactions between a number of highly context-dependant causal factors, which are as yet not fully understood (Blaustein & Kiesecker 2002; Stuart *et al*. 2004). Likely causes put forward for the observed declines include direct anthropogenic effects such as habitat conversion and loss, overexploitation, introduction of invasive species, and contamination by farming run-off (Blaustein & Kiesecker 2002; Collins & Storfer 2003; Lips *et al*. 2008). However, there are also many documented cases of so-called "enigmatic" declines, where suitable habitat remains and the causes of declines are not fully understood (Stuart et al., 2004). Such enigmatic declines are of particular concern, as the standard conservation method of habitat protection does not ensure species survival (Collins & Storfer 2003). Global climate change and disease have emerged as the most commonly cited causes of enigmatic amphibian declines, both separately and in synergy (Blaustein & Kiesecker 2002; Stuart *et al*. 2004; Wake 2007; Lips *et al*. 2008).

Amphibians are particularly vulnerable to changes in climate due to the direct effect of temperature on physiological processes of ectotherms; their susceptibility to dessication; the reliance of many species on both aquatic and terrestrial habitat across multiple life-history stages; and their predicted limited ability to disperse to more suitable areas in response to changing conditions (Corn 2005; Deutsch *et al*. 2008; Blaustein *et al*. 2010). Phenological shifts resulting in earlier breeding, in response to a warming climate, have already

been observed in a range of temperate amphibians (Parmesan 2006). In the UK, Beebee (2009) reported shifts to earlier breeding in the natterjack toad, the pool frog and all native newts species over 17 years in the south of England. The common frog has also spawned progressively earlier in both the UK (Scott *et al*. 2008; Phillimore *et al*. 2010) and France (Neveu 2009). However, not all species have responded to warming climates with phenological shifts: the common toad in the UK (Beebee 1995; Arnfield *et al*. 2012), black spotted pond frogs in Japan and Korea (Primack *et al*. 2009); and four species of amphibian studied in North America (Blaustein *et al*. 2001), have not shown a trend for earlier spawning. Geographical range shifts to higher altitudes in response to rising temperatures have been recorded in tropical amphibian species (Raxworthy *et al*. 2008) and the first climate change linked extinctions of any taxa have been observed in mountaintop amphibian species in Costa Rica (Pounds & Crump 1994; Parmesan 2006). Although a causative relationship between climate change and amphibian declines is hard to prove given the observational data on which it is based (Blaustein *et al*. 2010), local extinction events and declines have been linked to both climate change trends and extreme climatic events (Blaustein *et al*. 2010). For example, McMenamin *et al*. (2008) observed severe declines of four once common amphibian species in Yellowstone National Park, USA, in response to recent climate warming and resultant wetland desiccation. Likewise, regional warming in Costa Rica (Pounds & Crump 1994; Whitfield *et al*. 2007), Queensland, Australia (Alford *et al*. 2007), and South Carolina, USA (Daszak *et al*. 2005), have been linked to major declines in amphibian species. Reading (2007) demonstrated the first causative link between a changing climate and amphibian mortality, showing that there was a significant relationship between female common toad fitness in terms of body size, fecundity and survival, and years that had above average temperatures. The broad range of observed responses to climate change highlights the uncertain and varied nature of the direct consequences of climate change on amphibians.

Potential indirect consequences of climate change on amphibians include habitat loss, changes in predator-prey interactions, alterations to community structure,

and changes in disease dynamics (Sekercioglu *et al*. 2008; Blaustein *et al*. 2010). The chyrid-thermal-optimum hypothesis states that changes in climate are driving environmental conditions towards the optimum for growth of the chytrid fungus, *Batrochochytrium dendrobatidis* (Bd; Pounds *et al*. 2006). Bd has been implicated in severe declines and extinctions of populations and species of amphibians worldwide since its discovery in the 1990s (for a review see Fisher *et al*. 2009). Although the link between climate change and spread of Bd is still disputed (Lips *et al*. 2006; Rohr *et al*. 2008), a recent paper by Raffel *et al*. (2012) is the first study to provide evidence that temperature fluctuations lead to reduced resistance to Bd in amphibians using both laboratory experiments and field observations from South America. Despite the focus of almost all recent amphibian epidemiology studies on Bd (Duffus 2009), multiple other pathogens, including ranavirus (Cunningham *et al*. 1996; Pasmans *et al*. 2008), *Aeromonas hydrophilia* (the causative agent of red-leg disease; Bradford 1991) and the pathogenic *Saprolegnia* water moulds (Bragg 1962; Blaustein *et al*. 1994; Fernández-Benéitez *et al*. 2008) have been linked to increased amphibian mortality. Such diseases have not been as widely studied as Bd and the consequences of climate change on the spread and virulence of amphibian diseases other than Bd are largely unknown.

1.5 Study system

1.5.1 Altitudinal gradients in Scotland

Latitudinal and altitudinal gradients are often used as surrogates for temperature gradients (Körner 2007) and are increasingly used for evaluating the potential consequences of climate change (Butterfield 1996; Fielding *et al*. 1999; Dunne *et al*. 2004; Karlsson *et al*. 2005; Trivedi *et al*. 2008). However, the relationship between latitude and temperature is non-linear in some study systems (e.g. across latitudes in Fennoscandia: Laugen *et al*. 2002, 2003b; Lindgren & Laurila 2009). Therefore, altitudinal gradients have been proposed as

model systems for studying the ecological effects of climate, due to rapid but predictable changes in the thermal environment over short geographical distances (Miaud & Merilä 2000; Körner 2007), with temperature decreasing on average by 6.5°C for every 1000m gain in altitude worldwide (Briggs *et al*. 1997). Altitudinal gradients can be used to assess responses to current temperature regimes (Dunne *et al*. 2004), which in turn can be used to make predictions about responses to future changes in climate at both population- and species-levels (Sork *et al*. 2010).

Scotland's upland landscapes are the result of a succession of historical glacial events (Werritty *et al*. 1994), creating the 284 Munros present across Scotland today. Munros are mountains over 3000 feet (914.4m), named after the first person to publish a list of such mountains, Sir Hugh Munro (published in 1891; Bennet & Anderson 2008). Although of modest height in a European context (Trivedi *et al*. 2008), with the highest mountain in Scotland obtaining a height of 1344m (Ben Nevis; Bennet & Anderson 2008), temperature is predicted to change by at least 5.9°C between the base and summit of a Munro (Briggs *et al*. 1997). Furthermore, the 284 Munros in Scotland offer the opportunity to look at multiple replicated altitudinal transects, situated within a limited geographical area. Finally, studies along altitudinal gradients in Scotland are not limited by habitat fragmentation due to human activities and are characterised by low human population densities (Thompson & Brown 1992; Swan *et al*. 1994; Trivedi *et al*. 2008), avoiding difficulties associated with trying to separate anthropogenic influences and habitat fragmentation from environmental influences. Therefore, the mountains of Scotland offer the ideal opportunity to assess the effect of temperature on population-level responses to climate.

1.5.2 The common frog (Rana temporaria)

The common frog (*R. temporaria*) is the most widespread temperate amphibian, with a native range that spans Europe (Merilä *et al*. 2000a). Adults range in size

from 60-90mm (Inns 2009) and are explosive communal breeders, with breeding taking place immediately after winter dormancy in response to a putative 5°C temperature activity threshold (Odin *et al*. 1983; Riis 1991; Laurila *et al*. 2001). Males congregate at freshwater breeding sites and call to elicit phonotaxis in females (Elmberg & Lundberg 1991) and on female arrival, males move rapidly to mate, with the first male to reach the female usually achieving amplexus (Elmberg 1987). Spawn (consisting of up to 2000 eggs per female) is laid in the shallowest, warmest part of the breeding pool, where egg masses join together to form communal spawn mats (Inns 2009). This communal spawning helps regulate and maintain temperatures for developing eggs (Håkansson & Loman 2004). The breeding congregation lasts for one to two weeks (Obert 1976), with tadpoles hatching one to three weeks after spawning (Scott *et al*. 2008). After breeding, adults disperse and are generally found in damp vegetation for the rest of the year (Inns 2009). Fully grown tadpoles measure around 35mm (Inns 2009) and take between three and five months to reach metamorphosis, depending on altitude and latitude (Scott *et al*. 2008). Juvenile and adult *R. temporaria* overwinter at the bottom of pools or buried in the substrate to buffer against extremely cold winter temperatures (Inns 2009) and there is some evidence that *R. temporaria* are freeze tolerant (Pasanen & Karhapää 1997), but this has been disputed (Voituron *et al*. 2005). *R. temporaria* within Europe have been described as consisting of a western and an eastern mitochondrial clade, corresponding to two separate glacial refugia (Palo *et al*. 2004b; Teacher *et al*. 2009b). Western clade individuals, consisting of the United Kingdom and parts of France, Germany, Spain and Switzerland, are postulated to have survived the last glacial maximum in a refugium in the Iberian peninsula (Teacher *et al*. 2009b). The Eastern clade consists of Fennoscandia, Italy, Poland, Austria, Estonia, Romania and Russia and parts of France, Switzerland, Germany and Spain (Palo *et al*. 2004b; Teacher *et al*. 2009b). Research on *R. temporaria* to date has been conducted predominantly in eastern clade areas.

R. temporaria in Fennoscandia belong to the eastern mitochondrial clade and have been extensively studied, particularly in relation to latitude (Merilä *et al*.

2000a; Laugen *et al*. 2002, 2003b; Alho *et al*. 2010). High levels of genetic differentiation and local adaptation in larval fitness traits have been recorded, even over short geographical distances (Miaud & Merilä 2000; Palo *et al*. 2003). Larval fitness and thus size at metamorphosis has consequences for adult survival (Altwegg & Reyer 2003) and has been found to be dependent on non-genetic maternal effects (predominantly via egg size, Laugen *et al*. 2002), genetic adaptation (Orizaola *et al*. 2010; Hangartner *et al*. 2011b) and environment experienced during development (Merilä *et al*. 2000b; Lind *et al*. 2011). Although locally adaptive phenotypes have been linked to environmental factors such as pH (Räsänen et al. 2005), active period (Laugen *et al*. 2003a) and dessication pressure (Lind & Johansson 2007), knowledge of the relationship between temperature and larval fitness adaptation is lacking due to a non-linear gain in temperature with latitude in Sweden (Laugen *et al*. 2003b). Furthermore, whether these findings hold true for western clade *R. temporaria* is as yet untested.

In Scotland, *R. temporaria* belong to the western European mitochondrial clade (Teacher *et al*. 2009b). However, Teacher *et al*. (2009b) found that, of three samples collected in South-East Scotland, two clustered more closely with Spanish than English samples, questioning the assumption that frogs colonized Scotland northwards from the Iberian refugium through England. Further work is needed to verify the phylogenetic history of Scotland's frogs in light of the small sample size and limited geographic area covered by that study. *R. temporaria* occurs throughout Scotland, including from zero to over a thousand metres along altitudinal gradients in Scotland's mountains (Inns 2009), and is the most abundant of only six native amphibian species in Scotland; two of which have high conservation status (the great crested newt, *Triturus cristatus*, and the natterjack toad, *Epidalea calamita*) and a third that is known to be declining (the common toad, *Bufo bufo*) (Inns 2009). Despite extensive study of *R. temporaria* in Fennoscandia, Scotland is the only country where delayed metamorphosis and overwintering of *R. temporaria* as larvae has been recorded, although the cues that cause this response are as yet unknown (Walsh *et al*.

2008). Although not generally threatened, some populations have seen declines since the 1960s, mostly due to habitat alteration and changes in farming practise (Scott *et al*. 2008; Inns 2009). Throughout the UK, *R. temporaria* have shifted their spawning date earlier in recent years in response to warming temperatures (Scott *et al*. 2008; Phillimore *et al*. 2010). However, few predictions have been made about the responses and susceptibility of amphibians in the UK to continuing climate change. The study of abundant species as an indication of responses of rare species allows a way to gather extensive information, without the limitation of small sample size and avoiding disturbance and removal of individuals from rare populations (Bevill & Louda 1999).

1.6 Aims of this thesis

The overall aim of this thesis was to assess population-level relationships with climate, in order to make predictions regarding susceptibility to a changing climate, focussing on *R. temporaria* in Scotland. In particular, there were four principle objectives for this thesis:

1) to predict responses of *R. temporaria* to ongoing climate change in Scotland by inferring historical and contemporary patterns of gene flow in relation to current variation in local thermal conditions.
2) to assess whether local adaptation occurs along altitudinal gradients and to identify the environmental selection pressures that drive divergent adaptation.
3) to assess the physiological and behavioural responses that facilitate survival in different thermal environments.
4) to investigate the distribution of pathogenic *Saprolegnia* water moulds sampled from *R. temporaria* egg masses.

In **Chapter 2**, I investigated the route, rate and mode of post-glacial colonisation of Scotland by *R. temporaria* using mitochondrial DNA, and contemporary patterns of gene flow and genetic diversity in *R. temporaria* in relation altitude and temperature using microsatellite markers. I also quantified the temperature experienced at high- and low- altitude breeding sites in Scotland and assessed whether there is a linear reduction in temperature with altitude. In **Chapter 3**, I asked whether local adaptation in terms of larval fitness traits had taken place in relation to altitude, and whether phenotypic plasticity varied with altitude, using common garden experiments. I then used locally adapted traits and environmental measurements to identify the environmental drivers of local adaptation to altitude. In **Chapter 4**, I investigated the physiological and behavioural responses that facilitate survival at high-altitude, low-temperature environments. In a common environment I assessed whether tadpoles from high-altitude differed physiologically from those from low-altitude in terms of routine metabolic rate and freeze tolerance. Using spawning date and temperature data recorded in the field, I examined whether adults at high-altitude extend the time available for larval growth by breeding at lower temperatures than low-altitude individuals. Finally, in **Chapter 5**, I examined whether species composition of the pathogenic water mould, *Saprolegnia,* collected from infected *R. temporaria* eggs, varied within- and between-sites and assessed whether presence of *Saprolegnia* species, or genetic distance between water mould samples, related to geographical or environmental parameters. In **Chapter 6**, I brought together the findings of my thesis to make predictions about the responses and susceptibility of *R. temporaria* in Scotland to a changing climate and discussed the broader implications of my work.

Chapter 2 – Using genetic variation to infer recent and historical associations with climate in the common frog, *Rana temporaria*

2.1 Abstract

Recent and historical species' associations with climate can be inferred using molecular markers. This knowledge of population- and species-level responses to climatic variables can then be used to predict the potential consequences of ongoing climate change. The aim of this paper was to predict responses of *Rana temporaria* to environmental change in Scotland by inferring historical and contemporary patterns of gene flow in relation to current variation in local thermal conditions. We first inferred colonisation patterns within Europe following the last glacial maximum by combining new and previously compiled mitochondrial DNA sequences. Second, we assessed fine-scale genetic variation in relation to current temperature gradients using microsatellites, from frogs sampled from altitudinal gradients. We found that the mitochondrial DNA sequences from our Scottish samples were identical to (92%), or clustered with, the common haplotype previously identified from Western Europe. This clade showed very low mitochondrial genetic variation, consistent with a leptokurtic model of range expansion, where low numbers of long-distance dispersers cause multiple founder events. No population structure was found using microsatellites within or between altitudinal gradients at any scale (3-50km; average F_{ST}= 0.02), despite a mean annual temperature difference of 4.5°C between low- and high-altitude sites. Levels of genetic diversity and heterozygosity were considerable but did not vary by site, altitude or temperature. The panmictic population observed, even along temperature gradients, is a potentially positive sign for *R. temporaria* persistence in Scotland in the face of a changing climate. This study demonstrates that even within taxonomic groups thought to be at high-risk from environmental change, levels of vulnerability can vary.

2.2 Introduction

Worldwide, ecological responses to recent climate change have been well documented in a broad range of terrestrial and marine taxa (Parmesan & Yohe 2003; Perry *et al*. 2005), with records of species-level responses predominantly falling into two categories: range movements and phenological changes (Visser 2008). Range movement responses are dependent on the dispersal ability of the species in question and the availability of suitable habitat (Perry *et al*. 2005; Gienapp *et al*. 2008). The underlying mechanisms of phenological changes are more difficult to determine (but see Loehr et al. 2010; Phillimore et al. 2010), but could be due to phenotypic plasticity (where environmental cues act on the phenotype without genotypic changes) (Merilä *et al*. 2000b) and/or natural selection (Loehr *et al*. 2010). The potential for species to respond to environmental change via migration, plasticity and/or evolution is an important consideration when developing conservation strategies (Hulme 2005; Sork *et al*. 2010). Such responses can be predicted based on historical and recent species- and population-level relationships with environmental factors (Sork *et al*. 2010; Hope *et al*. 2011).

Predictions of future responses to climate change, using inferred historical responses to postglacial warming based on fossil records and, more recently, using molecular methods (Petit *et al*. 2008), have been formed for a range of plant (eg. worldwide tree species: Petit et al. 2008; American beech and red maple: McLachlan et al. 2005) and animal species (eg. mountain voles and northern pocket gophers: Hadly et al. 2004; freshwater gastropods: Cordellier & Pfenninger 2009; Holarctic shrews: Hope et al. 2011). Genetic variation between organisms from different geographic locations within modern ranges can be informative about routes, rates and modes of range expansion following the last glacial maximum (Palo *et al*. 2004b; McLachlan *et al*. 2005) and reflects both historic patterns of colonisation and recent demographic processes (Hewitt 1999). Placing current population structure within a phylogeographic background

creates a context from which ecological factors can be related to genetic patterns (Hewitt 1999; Palo *et al*. 2004b).

Environmental gradients have been widely used to explore the relationship between ecological variables and organismal traits (for review see Fukami & Wardle 2005) and are increasingly being used to predict impacts of climate change (Dunne et al. 2004). By exploring the association of rapidly evolving genetic variation (using DNA markers such as microsatellites) and climatic variation, it is possible to infer population-level demography in relation to local environmental conditions (Sork *et al*. 2010). Spatial structure can then be used to predict responses to temporal changes in environmental conditions (Sork *et al*. 2010), so called "space-for-time" analyses (Dunne *et al*. 2004; Fukami & Wardle 2005), and to evaluate species-level vulnerabilities (Sork *et al*. 2010). For example, Sork *et al*. (2010) combined knowledge of historical colonisation and spatial genetic variation in relation to fine-scale climatic variation along a range-wide latitudinal gradient to predict the potential for tolerance, evolution or migration of valley oak (*Quercus lobata*) in response to future climate change. Similarly, environmental gradients have been used to relate genetic patterns to environmental variables in light of a changing climate in butterflies (Zakharov & Hellmann 2008), redband trout (Narum *et al*. 2010), tropical rainforest trees (Rossetto *et al*. 2009), lizards (Nunes *et al*. 2011) and insects (Hodkinson 2005 and references therein), among others. Altitudinal gradients have been proposed as particularly suitable for studying responses to climatic variables due to the rapid change in environmental conditions over short geographical distances (Miaud & Merilä 2000; Körner 2007). However, it is important to quantify at a local level the relationship between altitude and the environmental variable of interest (for example, temperature) to ensure suitability as a surrogate, a step often lacking in environmental gradient studies (Stahlberg *et al*. 2001; Körner 2007).

Since temperature directly influences physiological processes in ectotherms (Carey & Alexander 2003; Laugen *et al*. 2003a), studying ectotherms along

temperature gradients offers an opportunity to examine how exposure to a climatic variable with a potentially strong selection pressure influences demography and genetic variation (Laugen *et al*. 2003a). Amphibians are predicted to be particularly affected by environmental change due to their dual-phase life history strategy, low dispersal abilities and high site fidelity (Carey & Alexander 2003; Sagvik *et al*. 2005). Changes in phenology and range shifts have already been observed in amphibians and species-level extinctions have been linked to climate change (Parmesan 2006). However, species- and population-level differences can exist within taxonomic groups in terms of vulnerability to changing environmental conditions (Walther *et al*. 2002; Somero 2010). Identifying variability in climate change response, alongside the causes and consequences, is important for understanding vulnerability (McCarty 2001). Species with broad distributions experience a range of environmental conditions and selective pressures, making them ideal candidates for studying spatial population variation (Miaud & Merilä 2000; Bonin *et al*. 2006).

The common frog, *Rana temporaria* (Anura: Ranidae), is the most widespread amphibian in Europe and hence subject to a wide variety of environmental conditions throughout its range (Laugen *et al*. 2003b). High levels of genetic differentiation and phenotypic variation in larval fitness-traits have been recorded in *R. temporaria* along latitudinal gradients in Fennoscandia, even over short geographical distances (Miaud & Merilä 2000; Palo *et al*. 2003), but population structure has rarely been assessed elsewhere in Europe (but see Brede & Beebee 2004; Safner *et al*. 2010). Furthermore, the relationship between temperature and genetic differentiation along latitudinal gradients in Fennoscandia have been confounded by a non-linear thermal cline (Laugen *et al*. 2003b). *R. temporaria* occurs along altitudinal gradients in Scotland from zero to over a thousand metres above sea level and is the most abundant of only six native amphibian species. The mountains of Scotland are an ideal system for studying the effect of local thermal environment on *R. temporaria* as they offer replicated altitudinal transects that have a linear relationship with temperature

(Trivedi *et al*. 2008), consisting of continuous habitat without human-induced barriers to dispersal (Thompson & Brown 1992).

The ultimate aim of this paper was to predict responses of *R. temporaria* to ongoing climate change in Scotland by inferring historical and contemporary patterns of gene flow in relation to current variation in local thermal conditions. Specifically, we addressed the following questions: 1) What was the route, rate and mode of post-glacial colonisation of Scotland by *R. temporaria*?; and 2) Are contemporary patterns of gene flow and genetic diversity in *R. temporaria* related to altitude and temperature?

2.3 Methods

2.3.1 Sampling and DNA extraction

For mitochondrial DNA sequencing, 39 metamorph and juvenile *R. temporaria* were collected in Scotland between August and September 2010 (Figure 2-1). Samples were collected from 15 low elevation sites chosen to cover a broad geographical area, as well as two high altitude sites (LO and BL). Individuals were transported back to the laboratory and euthanized by emersion in 10 mg l^{-1} MS222 for fifteen minutes, according to Schedule 1 UK Home Office methods. Muscle tissue was dissected from hind legs and stored in RNA-later (Qiagen Inc., Crawley) until DNA extraction.

For microsatellite genotyping, tissue samples were collected from paired high- (over 700m above sea level) and low-altitude (below 300m) sites from five mountain transects within Scotland (Figure 2-2). Site names refer to the study mountain and whether high- or low-altitude, e.g. Ben Lomond High. The study was set within a limited geographical area (maximum distance between sites

was 50km), to minimise the effect of latitude and longitude and highlight the effect of altitude. Within this area, study mountains were chosen based on presence of known high- and low-altitude breeding sites, mountain height and accessibility. A single egg was collected from thirty spawn clumps, each clump putatively corresponding to a single a female (Lind & Johansson 2007). Eggs were brought back to the laboratory and allowed to develop to Gosner stage 22 (Gosner 1960). Tadpoles were euthanized by emersion in 10 mg l^{-1} MS222 for 15 minutes. Tissue samples from tadpole tail clips were stored in RNA-later (Qiagen Inc., Crawley) until DNA extraction. For two sites (Beinn Ime Low and Meall Nan Tarmachan Low) eggs were collected from only ten spawn clumps but additional buccal swabs (Barloworld Scientific) were collected from 30 and 22 breeding adults, respectively. Although ideally adults and tadpoles would have been sampled from all sites, low encounter rates with adults at eight of the sites meant that this was not possible.

DNA extractions were carried out using DNeasy Blood and Tissue Extraction kits (Qiagen Inc., Crawley) following the standard protocol, but performing two separate elutions by adding 50 μl (muscle and tail tissue) or 20 μl (buccal swabs) of Buffer AE and incubating at room temperature for five minutes, before centrifuging at 8000 rpm and 14000 rpm, respectively, for one minute.

2.3.2 Historical phylogeography based on mtDNA sequences

2.3.2.1 mtDNA Sequencing

For assessment of broad phylogeographic patterns, mitochondrial DNA was amplified via conserved primers developed for use with ranids that target a 605 base pair segment of cytochrome b: L14850 (5° - TCTCATCCTGATGAAACTTTGGCTC-3°) and H15410 (5° - GTCTTTGTAGGAGAAGTATGG-3°)(Tanaka *et al*. 1994; Teacher *et al*. 2009b).

Cytochrome b was chosen to enable comparisons with previously published phylogeographic studies (Palo *et al*. 2004b; Teacher *et al*. 2009b). Polymerase chain reactions (PCR) were performed in a 20μl reaction volume containing: 2.5 mM $MgCl_2$ (Invitrogen), 1 x PCR Buffer (Invitrogen), 0.2 mM dNTP (Invitrogen), 0.1 μM forward primer, 0.1 μM reverse primer, 0.5 units of Taq polymerase (Invitrogen) and 1 μl of DNA template. Initial denaturisation took place at 95°C for 6 minutes; followed by 30 cycles of 95°C for 30 seconds, 52°C for 40 seconds and 72°C for 40 seconds; with a final extension step of 72°C for 7 minutes. Amplified samples were cleaned with ExoSAP-IT (USB, Cleveland), according to the manufacturer's instructions, and sent to the GenePool core genomics facility at the University of Edinburgh, where they were sequenced on an ABI 3730 automated sequencer.

2.3.2.2 Sequence alignment and haplotype network construction

Sequences were aligned and base-calling errors corrected using Sequencher v4.5 (Gene Codes Corporation, Ann Arbour) and matched to published haplotype sequences in Genbank using BLAST. Sequences described in Teacher *et al*. (2009b) (65 sequences sampled from 15 countries across Europe, accession numbers FJ030808- FJ030872) and Palo *et al*. (2004b) (seven sequences sampled from 12 countries throughout Europe; accession numbers AY619558-AY619564) were downloaded and aligned with the new Scottish sequences using MacClade v4.06 (Maddison & Maddison 2003). Alignments were trimmed to the length of the shortest sequences available. Collapse v1.2 (Posada 1998) was used to collapse sequences into unique haplotypes. Although the original papers reconstructed phylogenetic trees to depict broad relationships among sequences (Palo *et al*. 2004b; Teacher *et al*. 2009b), many haplotypes are separated by only a single nucleotide substitution. We thus reanalysed the combined dataset using a statistical parsimony-based approach (with a 95% connection limit) to construct a haplotype network, as implemented in TCS v1.18 (Clement *et al*. 2000).

2.3.3 Contemporary population structure in relation to altitude and temperature

2.3.3.1 Temperature variation within and between altitudinal gradients

In order to assess the role of temperature in influencing patterns of gene flow and genetic diversity within and between altitudinal gradients, we quantified temperature variation by site. Between March 2010 and October 2011, Thermocron i-buttons (Dallas Semiconductor/ Maxim, London) recorded air temperature every two hours. Data was downloaded to a laptop using a USB i-button adapter (Dallas Semiconductor/ Maxim, London) and the software, Thermodata viewer (Thermodata pty Ltd., Melbourne). *R. temporaria* spawn immediately after winter dormancy, in response to temperature cues (Beebee 1995). Therefore, spring is an important time in the *R. temporaria* lifecycle and will dictate breeding timing and early developmental environment (Loman 2002). Thus, mean spring temperatures as well as mean annual temperatures were used in analyses. Mean annual temperature was calculated by site in R v2.12.1 (R core development team), first by calculating daily mean air temperature, then averaging over 365 days. Mean spring temperatures were calculated by averaging the monthly mean temperatures for March, April and May per site. A linear regression was performed for mean annual temperature and mean spring temperature against altitude (m).

2.3.3.2 Microsatellite genotyping

Twenty four *R. temporaria* microsatellite loci were chosen from the 145 described by Matsuba & Merilä (2009) based on levels of polymorphism, core motif repeat (tri- and tetra-nucleotide repeats only) and allelic size range to enable multiplexing. Reliability of amplification was first assessed using unlabelled primers and DNA from three individuals representing a broad

altitudinal and latitudinal range (Ben Lomond High, Beinn Ime Low and RO; Figures 2-1 and 2-2). PCR was carried out in a 20μl reaction volume containing: 2.5 mM $MgCl_2$ (Invitrogen), 1 x PCR Buffer (Invitrogen), 0.2 mM dNTP (Invitrogen), 0.1 μM forward primer, 0.1 μM reverse primer, 0.5 units of Taq polymerase (Invitrogen) and 1 μl of DNA template. PCR was performed on Peltier Thermal Cyclers (MJ Research) using the protocol: 95°C for 15 minutes; followed by 30 cycles of 94°C for 1 minute, 58°C for 90 seconds and 72°C for 1 minute; with a final extension step of 60°C for 30 minutes.

Twelve loci that amplified reliably were selected and divided into two multiplex panels (Table 2-1). The forward primer of each pair was labelled with the fluorescent dyes (MWG/Operon, Eurofins): ATTO550 (yellow), FAM (blue) and HEX (green); to be used with a ROX-labeled (red) GS500 size standard. Samples were amplified alongside negative controls by multiplex PCR using Qiagen Multiplex PCR mixes (Qiagen Inc, Crawley) using the default reagent concentrations recommended by the kit instruction manual. PCR was performed using the protocol: 95°C for 15 minutes; 35 cycles of 94°C for 30 seconds, 58°C for 90 seconds and 72°C for 90 seconds; and a final extension step of 72°C for 10 minutes. PCR products were genotyped on an ABI 3730 (by the Sequencing Service, University of Dundee) and analysed using GENEMAPPER v3.7 (Applied Biosystems). Ten percent of samples were re-amplified and genotyped to verify results. Loci with data missing for more than 10% of individuals were excluded from analyses. Each population and locus was tested for deviation from Hardy-Weinberg equilibrium, null alleles and linkage disequilibrium using Arlequin v3.5 (Excoffier & Lischer 2010) and Microchecker v2.2.3 (Van Oosterhout *et al*. 2004); significance was assessed after Bonferroni correction for multiple tests. Loci that were flagged as showing null alleles or with significant deviation from Hardy-Weinberg equilibrium in multiple populations were removed from further analysis. Pairs of loci showing linkage disequilibrium were compared with the *R. temporaria* linkage map published by Cano *et al*. (2011), any pairs of loci that were on the same chromosome were removed from the analysis.

2.3.3.3 Analysis of spatial patterns of genetic diversity

Individual- and population-level microsatellite summary statistics were calculated as allelic richness (A_R), expected heterozygosity (H_e) and observed heterozygosity (H_o) using Microsatellite Analyser (Dieringer & Schlotterer 2003). Separate Analyses of Molecular Variance (AMOVA) based on population, altitudinal grouping, and genetic clusters identified by STRUCTURE (see below), were run in Arlequin v3.5 with 16000 permutations to evaluate sources of genetic variation. General linear models were used in R v2.12.1 to test whether individual-level diversity varied by altitude, site, or their interaction; or by temperature, using mean annual temperature and mean spring temperature. Separate tests were conducted for each summary statistic: A_R, H_e and H_o.

Considerable debate exists in the literature over the relative merits of using F_{ST} and Jost's D (Jost 2008) as measures of genetic diversity (Leng & Zhang 2011). F_{ST} has been criticised due to its sensitivity to within-population genetic variation (Jost 2008). However, recently D_{est} has been criticised as it is independent of population size and thus infers little about population demography (Whitlock 2011; Meirmans *et al*. 2011). Given these issues, we considered both genetic differentiation measures to elucidate population structure (Leng & Zhang 2011; Meirmans *et al*. 2011). Pairwise genetic differentiation between populations were estimated using F_{ST} values in Arlequin v3.5 and significance obtained through permutation tests adjusted using Bonferroni corrections. Pairwise population D_{est} (Jost 2008) was calculated using SMOGD v 1.2.5 (Crawford 2010). Distances between sites were measured using ArcGIS 10 (Esri, 1995-2012). The presence of a pattern of isolation by distance was tested in Arlequin v3.5 via a partial Mantel test using F_{ST} as the dependent variable and geographic distance and altitudinal difference (Table A1, Appendix A) as independent variables, with 10,000 permutations. The presence of a pattern of isolation by temperature was tested using Mantel tests of correlation between genetic distance (F_{ST}) and mean annual temperature/ mean spring temperature differences between each site.

STRUCTURE v2.3.3 (Pritchard *et al*. 2000) was used to infer the presence of genetic clusters (putative populations) based on the multi-locus genotype data. The programme was run assuming admixture and correlated allele frequencies with 100,000 burn-in cycles and 1,000,000 Markov Chain Monte Carlo runs (MCMC). The number of potential populations within the sample (K) was considered for 1-15, with ten replicates per K. This was repeated with and without sample origin priors. Likelihood values and variance among the ten replicates per K plus ΔK were calculated using STRUCTURE HARVESTER v 0.6.8 (Earl & VonHoldt 2011). ΔK measures the rate of change in the log probability of the data between successive K values (Evanno *et al*. 2005). The number of genetic clusters that best fit the data was decided based on the likelihood, variance and ΔK statistics. A hierarchical approach was used to assess whether there was evidence of fine-scale genetic structure when highly differentiated populations were removed from the analyses (Blouin *et al*. 2010).

2.4 Results

2.4.1 Historical phylogeography based on mtDNA sequences

Four haplotypes were identified from the 39 individuals sequenced for a 481 bp region of cytb from 16 sites across Scotland. Thirty-six individuals displayed the same haplotype, which was also the most commonly observed haplotype in western European samples surveyed by Teacher *et al*. (2009b; Haplotype 1) and Palo *et al*. (2004b; West 1). Three haplotypes did not exactly match any previously published sequences but these haplotypes each differed from the common haplotype by only a single synonymous base pair change at the third codon position. Novel haplotypes were observed in single individuals from three different populations (CF, TM and GA; Figure 2-1) that each also contained the common haplotype.

Consistent with previous phylogenetic analyses (Palo *et al*. 2004b; Teacher *et al*. 2009b), European-wide samples showed deep divergence between east and west, resulting in two genetically disconnected clades in the haplotype network, although some regions include haplotypes from both clades (Table 2-2). The Eastern clade consisted of all haplotypes from Fennoscandia, Italy, Poland, Austria, Estonia, Romania and Russia plus some samples from France, Switzerland, Germany and a single sample from Spain. Since the new Scottish samples all fell into the western clade, we focused on this clade for further analyses (Figure 2-3). Haplotype network analysis revealed very low levels of variation between haplotypes in this clade, with the majority only showing a single synonymous third codon base pair substitution from the common western haplotype (Figure 2-3). Three samples described in Teacher *et al*. (2009b; from Scotland, Spain and France) showed two synonymous third codon base-pair substitutions from the common haplotype. The number of haplotypes observed in each country varied between one and six, with Scotland showing the highest, but also the highest number of samples (Scotland: n=39, mean number of samples per country excluding Scotland=8.66; Table 2-2).

2.4.2 Contemporary population structure in relation to altitude and temperature

2.4.2.1 Temperature variation within and between altitudinal gradients

Mean annual temperature was on average 4.51°C lower, and mean spring temperature on average 3.85°C lower, at high- than low-altitude sites (Table 2-3). Mean annual temperature and mean spring temperature both showed a significant negative linear relationship with altitude (r^2=0.98, p=0 and r^2=0.87, p=0, respectively; Figure A1, Appendix A). Based on the regression analysis, mean annual temperature was predicted to decrease by 0.65°C and mean spring temperature by 0.53°C for every 100m gain in altitude.

2.4.2.2 Microsatellite genotyping

Nine sites (254 samples in total) were genotyped at 12 microsatellite loci. Unfortunately, samples from Ben Lomond Low could not be confidently typed despite multiple attempts, potentially due to low quality DNA. Due to varying levels of mortality, between 17 and 40 individuals were typed from each of the remaining nine sites (Mean=28.2; Table 2-3). Three loci (BFG157, BFG250 and BFG254) were removed from the analysis as they had failed in over 10% of individuals. Locus BFG048 was identified as having a null allele in Beinn Dubhchraig High and being significantly out of Hardy-Weinberg equilibrium in this population, as well as in both Beinn Ime sites (<0.0056 after Bonferroni correction), and was removed from analyses. Locus BFG258 was significantly out of Hardy-Weinberg equilibrium in Ben Lomond High (p=0.0026) but as this was found in only a single population, we did not remove the loci from further analyses. Using the *R. temporaria* linkage map (Cano *et al*. 2011), no loci flagged as potentially being in linkage disequilibrium (after Bonferroni correction) were found to be on the same chromosome. However, BFG106 and BFG207 were not shown on the published linkage map and BFG106 showed significant linkage disequilibrium with BFG161 in Beinn Ime Low and BFG207 showed significant linkage disequilibrium with BFG099 in Ben Lawers High. Nevertheless, since this was found in only a single population for each, we included these loci in the analyses.

2.4.2.3 Analysis of spatial patterns of genetic diversity

All populations exhibited considerable levels of genetic diversity and heterozygosity (A_R=5.75±0.87, H_e=0.64±0.036; Table 2-3). Lack of significant deviation from Hardy-Weinberg equilibrium (except for BFG258 in Ben Lomond High) and levels of observed heterozygosity (H_o=0.64±0.034; Table 2-3) were consistent with random breeding. Analyses of molecular variance showed that most variation was found within rather than between populations

(within=97.78%; between=2.22%), within rather than between high- and low-altitude groupings (within=99.56%; between=0.44%) and within rather than between genetic clusters identified by STRUCTURE analysis (within=97.79%; between=2.21%). No significant differences in genetic diversity or individual heterozygosity were found based on site (A_R: p=0.89; H_e: p=0.51; H_o p=0.7), altitude (A_R: p=0.99; H_e: p=0.85; H_o p=0.99), or their interaction (A_R: p=0.95; H_e: p=0.71; H_o p=0.84). No significant variation with temperature was found for genetic diversity or individual heterozygosity with either mean annual temperature (A_R: p=0.49; H_e: p=0.95; H_o p=0.46) or mean spring temperature (A_R: p=0.46; H_e: p=0.87; H_o p=0.31).

The pairwise F_{ST} values were universally low (mean=0.02 ± 0.017), showing little evidence of population subdivision (Table 2-4). Nevertheless, pairwise F_{ST} values were significant, following Bonferroni correction, for 14 out of 36 comparisons. The highest level of divergence was found for the Meall nan Tarmachan sites: both high- and low-altitude sites were significantly different from most other populations (ten of the significant comparisons; Table 2-4). However, Meall nan Tarmachan High did not show a significant difference from Ben Lawers Low, and Meall nan Tarmachan Low was not significantly differentiated from Ben Lawers High or Low. Meall nan Tarmachan is geographically closer to Ben Lawers than any of the other study mountains, with less than 5km separation (Figure 2-2; Table 2-4). Even though F_{ST} values between these populations were significant, they were still low (highest F_{ST}=0.07, between Meall nan Tarmachan Low and Beinn Ime High; mean significant F_{ST}=0.037 ± 0.012). There were no cases of significant high- and low-altitude differentiation within a study mountain and genetic distance did not correlate with differences in altitude (Mantel's r=-0.09, p=0.731). However, F_{ST} based isolation by distance was significant in terms of geographic distance, with genetic distance and geographic distance showing a significantly positive relationship (Mantel's r=0.423, p=0.003), but only explaining 17.9% of the observed genetic distances. Isolation by temperature was non-significant for mean annual temperature (Mantel's r=-0.086, p=0.651) and

mean spring temperature (Mantel's r=-0.050, p=0.577). Jost's D_{est} showed similar patterns as those based on F_{ST} (Table A1, Appendix A).

From the Bayesian cluster analysis using STRUCTURE, ΔK gave a clear strong peak at K=2, suggesting that the nine sites formed two genetic clusters (Figure 2-4A). This was supported by the likelihood values, which peaked at K=2 and K=3, but only K=2 showed high consistency over repeats, leading to a low variance (Figure 2-4B). Based on K=2, a STRUCTURE barplot revealed that samples from Meall nan Tarmachan formed a separate genetic cluster from all other sites (Figure 2-4C). This is in line with the significant differentiation seen at this site using F_{ST}. However, Meall nan Tarmachan still showed high levels of admixture with the genetic cluster common to the other sampled sites (Figure 2-4C). These results did not vary according to whether site of origin was or was not included as a prior in the analysis, although ΔK using origin as a prior gave a stronger peak at K=2 than without, as expected when population differentiation is low (Hubisz *et al.* 2009). Hierarchical analysis, excluding Meall nan Tarmachan, did not reveal any hidden subdivision between the other sites.

2.5 Discussion

2.5.1 Historical phylogeography based on mtDNA sequences

R. temporaria within Europe have been described as consisting of a western and an eastern clade, corresponding to two separate glacial refugia (Palo *et al.* 2004b; Teacher *et al.* 2009b). We sampled from a broad geographical area within Scotland, and found that 92% of samples were identical to the common western haplotype, seen throughout western Europe (Figure 2-1). Three novel haplotypes were identified from distant locations within Scotland, none of which matched the haplotypes previously identified in Scotland by Teacher *et al.* (2009b). Teacher *et al.* (2009b) found that, of three samples collected in

southern Scotland, two clustered more closely with Spanish than English samples and none matched the common western haplotype, questioning the assumption that they had recolonized northwards from the Iberian refugium through England. However, overall, the phylogeny was not well-resolved, with the node for the common western haplotype showing particularly low support values with all tree-building methods. In our study, all novel haplotypes differed by only a single base pair from the common western haplotype, meaning that the majority of Scottish samples were the same haplotype as those found in England and more closely related to English than Spanish *R. temporaria* (Figure 2-3).

The low mitochondrial diversity within the western clade as a whole (maximum two base pair separation from the common haplotype; Figure 2-3) points to rapid post-glacial colonisation. Leptokurtic expansion, where low numbers of long-distance dispersers cause multiple founder events, is expected to be accompanied by loss of genetic diversity (Schmitt 2007) and would be consistent with patterns seen in the western clade. The lack of genetic variation among mitochondrial sequences does not allow us to reconstruct colonisation routes to Scotland, but does allow us to confidently interpret patterns of current fine-scale population structure in Scotland in the absence of confounding historic variation.

2.5.2 Contemporary population structure in relation to altitude and temperature

The low dispersal ability and high site philopatry of amphibians leads to expectations of high levels of genetic differentiation among populations even over short geographical distances (Sagvik *et al*. 2005; Safner *et al*. 2010) and has been demonstrated in numerous species (e.g. *R. sylvatica:* Newman & Squire 2001; *R. cascadae:* Monsen & Blouin 2003; *R. arvalis:* Knopp & Merilä 2009; *Bufo bufo:* Brede & Beebee 2004), including *R. temporaria* (Sweden: Johansson *et al*. 2006; Richter-Boix *et al*. 2010; Fennoscandia: Palo, *et al*. 2004b) . No evidence

of genetic isolation between populations was found at any scale in our system, despite assessing population structure from the scale of 3km within altitudinal gradients to up to 50km between mountains (Table 2-4). Pairwise F_{ST} estimates between populations ranged from -0.012 to a high of 0.07, with an average of 0.02 (Table 2-4). F_{ST} values of 0.1 are generally interpreted as evidence for weak population structure (Meirmans & Hedrick 2011); all our values of F_{ST} were substantially below this threshold, including those involving Meall nan Tarmachan, which formed a distinctive genetic cluster in the STRUCTURE analyses (Figure 2-4). Meall nan Tarmachan is not obviously geographically isolated from all the other mountains and in fact there is less distance between Meall nan Tarmachan and Ben Lawers than between any other two study mountains (less than 5km; Table 2-4, Figure 2-2). The partially separated Meall nan Tarmachan cluster could be due to gene flow with a population not sampled for this study. More extensive sampling, particularly north of Meall nan Tarmachan, would be needed to look for admixture with other populations. Although a weak but significant pattern of isolation by distance was found within the study system as a whole, most molecular variance was observed within rather than between populations or genetic clusters. This lack of population structuring suggests that there are no barriers to gene flow at any scale within our system.

Latitudinal and altitudinal gradients are often used as surrogates for temperature gradients (Körner 2007), but the assumption that temperature decreases linearly with increasing altitude and latitude is rarely quantified on a local scale (Stahlberg *et al.* 2001; Körner 2007). In our study system, altitude and temperature showed a significant negative linear relationship (Figure A1, Appendix A), making this system suitable for comparisons of genetic- and temperature-variation. Our data, showing a decrease in mean annual temperature of 0.65°C for every 100m gain in altitude, is in line with globally predicted lapse rates of -6.5°C per 1000m gain in altitude (Briggs *et al.* 1997). No significant population differentiation was found within any of the altitudinal gradients and there were no isolation by altitude or temperature effects found

using Mantel tests, despite a difference of 4.51°C in mean annual temperature and 3.85°C in mean spring temperature between high- and low-altitude sites. Considerable levels of genetic diversity and heterozygosity were found at all sites and there was no evidence of inbreeding at any site (Table 2-3). Most molecular variance was within rather than between altitudinal groupings and no significant differences in genetic diversity or heterozygosity were found based on site, altitude or temperature parameters. This is surprising as, although there is continuous terrestrial habitat between the high- and low-altitude sites within a mountain, there are usually no breeding pools at intermediate elevations due to the steepness of the incline. To account for the observed lack of population isolation by altitude, dispersers would have to move directly from high- to low-altitude (or visa versa) between breeding seasons, or between metamorphosis and their first breeding season. As mean annual temperatures decreased by 0.65°C and mean spring temperatures decreased by 0.53°C for every 100m gain in altitude, dispersers must have the ability to cope with substantial changes to their thermal environment. Our results suggest that *R. temporaria* has a greater dispersal ability and is less site philopatric than previously postulated (Sagvik *et al*. 2005; Safner *et al*. 2010) and that individuals originating in one temperature regime can successfully breed in another.

Although it is unusual for an amphibian species to show such weak population structuring at this scale, it is in line with the low variability in mitochondrial DNA seen throughout the western clade, and with the small number of studies that have looked at fine-scale genetic differentiation of *R. temporaria* from other areas putatively within the western clade. In the northern French Alps, Safner et al. (2010) sampled 11 populations within 135km^2, between elevations of 283m and 820m, and found population pairwise F_{ST} values that were higher than in our system, but still low, with a mean of 0.065 (Safner *et al*. 2010). In southern rural England, Brede and Beebee (2004) found low levels of population differentiation (mean pairwise F_{ST}=0.051) between populations over scales from 2.5 to 12km and Teacher *et al*. (2009a) did not find significant neutral genetic structuring between ten populations in a 100km^2 sampling area. Although

Hitchings & Beebee (1997) found that urban populations of southern English *R. temporaria* were differentiated from one another (median F_{ST}=0.35) over distances averaging 2.3km, there was only weak differentiation among rural populations over much greater distances (average distance=41.3 km; median F_{ST}=0.11). The cause of the urban population structuring was postulated to be anthropogenic barriers. The low population differentiation observed in western clade *R. temporaria* is in contrast to the high degree of population subdivision commonly observed in eastern clade areas, even over short geographical distances (Palo *et al*. 2004b). For example, Palo *et al*. (2004b) found high population structuring in Fennoscandia across scales ranging from 3.5km to 2200km, with an overall F_{ST} value of 0.23 (see also Palo *et al*. 2003, 2004a; Johansson *et al*. 2006; Richter-Boix *et al*. 2010).

The cause of the putative *R. temporaria* eastern-western clade division in levels of population structuring is unclear. The eastern clade has lower haplotype diversity than the western clade (Palo *et al*. 2004b) and Fennoscandia was colonised later than western Europe (due to its more northerly location it remained covered by ice sheets for a longer period; Hewitt 1999), suggesting that it should show lower inter-population diversity rather than higher. More fragmented landscapes and barriers to dispersal could account for increased population isolation in the eastern clade, but the study by Palo *et al*. (2004b) included pristine northern Fennoscandian habitats; thus, this explanation seems unlikely. Differences between clade dispersal abilities could cause different patterns of structuring, but levels of diversity and the star-like phylogeny in both clades are consistent with rapid post glacial colonisation (eastern clade: Palo *et al*. 2004b; western clade: this study). Alternatively, levels of neutral population variation have often been used as an indicator of adaptive genetic divergence (Leinonen *et al*. 2008). Thus, the presence of local adaptive genetic divergence in the eastern but not western clade is one hypothesis for why eastern clade populations show higher subdivision. However, inferring potentially adaptive divergence from neutral genetic divergence has been widely criticised (e.g. McKay & Latta 2002). Therefore, experimental assessment of the presence of

local adaptation is needed in the western clade for comparisons with eastern clade *R. temporaria*, where local adaptation has been demonstrated in a range of life history traits (Laugen *et al*. 2003b; Lind & Johansson 2007). Results from experimental analysis of Scottish *R. temporaria* show local adaptation in larval fitness traits despite lack of population structuring (Chapter 3). Thus, low genetic structure does not necessarily imply lack of adaptive divergence in western clade *R. temporaria*. Therefore, the cause of the *R. temporaria* eastern-western clade division in levels of neutral genetic divergence remains to be explained.

2.6 Conclusion

Recent and historical associations with climate can be inferred using molecular markers. This knowledge of population- and species-level responses to climatic variables can then be used to predict the potential consequences of ongoing climate change. The panmictic population observed in our study even along altitudinal gradients is a potentially positive sign for *R. temporaria* persistence in Scotland in the face of a changing climate. The high genetic diversity and lack of inbreeding observed, coupled with on-going gene flow, will provide flexibility under changing climatic selection regimes. The lack of population structuring in our system suggests lower site philopatry and higher dispersal ability than recorded for other amphibians or indeed for *R. temporaria* in other parts of their range. Rapid post-glacial colonisation was also inferred to have potentially consisted of low numbers of long distance dispersers. Therefore, given available habitat, *R. temporaria* should have the potential to evade climate change through range shifts. However, further research is needed to assess how *R. temporaria* will potentially be affected by changes in climatic variables other than temperature.

Table 2- 1: Multiplexed microsatellite panels showing panel grouping, locus name, fluorescent dye used to label forward primer and published (Matsuba & Merilä 2009) allelic size range.

Panel	Locus	Dye	Size range
A	BFG053	FAM	147-183
A	BFG106	ATTO550	142-198
A	BFG142	HEX	299-363
A	BFG161	HEX	188-229
A	BFG250	ATTO550	249-306
A	BFG258	FAM	229-329
B	BFG048	FAM	355-374
B	BFG099	ATTO550	120-166
B	BFG146	HEX	284-356
B	BFG157	ATTO550	250-303
B	BFG207	FAM	175-194
B	BFG254	HEX	149-193

Table 2- 2: Western European clade mtDNA haplotype details, including number of samples (n samples) and number of haplotypes (n haplotypes).

Country	n samples	n haplotypes
Scotland	39	6*
Ireland	19	2
England	11	2
France**	8	4
Germany**	7	1
Spain**	4	3
Switzerland**	3	2
Total	91	12

* Three haplotypes newly found by this study

**Countries where eastern clade haplotypes have also been identified

Table 2- 3: Summary of populations sampled for microsatellite genotyping, including altitude (m), mean annual temperature (Annual temp; ˚C), mean spring temperature (Spring temp; ˚C), number of samples per site (n), allelic richness (A_r), observed heterozygosity (H_o) and expected heterozygosity (H_e). Standard deviations are indicated for mean values.

Site	Altitude	Annual temp	Spring temp	n	A_R	H_o	H_e
Beinn Dubhchraig High	900	3.47±5.99	3.36±1.78	30	5.17 ± 0.33	0.64 ±0.2	0.62 ± 0.22
Beinn Dubhchraig Low	197	8.18±7.34	7.33±2.73	28	5.83 ± 0.44	0.66 ± 0.21	0.62 ± 0.23
Beinn Ime High	703	NA*	NA*	30	5.22 ± 0.43	0.57 ± 0.21	0.59 ± 0.21
Beinn Ime Low	155	9.03±5.23	8.17±2.17	40	5.06 ± 0.41	0.63 ± 0.21	0.62 ± 0.22
Ben Lawers High	990	3.10±4.37	4.87±1.22	26	5.62 ± 0.19	0.7 ± 0.19	0.64 ± 0.2
Ben Lawers Low	215	7.75±6.12	6.93±2.57	17	4.88 ± 0	0.74 ± 0.2	0.63 ± 0.2
Ben Lomond High	720	5.37±5.87	4.20±2.46	30	5.78 ± 0.44	0.59 ± 0.22	0.63 ± 0.21
Ben Lomond Low	77	9.49±6.17	8.20±2.68	0**	NA	NA	NA
Meall nan Tarmachan High	900	3.97±5.53	3.08±2.85	21	6.54 ± 0.32	0.68 ± 0.16	0.66 ± 0.17
Meall nan Tarmachan Low	223	7.98±6.67	8.00±3.04	32	7.63 ± 0.86	0.7 ± 0.15	0.72 ± 0.14

*Temperature data unavailable for Beinn Ime High due to datalogger error
**Genetic data not available for Ben Lomond Low

Table 2- 4: Comparison of pairwise genetic distances based on F_{ST} from microsatellite markers (lower triangles) with geographic distances (km; upper triangle). Numbers in bold are significant after bonferroni correction ($p < 0.0056$).

	Dubhchraig High	Dubhchraig Low	Ime High	Ime Low	Lawers High	Lawers Low	Lomond High	Meall nan Tarmachan High	Meall nan Tarmachan Low
Dubhchraig High	--	4.52	18.26	21.22	36.08	33.86	24.17	31.24	32.85
Dubhchraig Low	-0.007	--	22.00	24.48	31.69	29.63	26.37	26.87	28.62
Ime High	0.027	**0.025**	--	4.54	49.70	46.30	11.57	44.93	45.45
Ime Low	0.005	0.000	0.020	--	50.01	46.32	7.44	45.35	45.53
Lawers High	0.003	0.001	**0.052**	0.021	--	4.70	47.39	4.87	4.98
Lawers Low	0.002	0.007	**0.037**	0.020	-0.012	--	43.31	4.25	1.04
Lomond High	0.005	0.019	0.018	**0.031**	0.012	0.005	--	42.99	42.63
Meall nan Tarmachan High	0.020	**0.025**	**0.038**	**0.028**	**0.022**	0.023	**0.029**	--	3.44
Meall nan Tarmachan Low	**0.036**	**0.046**	**0.070**	**0.041**	0.004	0.004	**0.033**	0.025	--

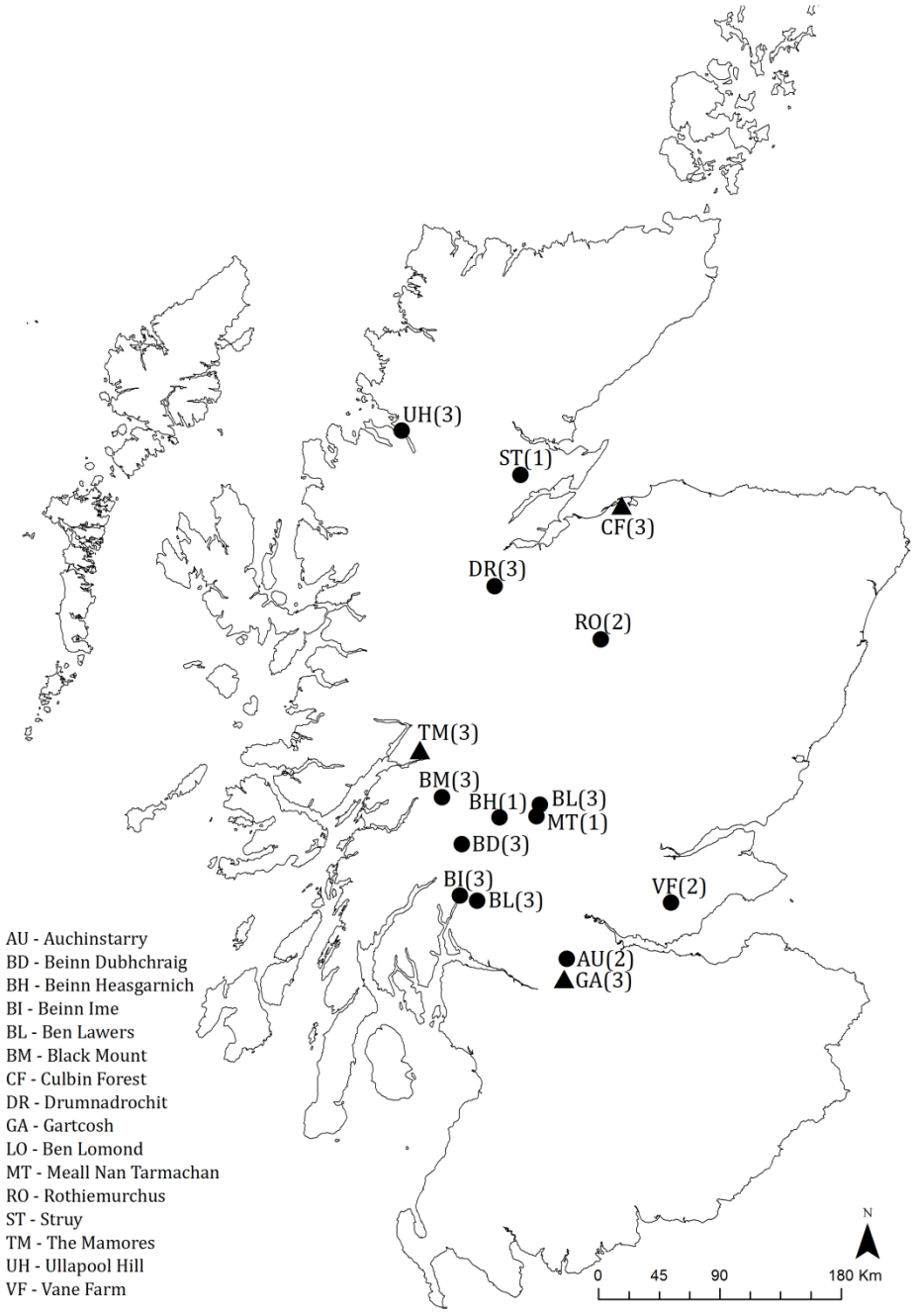

Figure 2- 1: Sample collection locations within Scotland for mtDNA sequencing. Numbers in brackets show the sample size for that location. Locations shown by a circle included only the common western haplotype. Locations shown by a triangle included the common haplotype as well as a single novel haplotype.

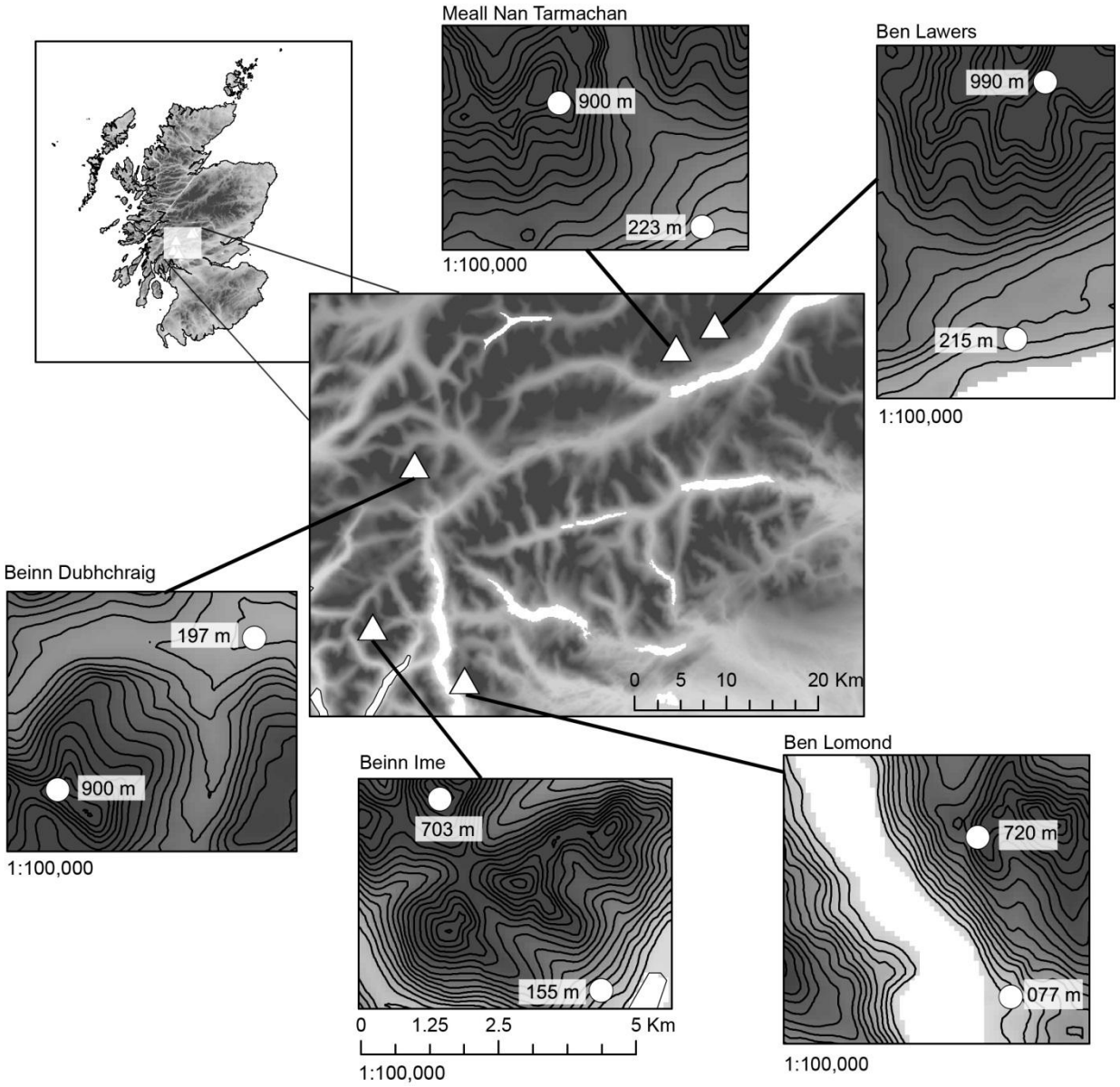

Figure 2- 2: Location of study mountains within west-central Scotland (inset) used to assess fine-scale population structure. Location and altitude of each high- and low-altitude study site is shown for each named mountain.

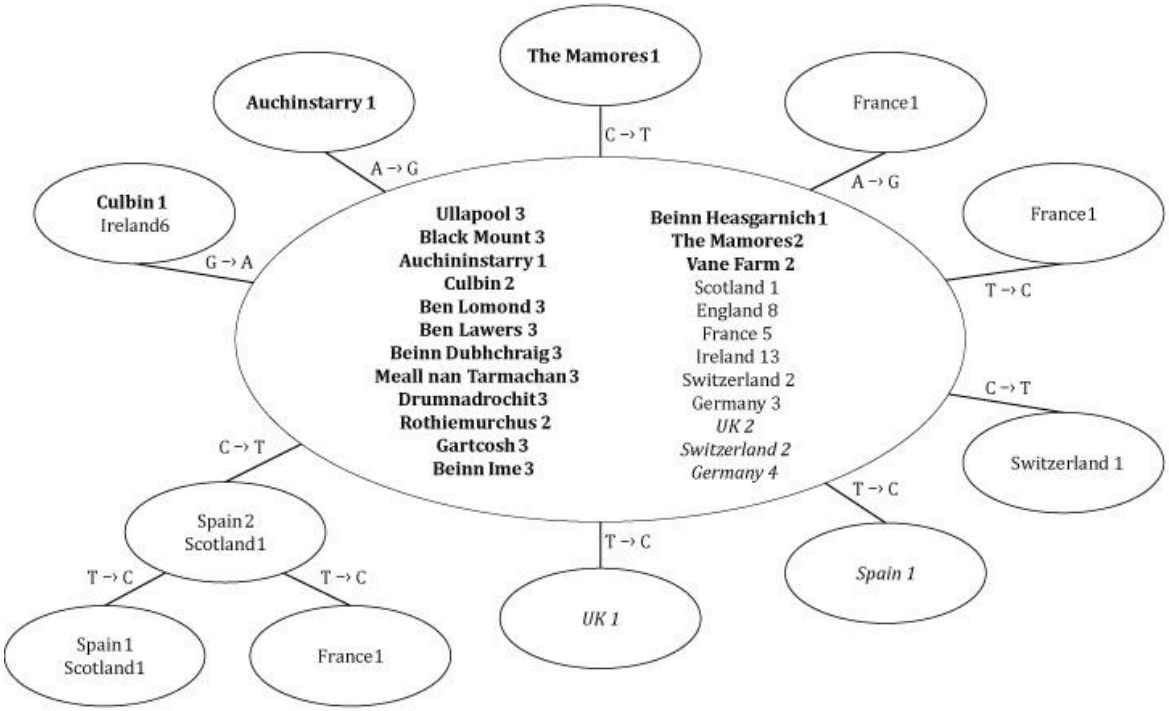

Figure 2- 3: Haplotype network analysis of cytb sequences from European western clade *R. temporaria*, based on samples from this study (bold text), Palo *et al*. (2004b; italics) and Teacher *et al*. (2009b; all others). Country of origin of samples is shown, followed by the number of samples from that location. Each unique haplotype is shown in an individual oval, within connecting lines showing the single base pair change between haplotype sequences. All base pair changes were synonymous and occurred at the third codon position.

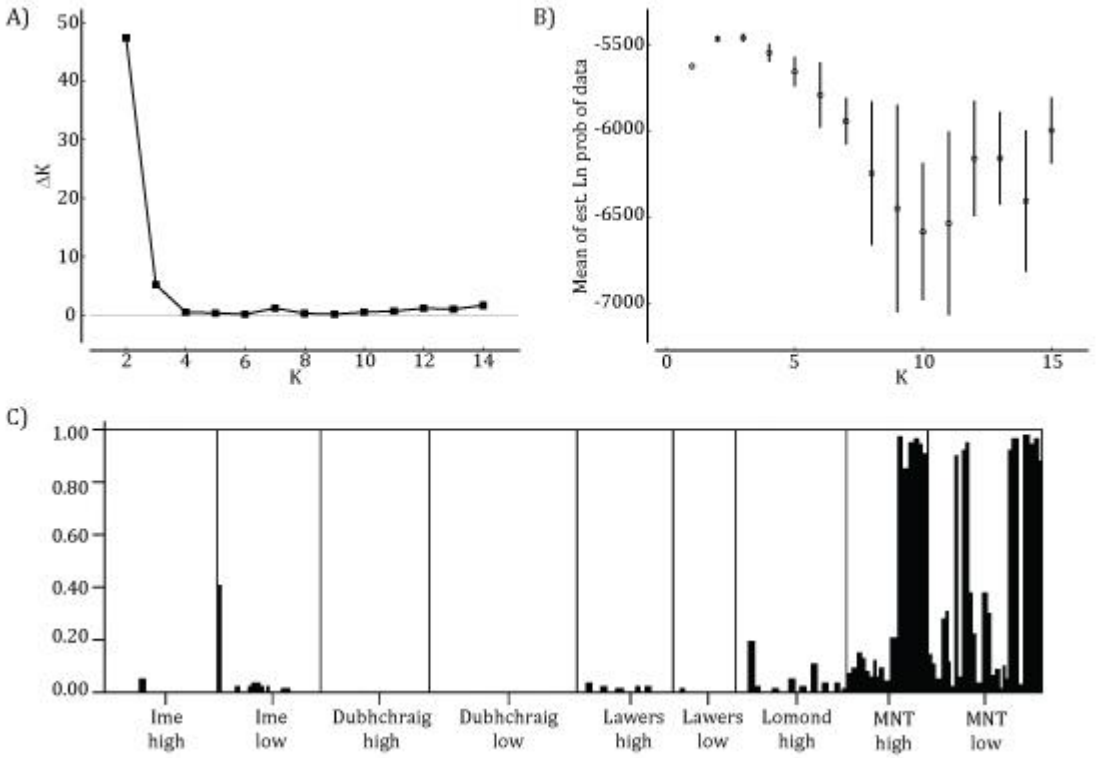

Figure 2- 4: Genetic structure based on Bayesian clustering analysis (STRUCTURE) of eight microsatellite loci. A) ΔK for each value of K (putative number of populations), averaged over ten replicates. The peak at K=2 shows the most likely number of genetic clusters within the sample. B) Likelihood probability profile estimated for K=1-15 showing the mean and variance for each value of K. K=2 showed the highest likelihood and had low variance, indicative of the best-fitting estimate. C) STRUCTURE barplot for K=2. Columns are individuals, with the proportion of an individual's genotype assigned to each cluster (K) denoted by black or white. Sampling locations are shown below the plot (the abbreviation MNT is used for Meall nan Tarmachan).

Chapter 3 – Local adaptation with high gene flow: spring temperature drives adaptation to altitude in the common frog (*Rana temporaria*)

3.1 Abstract

Phenotypic variation in relation to spatial environmental heterogeneity can be used to make predictions about responses to temporal environmental change. Both environmental and genetic influences can result in phenotypic variation, and assessing the contributions of local adaptation and phenotypic plasticity to phenotype is key to understanding the effect of environmental variation on populations. Identifying the selective pressures that drive phenotypic divergence is an important next step, but is often lacking in studies of local adaptation. The common frog (*Rana temporaria*) occurs along altitudinal gradients in Scotland and high levels of gene flow between high- and low-altitudes breeding sites have previously been demonstrated. The overall objective of this study was to assess whether local adaptation occurs along altitudinal gradients in the face of high gene flow and to identify the environmental selection pressures that drive divergent adaptation. I first quantified phenotypic variation in *R. temporaria* from five paired high- and low-altitude sites in Scotland. Three common temperate treatments (10°C, 15°C and 20°C) were used to assess phenotypic differentiation and phenotypic plasticity variation in relation to altitude in terms of larval fitness traits. Local adaptation to altitude was assessed using Q_{ST}-F_{ST} analyses (using F_{ST} values previously estimated using microsatellites) and adaptive phenotypic divergence was then related to environmental parameters using Mantel tests, to look for drivers of selection. I found that *R. temporaria* show evidence of local adaptation in all larval fitness traits measured: metamorphic weight, SVL gain, larval period and growth rate. However, only variation in larval period and growth rate was consistent with adaptation to

altitude. Moreover, this was only evident in the three mountains with the highest high-altitude sites (at least 900m), suggesting the possibility of a threshold for local adaptation. This variation was correlated with spring temperature, suggesting that temperature acts as a strong environmental selection pressure influencing local adaptation along altitudinal gradients, even in the face of high gene flow.

3.2 Introduction

Both spatial and temporal environmental heterogeneity have the potential to influence individuals within a species, resulting in phenotypic variation (Endler 1977; Allendorf & Luikart 2007). Recently, how temporal environmental variation influences phenotypes has come under increased scrutiny in light of a changing climate (Perry *et al*. 2005; Parmesan 2006; Savolainen *et al*. 2007). However, temporal studies are limited by the length of time required for data collection and/or the lack of experimental control when using historical records (Dunne *et al*. 2004). Spatial variation in relation to environment can be used to predict temporal responses to a changing environment when time is short (e.g. due to threats of rapid environmental change), or historical data are unavailable (Dunne *et al*. 2004; Fukami & Wardle 2005). Furthermore, spatial studies within heterogeneous environments make it possible to relate phenotype to multiple contemporary environmental pressures simultaneously (Hangartner *et al*. 2011b). However, spatial phenotype-by-environment correlations are rarely quantified and contrasted at a local level (Whitlock 2008) and incorporation of a range of fine-scale environmental data would elucidate the environmental parameters that act as agents of selection.

Observable differences in phenotype are a function of both genetic control and environmental induction (Allendorf & Luikart 2007). Natural selection can act to adapt populations to the local environment, here defined as a fitness advantage of local genotypes over genotypes originating in other environments (Miller *et al*.

2011). However, phenotypic divergence can also be caused by phenotypic plasticity: the ability of a single genotype to produce different phenotypes depending on the environment experienced (Via & Lande 1985). Therefore, observable differentiation between populations in the wild (or lack thereof) does not necessarily indicate local adaptation (Conover & Schultz 1995; Laugen *et al*. 2003b). Typically, the causes of phenotypic variation are assessed by removing the effect of environment via common garden and/or reciprocal transplant experiments (Merilä *et al*. 2000a; Leinonen *et al*. 2011). However, to understand the adaptive basis of genetic variation, neutral genetic processes (such as genetic drift) must also be accounted for in the observed phenotypic differentiation (Loman 2003; Hangartner *et al*. 2011b).

A common method to account for neutral variation has been to compare population divergence based on quantitative traits (Q_{ST}) with that based on putatively neutral genetic loci (F_{ST}) (Whitlock & Guillaume 2009; Lind & Johansson 2011). Comparison of Q_{ST} with F_{ST} tests whether the quantitative trait divergence is greater than that expected from neutral genetic variation alone (genetic drift) (Alho *et al*. 2010). A greater Q_{ST} than F_{ST} is taken as evidence for divergent natural selection; if Q_{ST} equals F_{ST}, genetic drift alone accounts for observed trait variation; and if Q_{ST} is less than F_{ST}, stabilising selection is inferred (McKay & Latta 2002; Alho *et al*. 2010; Lind & Johansson 2011). Q_{ST} vs. F_{ST} analyses, although widely used in evolutionary biology (see Leinonen *et al*. 2008 for a meta-analysis), are the subject of an on-going debate with regards to their utility as indicators of adaptation (see O'Hara & Merila 2005; Whitlock & Guillaume 2009; Ovaskainen *et al*. 2011; Edelaar *et al*. 2011 for further discussion). Recent adaptation studies have attempted to improve robustness of Q_{ST} vs. F_{ST} analyses by incorporating the following improvements: 1) Contrasting the population pairwise matrices of Q_{ST} and F_{ST}, rather than a single value of Q_{ST} and F_{ST} averaged across all sites, thereby avoiding biases due to potentially different distributions of the two estimators (Alho *et al*. 2010; Antoniazza *et al*. 2010); 2) calculating Q_{ST} within multiple common environments to avoid genotype-by-environment interactions that can confound comparisons with F_{ST}

(Hangartner *et al*. 2011b); 3) including at least ten populations to reduce the confidence intervals around Q_{ST} estimates (Antoniazza *et al*. 2010; Ovaskainen *et al*. 2011); and 4) using Q_{ST} vs. F_{ST} analyses as an exploratory tool to identify traits putatively under selection, which can then be used to explore the selective forces acting on phenotypic divergence in more detail (Edelaar & Björklund 2011; Hangartner *et al*. 2011b), a step frequently lacking in local adaptation studies (Whitlock 2008).

Local adaptation is typically thought to occur through divergent natural selection acting on isolated populations (Miaud & Merilä 2000; Palo *et al*. 2003; Bonin *et al*. 2006). Under this view, high levels of gene flow could swamp the effect of local natural selection through introduction of maladaptive alleles from differentially adapted populations (Allendorf & Luikart 2007; Bridle & Vines 2007; North *et al*. 2010). However, there is increasing empirical evidence that microevolution also can take place in the face of gene flow (Endler 1977; Bonin *et al*. 2006; Richter-Boix *et al*. 2010, 2011; Ribeiro *et al*. 2011; Cristescu *et al*. 2012). The level of gene flow required to inhibit local adaptation depends on the strength of selection acting on a trait (Savolainen *et al*. 2007; Young *et al*. 2011). Indeed, it has been suggested that directional selection on important life-history traits can maintain divergence between populations at adaptive loci, whilst allowing homogenisation in other parts of the genome (Lande 1976; Richter-Boix *et al*. 2011). There is also debate as to whether phenotypic plasticity is itself an adaptive trait, or merely a by-product of fluctuating selection (Via 1993). Phenotypic plasticity can certainly lead to fitness advantages in heterogeneous environments (Lind *et al*. 2011), although the costs of, and limits to, plasticity are poorly understand (Van Buskirk & Steiner 2009). Combining analyses of local adaptation and phenotypic plasticity to draw conclusions about the basis of phenotypic variation in heterogeneous environments can help to elucidate the relative roles of genotype, environment and their interaction (Lind & Johansson 2007).

Species that inhabit heterogeneous environments are subject to spatially varying selection pressures (Miaud & Merilä 2000). Environmental gradients, where parameters vary in a systematic way, are ideal for studying interactions of phenotype and environment (Fukami & Wardle 2005). Altitudinal gradients have been proposed as particularly suitable for studying selection pressures imposed by climatic variables, due to the rapid change in environmental conditions over short geographical distances (Miaud & Merilä 2000; Körner 2007). In particular, temperature has been found to decrease by 6.5°C for every 1000m gain in elevation globally (Briggs *et al*. 1997) and acts as a strong selective pressure on ectotherms, due to the direct effect of ambient thermal conditions on physiological processes (Carey & Alexander 2003; Laugen *et al*. 2003a).

The common frog, *Rana temporaria* (Anura: Ranidae), occurs throughout Europe and has been demonstrated to be locally adapted along a latitudinal gradient in Fennoscandia for a range of larval fitness traits (Laugen *et al*. 2003b; Palo *et al*. 2003; Loman 2003). Larval fitness and thus size at metamorphosis has consequences for adult survival (Lind & Johansson 2011) and is dependent on non-genetic maternal effects (Laugen *et al*. 2002), local adaptation (Orizaola *et al*. 2010; Hangartner *et al*. 2011b) and environment experienced during development (Merilä *et al*. 2000b; Lind *et al*. 2011). However, the influence of temperature on larval fitness traits is not fully understood, due to the non-linear temperature-latitude relationship within the Fennoscandian study area (Laugen *et al*. 2002). In Scotland, *R. temporaria* breed from zero to over a thousand metres above sea level and are the most abundant of only six native amphibians (Inns 2009). The mountains of Scotland offer replicated altitudinal transects, with a minimum of fragmentation by human activities and continuous habitat suitable for *R. temporaria* (Thompson & Brown 1992; Trivedi *et al*. 2008), avoiding difficulties associated with trying to separate anthropogenic influences and habitat fragmentation from environmental influences. I have previously established that temperature and altitude have a negative linear relationship in Scotland, with temperature decreasing by the predicted 6.5°C per 1000m, and that there is no evidence of population structuring of *R. temporaria* within or

between altitudinal gradients at a scale of up to 50km (average pairwise F_{ST}= 0.02; Chapter 2).

The overall objectives of this study were to assess whether local adaptation occurs along altitudinal gradients in the face of high gene flow and to identify the environmental selection pressures that drive divergent adaptation. Specifically, I aimed to answer the following questions: 1) Do quantitative traits and phenotypic plasticity vary in relation to altitude?; 2) Are populations locally adapted to altitude?; and 3) What are the environmental drivers of local adaptation to altitude?

3.3 Methods

3.3.1 Sampling

Within west central Scotland, five altitudinal gradients were chosen for study based on presence of known high- and low-altitude *R. temporaria* breeding sites, mountain height and accessibility (Figure 2-2). The study was set within a limited geographical area (maximum distance between study mountains was 50 km; distance measured as straight line distance using ArcGIS v10; Esri, 1995-2012) in order to minimise the effect of latitude and longitude and highlight the effect of altitude. Within each of the five mountains, a high-altitude (over 700m above sea level) and a low-altitude (below 300m) breeding pool was designated for use in the study, giving ten breeding sites in total. Site names refer to the study mountain (Mountain) and whether high- or low- altitude (Altitude); e.g. Ben Lomond High. All breeding pools consisted of small bodies of permanent standing water where *R. temporaria* tadpoles had been observed during the previous year, except Ben Lomond Low, which was an area of slow flow within a small stream.

For common garden experiments, two thirds of each of ten separate *R. temporaria* egg masses were collected from each study site during the 2011 breeding season (March-May 2011). Egg masses were defined as a group of eggs within a communal spawning area considered to be from a single mother based on the developmental stage of the eggs and size of the jelly capsules relative to surrounding masses (Griffiths & Raper 1994; Håkansson & Loman 2004). Eggs were collected soon after laying, before having reached Gosner stage 10 (Gosner 1960). Spawn clumps were placed in individual containers filled with source pond water. Spawn was transported immediately back to the lab in cool bags, with the aim of keeping the eggs at below 4°C during transport.

3.3.2 Quantitative trait variation and phenotypic plasticity in relation to altitude

3.3.2.1 Common garden experiments

On arrival in the Scottish Centre for Ecology and the Natural Environment (SCENE) laboratory, a subset of ten eggs were removed from each egg mass in order to identify developmental stage and measure egg diameter to the nearest 0.1 mm. Egg size can be used as a measure of maternal effects in *R. temporaria* (Gosner 1960; Laugen *et al.* 2002). The remainder of the egg masses were maintained at 10°C until hatching (Gosner stage 22). A randomly selected subset of thirty of the putatively full-sibship tadpoles (Lind & Johansson 2007) were removed from each clump and placed in groups of five in six individual 1331cm^3 (1.31L) plastic baskets. Two baskets per spawn clump (ten tadpoles) were placed in each temperature treatment room, with air temperatures set at 10°C, 15°C and 20°C, respectively. In each treatment, tadpole baskets from each site were placed in individual large tanks. In total, this gave ten tadpoles per spawn clump, from ten spawn clumps per site, from ten sites, replicated in each of the three temperature treatments (3000 tadpoles in total). Water quality was maintained using an intermittent flow-through system, where water was slowly

added for two hours every two days. Immediately after flow-through, tadpoles were fed *ad libitum* with a 1:2 mixture of finely ground dried fish and rabbit food. The amount of food provided increased with tadpole development to ensure that excess still remained after two days. As tadpoles got close to metamorphosis, it became necessary to completely change the water in the tanks once a week to ensure water quality. As water was sourced directly from a nearby loch, its temperature varied throughout the experiment. Therefore, during complete water changes, water was allowed to adjust to treatment room temperature before tadpoles were added to it and flow-through was kept slow enough that tank temperature did not vary by more than 1.5°C during cleaning (measured using submerged thermometers in each tank). The light regime was maintained at 12 hours light: 12 hours darkness throughout the experiment.

At the start of the experiment, at hatching (Gosner stage 22), three tadpoles per spawn clump were measured for snout-vent length (SVL) to the nearest mm and wet weight to the nearest 0.1g, and returned for use in the experiments. All tadpoles were allowed to develop until they reached metamorphosis (the end point for the experiment), observed as front leg emergence (Gosner stage 42). SVL and wet weight was measured for all surviving tadpoles. Survival was recorded as the number of tadpoles remaining at the end of the experiment out of the initial number placed in the tanks (tadpoles that died were removed from tanks throughout the experiment). SVL gain and weight at metamorphosis were calculated by subtracting SVL and weight at the beginning of the experiment (an average per site using the three individuals measured at hatching, due to low observed variability in size at this early stage) from SVL and weight at the end of the experiment per individual. Larval period was recorded as the number of days from hatching to metamorphosis. Growth rate was calculated as metamorphic weight divided by larval period.

3.3.2.2 Statistical analyses

All statistics were performed in R v2.12.1 (R core development team). To explain the variation in quantitative trait values observed in relation to altitude, a generalized linear mixed model consisting of altitude as the fixed factor of interest (as a categorical variable: low or high), with treatment as a fixed factor (10°C, 15°C or 20°C), mountain as a random effect and per-site survival as a covariate (to account for the variation in survival between sites), was applied to each trait. Each model parameter and all their interactions were sequentially removed from the model and a likelihood ratio test used to evaluate parameter significance. Only parameters that significantly changed the log likelihood when removed from the model were included in the final model. A Tukey's HSD test (Tukey 1953), with associated chi-squared test, was carried out using the final model to evaluate significant differences in pairwise comparisons of means. Phenotypic plasticity was assessed as the ability of a single genotype to show multiple phenotypes in different environments (Merila *et al*. 2000a). Reaction norms for each site (by mountain and altitude) were plotted for the larval trait mean against the temperature treatment, for each of the following quantitative traits: metamorphic weight, larval period, SVL gain, growth rate, and survival.

3.3.3 Local adaptation in relation to altitude

3.3.3.1 Calculating Q_{ST}

A generalized linear mixed models approach was used to assess within- and between-site trait variation for calculation of Q_{ST}. Site and family were considered as random effects of interest (to be extracted for further calculations); with egg size as a covariate and treatment as a fixed factor. Egg size has been found to account for a large proportion of variation resulting from non-genetic maternal effects in *R. temporaria* (Laugen *et al*. 2002) and inclusion

in the model can be used to reduce this as a confounding variable when using wild collected eggs (Lind & Johansson 2007). Treatment was considered as a fixed factor to account for any variation due to genotype x environment interactions (Laugen *et al*. 2005; Lind & Johansson 2011). Normality of trait distributions was tested using Shapiro-Wilk normality tests. Traits that showed non-normality ($p<0.05$) were log transformed to homogenise variances (as per Hangartner *et al*. 2011b). Between-site variance (V_b; variation due to site) and between-family variance (V_f; variation due to family) were extracted from the models as sums of squares. V_f (due to family) was then converted to V_w (within-site variance) in accordance with the full-sibling design ($V_w = 2V_f$; Lynch & Walsh 1998). Quantitative trait divergence (Q_{ST}) values were calculated for each larval trait over all populations and between all population pairs, using the formula Q_{ST} trait = $V_b/(2V_w + V_b)$ (adapted from Whitlock 2008; Hangartner *et al*. 2011b).

3.3.3.2 Q_{ST}-F_{ST} Comparisons

Global F_{ST} (F_{ST}-G) and pairwise F_{ST} between each site (F_{ST}-P) were calculated in Chapter 2, based on eight microsatellite markers; those values are used here for comparison with Q_{ST}. Global Q_{ST} (Q_{ST}-G) was first compared with F_{ST}-G to assess the direction of the relationship within the system as a whole (i.e. whether individuals were under divergent, stabilising or no selection). Second, a multivariate analysis (using a Mantel test; Mantel & Valand 1970) was used to measure dependency between the F_{ST} and Q_{ST} matrices of site pairwise divergence (F_{ST}-P and Q_{ST}-P). Mantel tests were implemented in Arlequin v3.5 (Excoffier & Lischer 2010) with 10,000 permutations.

3.3.4 Environmental drivers of local adaptation to altitude

3.3.4.1 Quantifying environmental parameters in relation to altitude

During the 2010 breeding season, high- and low-altitude sites were visited and Thermocron i-buttons (Dallas Semiconductor/Maxim, London) were placed to record air temperature measurements every two hours. Data were downloaded to a laptop every six months using a USB i-button adapter (Dallas Semiconductor/Maxim, London) and the software, Thermodata viewer (Thermodata pty Ltd., Melbourne). Dataloggers were removed from the field in October 2011. The water parameters pH (to 0.01 pH), conductivity (to 1 μS cm^{-1}) and total dissolved solids (to 1 ppm) were recorded at three points around the edge of each site pool using an HI 98129 Waterproof pH/EC/TDS/Temperature Tester (Hanna instruments, Leighton Buzzard). Measurements were taken in each season that *R. temporaria* are active (spring, summer and autumn), giving three measurements per site through the year. Dissolved oxygen content (to 0.1 mg l^{-1}) was recorded during sample collection in spring 2011, at three locations around the edge of each site pool, using a Jenway 9071 portable DO_2 meter (Jenway, Stone).

Mean annual temperature was calculated by site in R v2.12.1 (R core development team), first by calculating daily mean air temperature, then averaging over 365 days. Maximum temperature difference (a measure of environmental heterogeneity) was calculated as the maximum minus the minimum temperature recorded per site. For seasonal means, monthly averages were calculated per site then averaged over March, April and May for spring; June, July and August for summer; September, October and November for autumn; and December, January and February for winter (adapted from Raffel *et al.* 2006; UK Meteorological Office). Water parameters were recorded as an average per site. Linear regression analysis was used to assess whether each

environmental parameter varied predictably with altitude (metres above sea level).

3.3.4.2 Correlated divergences in adaptive traits and environmental parameters

First, divergence due to neutral genetic variation (i.e. non-adaptive trait variation) was removed from the population pairwise quantitative trait divergence matrices by subtracting F_{ST} from Q_{ST} (Q_{ST}-P - F_{ST}-P), leaving only adaptive trait divergence matrices. Only traits that showed evidence of local adaptation in relation to altitude, and the mountains where this was observed, were used in the matrices. Second, pairwise environmental differences between sites were computed to construct environmental divergence matrices for parameters that showed a significant relationship with altitude. Mantel tests of dependency, consisting of one dependent variable and one or more independent variable(s), were then carried out in Arlequin v3.5 (Excoffier & Lischer 2010) using 10,000 permutations to assess correlation between adaptive trait- and environmental-divergence. If more than one of the environmental parameter matrices significantly correlated with trait divergence in the Mantel tests, partial Mantel tests were conducted with multiple environmental matrices simultaneously to assess which environmental parameter explained more of the trait divergence and to eliminate any significance biases created by carrying out multiple Mantel tests. If an environmental parameter matrix still significantly correlated with adaptive trait divergence following the partial Mantel test ($p<0.05$), quantitative trait means were plotted against environmental parameter measurements per site and fitted with linear regression lines in R in order to visualise the direction of the relationship.

3.4 Results

3.4.1 Quantitative trait variation and phenotypic plasticity in relation to altitude

3.4.1.1 Quantitative trait variation

Complete mortality was observed with DUBLOW tadpoles in the 10°C treatment and with LAWHIGH tadpoles in the 20°C treatment. Therefore, larval trait data were available for 9 populations at 10°C and 20°C and 10 populations at 15°C (Table 3-1). The likelihood ratio test revealed that mountain, altitude and treatment, but not per-site survival, significantly changed the log likelihood when removed from the model. Therefore, mountain, altitude, treatment and their interaction were retained in the final model. Based on Tukey's HSD tests, larval period differed significantly between altitudes in all mountains and treatments (Table 3-2). However, only DUB, LAW and MNT had consistently shorter larval periods at high- than low-altitude in all temperature treatments (Tables 3-1, 3-2). In contrast, for IME and LOM, the direction of the relationship varied by temperature treatment (Table 3-2). Similarly, growth rate was consistently higher at high-altitude in DUB, LAW and MNT (5/7 interactions were significant; Table 3-2). In contrast, the growth rates in IME and LOM were not significantly different by altitude (Table 3-2). There was a significant effect of altitude on metamorphic weight, but this was only significant for LAW and MNT at 15°C (Table 3-2). A significant difference in SVL gain between high- and low-altitudes was only found in individuals from LOM and only at 15°C and 20°C. However, for LAWLOW and LOMLOW at 10°C quantitative trait values are based on only a single surviving individual (Table 3-1).

3.4.1.2 Phenotypic plasticity

In general, the slopes of the thermal reaction norms for each site were highly variable and comparisons between low- and high-altitude sites did not show consistent patterns across mountains or phenotypic traits. Metamorphic weight decreased with increasing temperature in individuals from all sites except for MNTHIGH (Figure 3-1a). For this site, there was a nonlinear relationship with temperature; tadpoles showed a higher metamorphic weight at 15°C than 10°C, but metamorphic weight was still lowest at 20°C (Table 3-1). No data were available for DUBLOW at 10°C or LAWHIGH at 20°C due to complete mortality, but metamorphic weight was still lower in the higher of the two temperature treatments measured. Larval period (days) also decreased with increasing treatment temperature at all sites, except for LOMLOW (Figure 3-1b). Larval period peaked at 15°C in LOMLOW, with the slope of the reaction norm varying greatly from those observed for the other sites (Figure 3-1b). Three mountains (DUB, MNT and LAW) had a longer larval period in all treatments for low-altitude than high-altitude sites, but the slope of the reaction norms did not vary between mountains or altitudes. LOM and IME high- and low-altitude sites had crossing reaction norms between altitudes, showing a reverse in the direction of the relationship depending on treatment. High-altitude sites had a longer developmental period at 10°C and 20°C, whereas low-altitude sites took longer to develop at 15°C for both LOM and IME (Table 3-2). SVL gain showed only slight variation with temperature at all sites and reaction norms were not different between high- and low-altitude sites (Figure 3-1c). Most sites had a slightly lower SVL gain at 20°C than 10°C, although IMEHIGH showed no difference in SVL gain between 10°C and 20°C (Table 3-2). LOMHIGH and IMEHIGH had peak SVL gain at 15°C, whereas IMELOW had the least SVL gain in the 15°C treatment compared to the other temperature treatments. Growth rate was higher at 20°C than 10°C for all sites and growth rate increased with temperature for both low and high sites for IME and MNT and for LOMHIGH (Figure 3-1d). However, both sites on DUB and LAW showed peak growth rates at 15°C and growth rate was lowest at 15°C for LOMLOW. DUB, MNT and LAW had a higher growth rate at

high- than low-altitude sites at all temperatures (high-altitude gained 0.0062 grams per day more than low-altitude individuals, on average; Table 3-1). In contrast, IME and LOM high- and low-altitude sites had crossing reaction norms (Figure 3-1d). LOMHIGH had a faster growth rate in the 15°C and 20°C treatments than LOMLOW, but at 10°C LOMLOW had a faster growth rate. The growth rate of IMEHIGH was faster than that of IMELOW at 10°C and 20°C, but the reverse was observed in the 15°C treatment (Figure 3-1d). Survival peaked at 15°C for LAWHIGH and MNTHIGH but at 20°C for all other sites (Figure 3-1e).

3.4.2 Local adaptation in relation to altitude

F_{ST}-G across this study system (excluding LOMLOW due to lack of neutral genetic data) has previously been estimated as 0.02 (Chapter 2). Q_{ST}-G values were 0.22 for metamorphic weight, 0.48 for growth rate, 0.30 for larval period and 0.28 for SVL gain. Q_{ST}-G values for all traits exceeded F_{ST}-G by at least ten fold, suggesting that divergent local adaptation had driven observed phenotypic differentiation between sites. Mantel tests comparing Q_{ST}-P and F_{ST}-P showed that Q_{ST}-P was not significantly explained by F_{ST}-P (Table 3-3; Tables B1-B4, Appendix B), further (and more robustly) suggesting that quantitative trait variation was not significantly explained by neutral genetic variation and that local adaptation had taken place (Table 3-4a).

3.4.3 Environmental drivers of local adaptation to altitude

3.4.3.1 Quantifying environmental parameters in relation to altitude

The mean annual air temperature across all sites was 6.8°C±2.2 (Table 3-5), with a 4.5°C temperature difference on average between high- and low-altitude populations (see Chapter 2). The maximum temperature recorded overall was

34.5°C at LAWLOW and the minimum was -18.5°C at DUBHIGH. The average maximum annual temperature difference (maximum-minimum temperature) was 41.1°C±6.6 (Table 3-5). Across sites, seasonal means were 6.01°C±2.12 in spring, 11.04°C±2.53 in summer, 5.32°C±2.46 in autumn and -0.46°C±1.96 in winter, with standard deviation around the mean remaining fairly constant throughout the year (Table 3-5). The average pH across all sites was 6.2±0.8 and was neutral to acidic at all sites, with the highest acidity at LOMHIGH (pH4.8). Conductivity and total dissolved solids showed high variability between sites (39μS±28; 23ppm±17), whereas dissolved oxygen content varied little between sites (10.1mg l^{-1}±1.2; Table 3-5).

Altitude of site showed a strong and significant regression with dissolved oxygen content (positive association; r^2=0.53, p<0.01), mean annual temperature (negative association; r^2=0.77, p<0.01) and mean seasonal temperature (negative association; spring: r^2=0.87, p<0.01; summer: r^2=0.98, p<0.01; autumn: r^2=0.93, p<0.01; winter: r^2=0.82, p<0.01). There was no significant relationship between altitude and pH (r^2=-0.12, p=0.83), conductivity (r^2=0.04, p=0.28), total dissolved solids (r^2=0.15, p=0.15), or maximum temperature difference (r^2=-0.10, p=0.64).

3.4.3.2 Correlated divergences in adaptive traits and environmental parameters

Only growth rate and larval period showed evidence of local adaptation in relation to altitude in individuals from DUB, LAW and MNT and were used to test for correlated divergences between adaptive traits and environmental parameters. Adaptive trait divergence in larval period was significantly correlated with mean spring temperature (r=0.43, p=0.03; Table 3-4b), with larval period increasing as mean spring temperature increased (Figure 3-2a). Adaptive trait divergence in growth rate was significantly correlated with between-site divergence in both mean spring temperature and mean winter temperature (r=0.74, p=0.01 and r=0.68, p=0.05, respectively) in the single

Mantel comparisons (Table 3-4b). This pattern did not change when the two were analysed simultaneously in a partial Mantel test (r=0.74, p=0.01 and r=0.69, p=0.05; respectively), with spring temperature showing a larger correlation coefficient. Growth rate decreased as mean spring temperature and mean winter temperature increased (Figure 3-2c, d).

3.5 Discussion

3.5.1 Quantitative trait variation and phenotypic plasticity in relation to altitude

3.5.1.1 Quantitative trait variation

The mountains DUB, LAW and MNT had a significantly shorter larval period and a consistently higher growth rate for individuals from high- compared to low-altitude sites in all temperature treatments, suggesting larval period and growth rate are locally adapted in relation to altitude in these mountains. In contrast, larval period was significantly shorter for IME and LOM high-altitude individuals in two temperature treatments but longer in the 15°C treatment, and growth rate was not significantly different, compared to low-altitude individuals (Table 3-2). DUB, LAW and MNT are the three highest mountains in this study system (high-altitude sites≥900m; IME and LOM high-altitude sites=703m and 720m, respectively). Therefore, my results suggest that the environmental conditions experienced at high-altitude IME and LOM do not result in detectable local adaptation to altitude; this could be a result of the absolute environmental conditions at the higher altitudes, or the relative difference between environmental conditions at high- vs. low-altitude among mountains. Although geographic distance is known to limit local adaptation in plants and animals (Galloway & Fenster 2000; Becker *et al*. 2006), this is the first study, to the best of my knowledge, which has identified a potential threshold for local adaptation

based on environmental parameters. Further research into the altitude, or altitudinal difference between sites, at which local adaptation begins to occur would be interesting for relating environmental conditions to adaptation, particularly in light of a changing climate. A potential source of bias in this study is the grouping of individuals into containers, leading to non-independence of individuals within the same container. Therefore, further research using larvae separated into individual containers would be useful to rule out within-container non-independence as a confounding factor.

Differences in larval period between sites at different altitudes and latitudes has often been attributed to a shorter period of growth (activity period) at high altitude/latitude (Lindgren & Laurila 2009; Orizaola *et al*. 2010; Dahl *et al*. 2011). Lower temperatures and shorter growing seasons are thought to favour faster growing individuals, who can complete metamorphosis before winter dormancy (Lindgren & Laurila 2010). However, metamorphic weight is an important fitness indicator and a higher metamorphic weight leads to an increased chance of survival as juveniles and adults (Altwegg & Reyer 2003). Therefore, the higher growth rate observed (weight gained per day) at the high-altitude sites of DUB, LAW and MNT, in conjunction with a shorter larval period, means that individuals can grow faster without metamorphosing at a smaller size. This is supported by the observation that no consistent significant differences in metamorphic weight or SVL gain were seen between high- and low-altitude sites in this system (Table 3-2). A positive relationship between latitude and growth rate has been well documented in *R. temporaria* along latitudinal gradients in Fennoscandia (e.g. Merilä *et al*. 2000b; Laugen *et al*. 2003a). My results suggest that altitudinal and latitudinal gradients are comparable in their influence on fitness traits and are potentially subject to the same selective pressures.

3.5.1.2 Phenotypic plasticity

Quantitative trait divergence by altitude was strongly influenced by genotype-by-environment interactions (Figure 3-1). All populations showed phenotypic plasticity (the ability of a single genotype to produce different phenotypes depending on the environment experienced; Merila *et al.* 2000b) in terms of metamorphic weight, larval period and growth rate, but not SVL gain or survival (Figure 3-1a, b, d, c and e, respectively). However, there was no consistent difference between the slopes of the reaction norms at high- vs. low-altitude sites for any of the traits (Figure 3-1), suggesting no difference in the level of phenotypic plasticity with respect to altitude. My results are in contrast to those of Sommer & Pearman (2003), who found that plasticity in metamorphic mass was greater for high- (1800m) than low-altitude populations in Switzerland. However, as their study only included a single high- and low-altitude site from two different mountains separated by over 200km, it is likely that many environmental factors varied over this scale other than those related to altitude. Environmental heterogeneity is generally considered a prerequisite for the evolution of phenotypic plasticity (Via 1993) and adaptive phenotypic plasticity has been identified in response to varying pool-drying regimes in *R. temporaria* (Lind & Johansson 2007). In terms of temperature, the difference between maximum and minimum values has been used as a measure of environmental heterogeneity (Orizaola & Laurila 2009). In my study, there was no relationship between this difference and altitude; lack of variation in plasticity with altitude is thus consistent with lack of variation in environmental heterogeneity.

3.5.2 Local adaptation in relation to altitude

Q_{ST}-G exceeded F_{ST}-G by at least a factor of ten in all traits on a global scale. Higher Q_{ST}-G than F_{ST}-G is interpreted as evidence of divergent selection (i.e. local adaptation; Lind & Johansson 2011). The lack of significant correlation

between Q_{ST}-P and F_{ST}-P when considered pairwise by site (Table 3-4a) also suggested that quantitative trait variation cannot be explained by neutral genetic variation alone and thus that populations are locally adapted. Although correlations of Q_{ST}-P and F_{ST}-P matrices are thought to give more robust results regarding presence of local adaptation than comparisons of global values, due to the lack of assumptions about the distributions of the parameters in the two matrices being compared (Alho *et al*. 2010; Hangartner *et al*. 2011b), I found that both the traditional approach of comparing global values and the improved approach of comparing pairwise values gave evidence of local adaptation. However, only growth rate and larval period were consistent in the direction of the difference in trait means between high- and low-altitude and only in the three highest mountains (DUB, LAW and MNT). Therefore, although there is evidence for local adaptation in all fitness traits in this system, only growth rate and larval period appear to be locally adapted specifically to altitude.

My results suggest that local adaptation has occurred within altitudinal gradients in Scotland despite the previous finding of extensive gene flow and lack of population structure (Chapter 2). High levels of gene flow are generally thought to inhibit local adaptation between sites by introducing alleles that are adapted to other locations and potentially maladaptive in the new location (North *et al*. 2010). However, local adaptation in the face of high gene flow has also been observed in *R. temporaria* in Sweden in response to varying pond canopy cover (Richter-Boix *et al*. 2010) and different pond drying regimes (Lind *et al*. 2011). As the level of gene flow that will inhibit local adaptation depends on the strength of the local selective force (Richter-Boix *et al*. 2010), the local adaptation to altitude of *R. temporaria* in Scotland, in the face of high gene flow, suggests that strong selective pressures are driving trait differentiation.

3.5.3 Environmental drivers of local adaptation to altitude

When corrected for phenotypic variation attributable to neutral genetic variance (i.e. Q_{ST}-P – F_{ST}-P; Table 3-4), between-site divergence in growth rate and larval period both showed a significant correlation with mean spring temperature (r=0.74, p=0.01 and r=0.44, p=0.03, respectively; Table 3-4b). Growth rate was also significantly, but less strongly, correlated with mean winter temperature (r=0.69, p=0.05, Table 3-4b). These results suggest that spring temperature is an important selective force driving local adaptation to altitude, with lower spring temperatures selecting for a shorter larval period and higher growth rate (Figure 3-2). *R. temporaria* breed immediately after winter dormancy in response to warming temperature cues (Beebee 1995; Inns 2009). Therefore, spring temperatures will dictate the timing of spawning and the early environment experienced by offspring. Although it has been speculated that the length of the activity period in *R. temporaria* drives faster development at high latitudes rather than low temperatures *per se* (Laugen *et al.* 2003b), this study is the first to explicitly test the potential drivers of divergent selection and these results suggest that local adaptation to altitude is driven at least partly by spring temperature.

3.6 Conclusion

Variation in spring temperature provides a strong environmental selection pressure that has influenced local adaptation even in the face of high gene flow in *R. temporaria*. Temperature is set to rise within the west of Scotland between 0.8°C and 4.4°C in the next 50 years (depending on emissions scenario and uncertainty range; UKCP09 2011). Therefore, ongoing global warming has the potential to cause fitness changes in populations of *R. temporaria*. Further research is needed to identify why only the highest mountains show local adaptation, and whether absolute temperature or temperature difference

between sites is driving divergence, in order to further elucidate the relationship between temperature changes and fitness.

Table 3- 1: Quantitative trait variation by temperature treatment (Treatment) and site (with associated site name abbreviation). Values per site (mean values are shown with their associated standard deviations) are shown for the diameter of *R. temporaria* eggs at collection (Egg size); the mass at metamorphosis minus the mass at hatching (Metamorphic mass); the number of days between hatching and metamorphosis (Larval period); the gain in snout-vent length between hatching and metamorphosis (SVL gain); the increase in mass per day during the larval period (Growth rate); and the percentage of tadpoles that survived from hatching to metamorphosis (Survival).

Site	Abbreviation	Treatment (°C)	Egg size (mm)	Metamorphic mass (g)	Larval period (days)	SVL gain (mm)	Growth rate (g/day)	Survival (%)
Beinn Dubhchraig High	DUBHIGH	10	2.25±0.24	0.843±0.172	111	11.83±0.93	0.008±0.002	7
	DUBHIGH	15	2.25±0.24	0.775±0.158	39	11.18±0.73	0.020±0.004	8
	DUBHIGH	20	2.25±0.24	0.344±0.193	22	8.24±1.28	0.016±0.009	41
Beinn Dubhchriaig Low	DUBLOW	10	2.19±0.14	NA	NA	NA	NA	0
	DUBLOW	15	2.19±0.14	0.624±0.170	76	10.20±1.05	0.008±0.002	62
	DUBLOW	20	2.19±0.14	0.391±0.111	57	8.78±1.22	0.007±0.002	90
Beinn Ime High	IMEHIGH	10	2.12±0.15	0.833±0.187	106	10.53±2.05	0.008±0.002	9
	IMEHIGH	15	2.12±0.15	0.680±0.161	49	11.42±1.13	0.014±0.003	17
	IMEHIGH	20	2.12±0.15	0.494±0.124	26	10.29±1.20	0.019±0.005	17

Table 3-1 continued

Beinn Ime Low	IMELOW	10	2.15±0.15	0.860±0.207	121	11.73±0.53	0.007±0.002	5
	IMELOW	15	2.15±0.15	0.669±0.151	45	10.46±0.97	0.015±0.003	39
	IMELOW	20	2.15±0.15	0.407±0.099	27	10.44±1.28	0.015±0.004	43
Ben Lawers High	LAWHIGH	10	2.22±0.19	0.619±0.103	106	10.77±0.93	0.006±0.001	21
	LAWHIGH	15	2.22±0.19	0.540±0.161	35	10.06±1.09	0.011±0.006	21
	LAWHIGH	20	2.22±0.19	NA	NA	NA	NA	0
Ben Lawers Low	LAWLOW	10	2.41±0.26	0.6±0	137	13.04±0.00	0.004±0.000	1
	LAWLOW	15	2.41±0.26	0.473±0.140	69	9.79±0.94	0.007±0.002	49
	LAWLOW	20	2.41±0.26	0.345±0.115	60	8.80±1.02	0.006±0.002	53
Ben Lomond High	LOMHIGH	10	2.43±0.13	1.050±0.255	102	10.08±2.02	0.010±0.003	10
	LOMHIGH	15	2.43±0.13	0.673±0.169	41	8.45±0.91	0.016±0.004	26
	LOMHIGH	20	2.43±0.13	0.451±0.201	25	6.82±1.59	0.018±0.008	37

Table 3-1 continued

Ben Lomond Low	LOMLOW	10	2.25±0.10	0.633±0.150	43	10.31±1.15	0.015±0.003	1
	LOMLOW	15	2.25±0.10	0.6±0	115	11.91±0.00	0.005±0.000	12
	LOMLOW	20	2.25±0.10	0.475±0.086	28	9.95±1.19	0.017±0.003	16
Meall nan Tarmachan High	MNTHIGH	10	2.44±0.24	0.729±0.146	116	11.29±1.51	0.006±0.001	28
	MNTHIGH	15	2.44±0.24	0.763±0.148	45	9.94±0.97	0.017±0.003	75
	MNTHIGH	20	2.44±0.24	0.505±0.158	29	9.28±1.14	0.017±0.005	44
Meall nan Tarmachan Low	MNTLOW	10	2.03±0.17	0.739±0.161	127	11.16±1.20	0.006±0.001	18
	MNTLOW	15	2.03±0.17	0.485±0.129	75	10.12±0.95	0.006±0.002	39
	MNTLOW	20	2.03±0.17	0.405±0.076	60	9.21±1.35	0.007±0.001	39

NA: Quantitative trait data not available due to complete larval mortality

Table 3- 2: Results of Tukey's HSD test of significant difference between the means of low- and high-altitude sites, by mountain and treatment, per trait. A positive difference between the means shows that individuals from low-altitude have a greater mean trait value than those from high-altitude, and a negative difference between the means shows that individuals from high-altitude have a greater mean trait value than those from low-altitude.

Trait	Mountain	Treatment	Difference between means	Lower 95% Confidence Interval	Upper 95% Confidence Interval	p
Larval period	DUB	10°C	NA	NA	NA	NA
	DUB	15°C	37.000	37.000	37.000	0.000*
	DUB	20°C	35.000	35.000	35.000	0.000*
	IME	10°C	15.000	15.000	15.000	0.000*
	IME	15°C	-4.000	-4.000	-4.000	0.000*
	IME	20°C	1.000	1.000	1.000	0.000*
	LAW	10°C	31.000	31.000	31.000	0.000*
	LAW	15°C	31.000	31.000	31.000	0.000*
	LAW	20°C	NA	NA	NA	NA
	LOM	10°C	13.000	13.000	13.000	0.000*
	LOM	15°C	-2.000	2.000	2.000	0.000*
	LOM	20°C	3.000	3.000	3.000	0.000*

Table 3-2 continued

	MNT	10°C	11.000	11.000	11.000	0.000*
	MNT	15°C	30.000	30.000	30.000	0.000*
	MNT	20°C	31.000	31.000	31.000	0.000*
Growth rate	DUB	10°C	NA	NA	NA	NA
	DUB	15°C	-0.012	-0.017	-0.006	0.000*
	DUB	20°C	-0.009	-0.012	-0.006	0.000*
	IME	10°C	-0.001	-0.009	0.007	1.000
	IME	15°C	0.001	-0.003	0.005	1.000
	IME	20°C	-0.004	-0.008	0.000	0.112
	LAW	10°C	-0.001	-0.016	0.013	1.000
	LAW	15°C	-0.012	-0.016	-0.007	0.000*
	LAW	20°C	NA	NA	NA	NA
	LOM	10°C	-0.005	-0.020	0.010	1.000
	LOM	15°C	-0.002	-0.007	0.003	1.000
	LOM	20°C	-0.001	-0.005	0.003	1.000
	MNT	10°C	0.000	-0.005	0.004	1.000
	MNT	15°C	-0.010	-0.014	-0.007	0.000*
	MNT	20°C	-0.011	-0.014	-0.007	0.000*

Table 3-2 continued

Metamorphic weight	DUB	10°C	NA	NA	NA	NA
	DUB	15°C	-0.151	-0.363	0.061	0.639
	DUB	20°C	0.048	-0.057	0.152	0.998
	IME	10°C	0.027	-0.277	0.331	1.000
	IME	15°C	-0.011	-0.176	0.155	1.000
	IME	20°C	-0.087	-0.246	0.072	0.967
	LAW	10°C	-0.019	-0.577	0.539	1.000
	LAW	15°C	-0.017	-0.322	-0.018	0.010*
	LAW	20°C	NA	NA	NA	NA
	LOM	10°C	-0.450	-1.022	0.123	0.415
	LOM	15°C	-0.040	-0.230	0.150	1.000
	LOM	20°C	0.024	-0.141	0.188	1.000
	MNT	10°C	0.010	-0.154	0.175	1.000
	MNT	15°C	-0.277	-0.400	-0.155	0.000*
	MNT	20°C	-0.099	-0.219	0.020	0.301

Table 3-2 continued

SVL gain	DUB	10°C	NA	NA	NA	NA
	DUB	15°C	-0.945	-2.650	0.760	0.963
	DUB	20°C	0.540	-0.300	1.381	0.830
	IME	10°C	1.202	-1.242	3.647	0.993
	IME	15°C	-0.952	-2.283	0.380	0.637
	IME	20°C	0.167	-1.108	1.443	1.000
	LAW	10°C	2.271	-2.215	6.758	0.989
	LAW	15°C	-0.545	-1.769	0.678	0.998
	LAW	20°C	NA	NA	NA	NA
	LOM	10°C	1.830	-2.767	6.427	1.000
	LOM	15°C	1.859	0.330	3.389	0.002*
	LOM	20°C	3.279	1.957	4.602	0.000*
	MNT	10°C	-0.131	-1.455	1.193	1.000
	MNT	15°C	0.177	-0.807	1.160	1.000
	MNT	20°C	-0.064	-1.028	0.900	1.000

*significant at $p \leq 0.05$

NA: Quantitative trait data not available due to complete larval mortality

Table 3- 3: Comparison of pairwise genetic distances based on F_{ST} from microsatellite markers (lower triangle) with Q_{ST} of growth rate (upper triangle).

	DUBHIGH	DUBLOW	IMEHIGH	IMELOW	LAWHIGH	LAWLOW	LOMHIGH	MNTHIGH	MNTLOW
DUBHIGH	--	0.423	0	0.002	0.054	0.563	0.047	0	0.577
DUBLOW	-0.007	--	0.408	0.593	0.121	0.081	0.567	0.407	0.034
IMEHIGH	0.027	0.025	--	0.003	0.052	0.566	0.021	0	0.758
IMELOW	0.005	0	0.020	--	0.093	0.721	0.017	0	0.758
LAWHIGH	0.003	0.001	0.052	0.021	--	0.235	0.118	0.050	0.208
LAWLOW	0.002	0.007	0.037	0.020	-0.012	--	0.682	0.491	0.026
LOMHIGH	0.005	0.019	0.018	0.031	0.012	0.005	--	0.010	0.704
MNTHIGH	0.020	0.025	0.038	0.028	0.022	0.023	0.029	--	0.465
MNTLOW	0.036	0.046	0.070	0.041	0.004	0.004	0.033	0.025	--

Table 3- 4: Mantel test results for: a) correlations between quantitative trait divergence (Q_{ST}-P) for each trait measured and neutral genetic variation (F_{ST}-P); and b) adaptive trait divergence (Q_{ST}-P - F_{ST}-P) and environmental parameters.

	Dependent matrix	Trait	Independent matrix	Mantel's r	p
a)	Q_{ST-P}	Metamorphic Weight	F_{ST}	-0.201	0.84
		Growth rate	F_{ST}	0.112	0.262
		Larval Period	F_{ST}	0.024	0.431
		SVL Gain	F_{ST}	-0.111	0.671
b)	Q_{ST-P} - F_{ST-P}	Growth rate	Dissolved Oxygen Content	-0.016	0.497
			Mean Annual Temperature	0.418	0.092
			Mean Spring Temperature	0.742	0.008*
			Mean Summer Temperature	0.51	0.063
			Mean Autumn Temperature	0.781	0.059
			Mean Winter Temperature	0.687	0.045*
		Larval Period	Dissolved Oxygen Content	-0.024	0.439
			Mean Annual Temperature	0.151	0.226

Table 3-4 continued

Mean Spring Temperature	0.427	0.026*
Mean Summer Temperature	0.233	0.161
Mean Autumn Temperature	0.267	0.066
Mean Winter Temperature	0.232	0.128

*significant at $p<0.05$

Table 3- 5: Environmental parameters by site, indicating altitude; temperature parameters: mean annual temperature (˚C), maximum temperature difference (maximum – minimum; ˚C), seasonal mean temperature (spring, summer, autumn and winter; ˚C); and water parameters: pH; conductivity (µS), total dissolved solids (ppm) and dissolved oxygen content (mg l^{-1}). All mean values are accompanied by their standard deviation.

Site	Altitude (m)	Annual Temperature	Temperature Difference	Spring Temperature	Summer Temperature	Autumn Temperature	Winter Temperature
DUBHIGH	900	3.47±5.99	34.5	3.36±1.78	8.46±0.72	2.64±4.57	-2.38±1.32
DUBLOW	197	8.18±7.34	52.5	7.33±2.73	13.21±0.76	6.59±5.30	-0.38±3.00
IMEHIGH*	703	NA	NA	NA	NA	NA	NA
IMELOW	155	9.03±5.23	35	8.17±2.17	12.94±0.85	8.38±3.18	2.45±2.31
LAWHIGH	990	3.10±4.37	32.5	4.87±1.22	7.80±0.70	2.48±4.55	-2.76±1.50
LAWLOW	215	7.75±6.12	46	6.93±2.57	12.30±0.79	6.17±4.38	0.16±1.88
LOMHIGH	720	5.37±5.87	43.5	4.20±2.46	9.71±0.63	4.13±4.40	-1.16±1.32
LOMLOW	77	9.49±6.17	41	8.20±2.68	14.05±0.51	8.55±4.04	2.18±1.87
MNTHIGH	900	3.97±5.53	38.5	3.08±2.85	7.94±0.62	2.44±4.54	-2.64±1.16
MNTLOW	223	7.98±6.67	46.5	8.00±3.04	12.97±1.45	6.52±4.72	0.42±2.08

*Temperature data not available due to datalogger failure

Table 3-5 continued

Site	pH	Conductivity	Dissolved Solids	Dissolved Oxygen
DUBHIGH	5.9±0.6	16±4	10±3	11.3
DUBLOW	5.3±1	45±50	30±27	9.8
IMEHIGH	6.0±0.5	41±38	22±18	10
IMELOW	6.3±1	24±9	17±4	8.2
LAWHIGH	7.0±0.8	26±12	11±4	12.2
LAWLOW	7.1±0.6	108±32	61±14	8.5
LOMHIGH	4.8±1.1	24±25	12±13	10.6
LOMLOW	6.1±0.7	16±4	10±3	9.3
MNTHIGH	6.6±0.2	29±19	17±12	10.2
MNTLOW	7.3±0.6	60±14	45±22	10.6

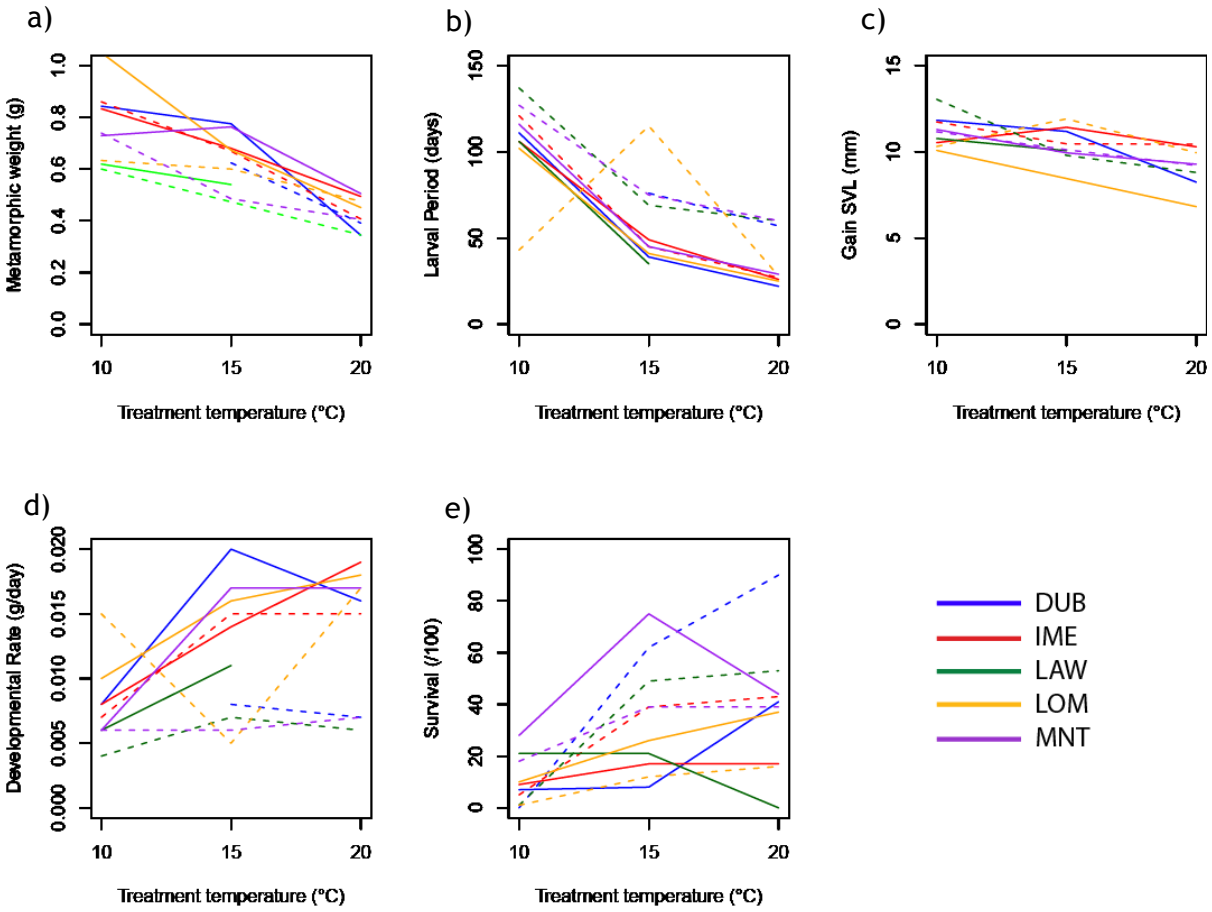

Figure 3- 1: Thermal reaction norms by site for each quantitative trait, demonstrating the relationship between treatment, mountain and altitude. Lines are solid for high-altitude populations and dashed for low-altitude populations on each mountain (see Table 3-1 for mountain name abbreviations). The slope of the line shows the level of phenotypic plasticity at each site (a steeper slope means higher phenotypic plasticity); the location of the line shows the value of the phenotypic trait in relation to other sites (lower down on the graph means a lower trait mean, relative to other sites); if lines are parallel, sites have a similar level of phenotypic plasticity. Values were not available for LAWHIGH at 20°C and DUBLOW at 10°C due to complete mortality during the experiment.

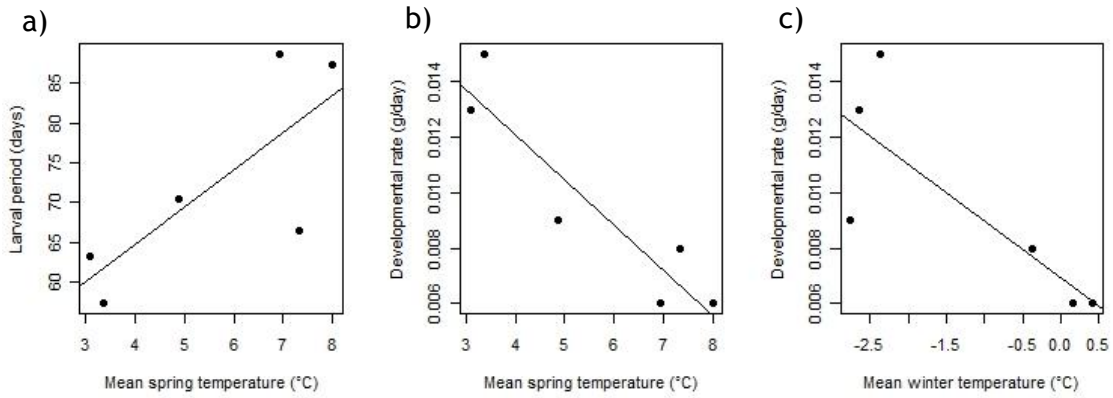

Figure 3- 2: Direction of the relationship between mean quantitative trait value and environmental parameters that showed significant correlations in the partial Mantel tests. Mean trait values were fitted against temperature with a linear regression line: a) larval period versus mean spring temperature: r^2=0.49, p=0.07, slope=4.69; b) growth rate versus mean spring temperature: r^2=0.81, p=0.01, slope=-0.002; c) growth rate versus mean winter temperature: r2=0.57, p=0.05, slope=-0.002.

Chapter 4 – Survival in low-temperature environments: routine metabolic rate, freeze tolerance and spawning temperature in relation to altitude

4.1 Abstract

Extreme environments can impose strong ecological and evolutionary pressures at a local level. Ectotherms are particularly sensitive to low-temperature environments, such as high-latitudes and altitudes, which can result in a reduced activity period, slowed physiological processes and increased periods of sub-zero temperatures. The overall aim of this study was to assess the physiological and behavioural responses that facilitate survival in low-temperature environments. In particular, I asked the questions: 1) do common frog (*Rana temporaria*) tadpoles sampled from high-altitude sites differ physiologically from those from low-altitude sites, in terms of routine metabolic rate and freeze tolerance?; and 2) do high-altitude *R. temporaria* adults extend the time available for larval growth by breeding at lower temperatures than low-altitude individuals? For the physiology experiments, spawn was collected soon after laying from five paired high- (above 700m) and low-altitude (below 300m) *R. temporaria* breeding sites in Scotland and raised in a common laboratory environment. Routine metabolic rate (RMR) was measured as millilitres of oxygen consumed using a closed respiratory tube system. Freeze tolerance of tadpoles was measured as glucose accumulation and survival following slow cooling to the point when all container water had frozen. Breeding date was assessed as the day spawn was first seen, and breeding temperature was determined using dataloggers at each site. I found that mountain, altitude and their interaction were significant in predicting RMR, with RMR lower for individuals sampled from high- than low-altitude sites within the three mountains with the highest high-altitude sites (≥900m). Glucose accumulation was not significantly different based on altitude, but this result

was limited in terms of model power. However, individuals from low-altitude survived freezing significantly better than those from high-altitude, across all mountains. Breeding did not occur below 5˚C at any site and there was no significant difference in breeding temperature between high- and low-altitude sites, leading to high-altitude individuals spawning 30 days later than those at low-altitude. My results suggest that high-altitude adult frogs experience longer periods of low-temperatures prior to spawning and that tadpoles are adapted physiologically to surviving at high-altitude via reduced RMR, but only at the highest breeding sites.

4.2 Introduction

Stressful environments (environments outside the optimum conditions for a particular species) can impose strong ecological and evolutionary pressures at a local level (Narum *et al*. 2010; Hangartner *et al*. 2011a). Population persistence depends on the ability of individuals to respond to environmental stress through adaptive, plastic or behavioural mechanisms that maximise fitness (Voituron *et al*. 2005). Extremes of pH (common frog; Hangartner *et al*. 2011b), water availability (wild mustard; Lee & Mitchell-Olds 2011), and temperature (redband trout; Narum *et al*. 2010) have been observed to drive adaptive population divergence. High-latitudes and altitudes experience low temperatures that can result in reduced activity periods and increased periods of freezing (Laugen *et al*. 2003b; Sears 2005; Voituron *et al*. 2005). Plastic and adaptive responses to low temperature environments have been widely recorded (for a review see Bullock 1955) and can result in cryptic divergence between populations inhabiting different temperature regimes (Conover & Schultz 1995). Temperature is often the major abiotic factor that influences physiological mechanisms in ectotherms (Sinsch 1984; Orizaola *et al*. 2010) and growth slows in response to cold environments (Conover & Schultz 1995). Reduced activity periods in low-temperature environments, in combination with low-temperature

driven growth-rate reductions, can result in lower sizes at important life-history events such as metamorphosis and reproduction (Lee *et al*. 2010). Smaller sizes can translate to lower fitness when weight is positively correlated with survival or reproductive success (Räsänen *et al*. 2005; Lee *et al*. 2010).

Assessing the mechanisms that facilitate survival in challenging environments is important for understanding how populations respond to ecological and evolutionary pressures, particularly in a globally changing climate (Tanino & Storey 2012). Potential responses to maximise size at important life history stages in low-temperature environments include altering metabolic rate (e.g. to allow more resources to be allocated to growth; Laugen *et al*. 2003b; Sears 2005), developmental period (e.g. delaying sexual maturity; Block & Somme 1983; Lencioni 2004; Walsh *et al*. 2008), or temperature activity range (e.g. breeding at lower temperatures; Moore 1939). Populations that inhabit high-altitude environments experience lower temperatures and shorter activity periods than their low-altitude neighbours and offer an excellent opportunity to assess how survival is facilitated in environments where growth is constrained (Körner 2007). Amphibians are a particularly good model for studying physiological and behavioural responses to growth constraints, as size at metamorphosis is positively correlated with survival in the subsequent terrestrial life-history stages (Altwegg & Reyer 2003; Lesbarrères *et al*. 2007).

Variation in metabolic rates between individuals is a common occurrence in nature (Killen *et al*. 2011), but the effects on fitness are still relatively unknown (Reid *et al*. 2012). Resting metabolic rate, here defined as the energetic cost of self-maintenance (Burton *et al*. 2011), has been linked to multiple physiological and behavioural traits including predator avoidance, foraging behaviour, swimming performance and growth (Lee *et al*. 2010; Killen *et al*. 2011). Growth imposes a significant physiological cost and can result in a trade-off with other physiological mechanisms (Alvarez & Metcalfe 2007; Arendt 2010; Lee *et al*. 2010), especially when resources are limited (Beck & Congdon 2000). As the vast majority of energy expenditure in ectotherms is maintenance costs (80-85%), small differences in resting metabolic rate can result in large differences

in energy available for growth (Beck & Congdon 2000). An increased growth rate can result in a larger size at important life history events and has been linked to a reduced resting metabolic rate in sagebrush lizards at high-altitude (Sears 2005), Sydney rock oysters from growth rate-selected stock (Bayne 2000) and snapping turtles (Steyermark 2002). However, attempts to assess the physiological trade-offs facilitating higher growth rates in larval common frog (*R. temporaria*) at high latitudes have found no link to reduced metabolic rates (Lindgren & Laurila 2009; Dahl *et al*. 2011). However, as temperature is not linearly related to latitude in Sweden (Laugen *et al*. 2003b), these results may mask the true nature of the temperature-metabolic rate relationship. Therefore, further research in a system with a linear temperature change is required to elucidate the relationship between resting metabolic rate, growth rate and temperature.

Another potential response to maximise size at important life-history events, is to increase the time available for growth prior to metamorphosis or reproduction, by delaying development (Lencioni 2004). The concept of delayed development, or diapause, has been commonly observed in insects, often in terms of cohort splitting where different cohorts within a population complete development at different times of the year, or even in different years (Lencioni 2004). In amphibians, the period immediately prior to metamorphic climax is accompanied by a loss of weight (Kuan & Lin 2011), but a lower weight decreases the chances of adult survival (Altwegg & Reyer 2003). Therefore, overwintering at a higher weight, but still at the larval stage, and metamorphosing the following year has the potential to increase survival, and has been recorded in a number of temperate amphibian species (Walsh *et al*. 2008; Kuan & Lin 2011). However, low winter temperatures at high-altitude can lead to prolonged periods of freezing (Trivedi *et al*. 2007). Therefore, in order to survive, overwintering larval amphibians must be able to respond to freezing temperatures via freeze avoidance (i.e. inhabiting environments that buffer individuals from freezing temperatures) or freeze tolerance (survival of extensive freezing of body fluids; Storey & Storey 1992; Costanzo *et al*. 1993; Voituron *et al*. 2005). Freeze tolerance depends on the ability to restrict ice

formation to extra-cellular areas, which is mediated by accumulation of low molecular weight carbohydrates in the blood (Costanzo *et al*. 1993; Voituron *et al*. 2005). Freeze tolerance has been linked to glucose accumulation in the blood, via release of liver glycogen, in *Rana sylvatica, R. lessonae* and *R. esculenta* (Costanzo *et al*. 1993; Voituron *et al*. 2005), and with glycerol accumulation in *Hyla versicolor* (Layne & Jones 2001). However, all previous studies have focussed on freeze tolerance in adult amphibians and the potential for freeze survival in the larval stage has, to the best of my knowledge, never been studied. The ability of a tadpole to survive freezing would extend the time available for growth to, and thus size at, metamorphosis in amphibians breeding in temperate climates.

An alternative response to larval growth constraints, rather than amphibian larvae adapting physiologically, would be for adults to adapt behaviourally. Adults have the potential to expand the growing season for larvae by breeding earlier in the year (Moore 1939). In temperate amphibians, breeding is closely linked to temperature (Beebee 1995) and frequently occurs immediately after winter dormancy (e.g. *Bufo bufo, R. chinensis, R. sylvatica and R. temporaria;* Duellman & Trueb 1986; Wells 2007). By adults becoming active and breeding at lower temperatures, larvae would have longer to grow and develop prior to winter dormancy. The longer time available for growth would allow larvae to reach a larger size at metamorphosis and thus have an increased chance of survival as adults (Altwegg & Reyer 2003).

The common frog (*R. temporaria*) is the most widespread amphibian in Europe and occurs from zero to over a thousand metres above sea level on the mountains of Scotland (Laugen *et al*. 2003b; Inns 2009). It is an explosive breeder, with communal spawning taking place immediately after winter dormancy (Inns 2009); a 5°C temperature threshold is generally considered to initiate activity and spawning (Odin *et al*. 1983; Laurila *et al*. 2001). *R. temporaria* larvae show increased growth rates in response to low temperatures experienced at high-latitudes and altitudes throughout its range (Chapter 3; Laugen *et al*. 2003b). Local adaptation to high-altitude environments has been

shown even in the face of high gene flow, suggesting that temperature exerts a strong selective pressure (Chapters 2, 3). However, there are also an increasing number of reports of *R. temporaria* overwintering as tadpoles in Scotland, although it is currently unclear whether this response is particularly linked to low-temperature environments (Walsh *et al*. 2008). The mountains of Scotland offer an excellent opportunity to study the responses that facilitate survival in low-temperature environments, as there is continuous habitat along altitudinal gradients, with temperature decreasing linearly by 0.65°C for every 100m gain in altitude (Chapter 2; Thompson & Brown 1992; Briggs *et al*. 1997). Individuals from high-altitude sites in Scotland experience substantially lower temperatures than their low-altitude counterparts, with an average mean annual temperature reduction of 4.5°C at high- compared to low-altitude breeding sites (Chapter 2).

The overall aim of this study was to assess the physiological and behavioural responses that facilitate survival in low-temperature environments. In particular, I asked the questions: 1) do tadpoles sampled from high-altitude sites differ physiologically from those from low-altitude sites, in terms of routine metabolic rate and freeze tolerance?; and 2) do high-altitude adults extend the time available for larval growth by breeding at lower temperatures than low-altitude individuals?

4.3 Methods

4.3.1 Sampling

Ten *R. temporaria* egg masses were collected soon after laying (Gosner stage 10 or below; Gosner 1960) from paired high- (above 700m) and low-altitude (below 300m) sites from five mountains within west central Scotland (Figure 2-2). Egg masses were collected during the 2011 breeding season, transferred to the laboratory and maintained until hatching as per the procedure outlined in

Chapter 3. In the laboratory, a randomly selected subset of ten of the putatively full-sibship tadpoles (Lind & Johansson 2007) were removed from each egg mass and placed in groups of five in two individual 11cm^3 plastic baskets. Baskets were placed in large tanks in a common 15°C treatment room. Water quality was maintained using a flow-through system and tadpoles were fed *ad libitum* with a 1:2 mixture of finely ground dried fish and rabbit food (for further details see Chapter 3).

4.3.2 Larval physiology in relation to altitude

4.3.2.1 Routine metabolic rate

Tadpoles were allowed to develop until hind leg toe differentiation became apparent, in the early stages of metamorphosis (Gosner stages 36-39). Twenty individuals per site were transferred to individual containers and allowed to acclimatise to laboratory conditions in 100% oxygenated water for an hour prior to commencement of experimental procedures. After this period, tadpoles were moved into 8ml respiration tubes filled with 100% oxygenated water and the lids immediately sealed. Tubes were placed in a dim, quiet location to reduce disturbance during the experiment. Respiration tubes remained closed for one hour. At the end of this period, the lid was removed and the oxygen saturation of the water was measured using an oxygen meter and probe (Strathkelvin Instruments, UK). The same procedure was carried out using a control tube containing no tadpole, to account for any oxygen consumption caused by microbial action. The oxygen meter was calibrated prior to each use using 100% and 0% oxygenated distilled water as standards. Distilled water was fully oxygenated using an aquatic bubbler and fully deoxygenated by adding sodium sulphite (Lewis 1970). The oxygen probe was maintained at a constant temperature to avoid biases caused by thermal fluctuation using a flowing water bath. The temperature of the water bath was monitored throughout using a

submerged thermometer. Once the experiment was completed, tadpoles were immediately blotted dry to remove excess water, weighed using an electric balance (to the nearest 0.1g) to account for size differences among individuals in metabolic rate calculations, and returned to 100% oxygenated water. Although tadpole activity levels were very low whilst sealed in the respiration tubes, some short bursts of spontaneous activity were observed. Therefore, the metabolic rate estimates are considered as routine, which includes resting metabolic rate plus any extra energy expenditure due to spontaneous activity and stress (Lindgren & Laurila 2009). Percentage of oxygen consumed was calculated by subtracting the oxygen saturation of the control tube (i.e. the oxygen used by microbial activity) from the oxygen saturation of each respiration tube (i.e. the total oxygen consumed by both tadpole and bacterial activity). Percentage saturation was converted to ml l^{-1} using standard conversion tables based on water temperature during the experiment (water temperature varied between 19-22°C depending on date of experiment). Routine metabolic rate (RMR) was calculated for each individual as millilitres of oxygen consumed per gram weight per hour (ml O_2 g^{-1} h^{-1}).

4.3.2.2 Freeze tolerance and glucose accumulation

Forty tadpoles per site were moved to individual containers at Gosner stage 36-39 and deprived of food for 48 hours. Glucose acts as a cryoprotectant in the closely related *R. sylvatica* (Costanzo *et al.* 1993; Voituron *et al.* 2005) and was consequently measured here in relation to freeze tolerance. In order to test for blood glucose levels using a hand-held meter (to 0.1 mmol l^{-1}; ACCU-CHEK Aviva, Roche, Indiana), 0.6µl of blood was required. Thus, due to their small size, it was necessary to euthanize tadpoles prior to sampling blood from the artery at the base of their tail. Therefore, twenty of the tadpoles from each site were euthanized by emersion in 10 mg l^{-1} MS222 for fifteen minutes, according to Schedule 1 UK Home Office methods, and measured for blood glucose level prior to freezing. The remaining twenty tadpoles were sealed within individual

containers containing 80ml of water and cooled to 4°C for 24 hours to cause inactivity. Containers were then gradually cooled, over a period of six hours, just until all the water in the container became completely frozen. Following this, tanks were gradually warmed (over a period of 14 hours) to 15°C and this temperature was maintained for one hour. All tadpoles were assessed for normal swimming behaviour at this point and the number of individuals that were still alive and exhibiting normal behaviour were recorded as the measure of freeze survival. These individuals were then euthanized and their blood glucose level recorded. Glucose accumulation per site was calculated as the mean blood glucose level post-freezing minus the mean pre-freezing blood glucose level.

4.3.2.3 Statistical analyses

To evaluate whether RMR, glucose accumulation or freeze survival varied by altitude (fixed factor of interest; considered as a categorical variable of low or high) a generalised linear model approach was used, as implemented in R v2.12.1 (R core development team). Mountain was included as a random factor in all models. Weight was also included as a linear covariate in the glucose accumulation and freeze survival models, but was included in the measurement of RMR (ml O_2 g^{-1} h^{-1}). For RMR and glucose accumulation the model was assessed under a normal distribution and for freeze survival a binomial distribution was used. Each model parameter and interaction was sequentially removed from the models and a likelihood ratio test used to evaluate parameter significance. Only parameters that significantly changed the log likelihood when removed from the model were included in the final model. Significant differences due to the model parameters that showed interactions were evaluated using a Tukey's HSD test (Tukey 1953). A post-hoc power analysis was carried out for RMR and glucose accumulation using G*Power v3.1.3 (Erdfelder *et al.* 1996); effect size was calculated by hand (using the formula in Krebs 1989).

4.3.3 Adult spawning behaviour in relation to altitude

4.3.3.1 Data collection

Air temperatures were recorded every two hours at each site using Thermocron i-buttons (Dallas Semiconductor/ Maxim, London) and downloaded to a laptop every six months using a USB i-button adapter (Dallas Semiconductor/ Maxim, London) and the software, Thermodata viewer (Thermodata pty Ltd., Melbourne), as per Chapters 2 and 3. Date of spawning was recorded as the day egg masses were first observed and collected at each site. The daily mean temperature on the day of egg mass collection was calculated for all sites. Although the majority of egg masses were at or below Gosner stage 10 on this date, and thus likely to have been laid no more than 100 hours previously (Herreid & Kinney 1967), it is possible that spawning activity started prior to this. Therefore, the daily mean temperatures and the overall average for the week prior to egg mass collection were also calculated. In addition, degree days to egg collection were calculated for each site (a statistic commonly used to predict flowering date in plants; Sork *et al*. 2010), using the following approach. From the 1st January 2011, degrees above the threshold for development (set at 5°C; Odin *et al*. 1983) were calculated per day using the formula: ((daily maximum temperature+daily minimum temperature)/2)-threshold value. The resulting values were summed to give the total degree days (Sork *et al*. 2010).

4.3.3.2 Statistical analyses

Student's t-tests were used to assess whether the temperature at which individuals spawned was significantly different at high- vs. low-altitude sites, based on the temperature on the date spawn were collected, the mean temperature over the week prior to sampling, and degree days over the

threshold 5˚C. The average daily temperature for the week prior to egg mass collection was plotted for each site and fitted with a linear regression line in R.

4.4 Results

4.4.1 Larval physiology in relation to altitude

4.4.1.1 Routine metabolic rate

Between eight and 20 individuals per site were measured for RMR, due to varying levels of mortality (Mean=16±5; Table 4-1). Mean RMR per site varied between 0.02 ml O_2 g^{-1} h^{-1} (LOMHIGH) and 0.10 ml O_2 g^{-1} h^{-1} (DUBLOW), with an overall average of 0.07±0.02 ml O_2 g^{-1} h^{-1} (Table 4-1). Mountain, altitude, and their interaction were found to be significant in predicting RMR. A Tukey's HSD test showed a significant difference between high- and low-altitude RMR in individuals from three of the mountains: DUB (diff=0.03, p=0.04), MNT (diff=0.02, p=0.03) and LOM (diff=-0.07, p<0.001). The difference between high- and low-altitude RMR was not significant for IME (diff=-0.01, p=0.54) and LAW (diff=0.01, p=0.95). The direction of the relationship varied between mountains, with individuals from DUB, LAW and MNT showing a trend for lower RMR at high- compared to low-altitude, whereas individuals from IME and LOM had higher RMR at high-altitude (Figure 4-1). A power analysis revealed a power of 0.69 and an effect size of 0.29 for this model. The power to confidently conclude that no significant interactions have been missed (a type II error), is generally set at 0.8 (Crawley 2005).

4.4.1.2 Freeze tolerance and glucose accumulation

The number of individuals tested for glucose accumulation and freeze tolerance varied between 10 and 24 (Mean=19±4; Table 4-1), due to variable tadpole mortality. No tadpoles from DUBHIGH were tested for glucose accumulation and freeze tolerance due to complete mortality prior to the experiment. The mean glucose accumulation for high-altitude sites was -0.4±0.4 mmol l^{-1} and for low was -0.15±0.6 mmol l^{-1} (Table 4-1). Only the effect of mountain on observed variation in glucose accumulation was significant in the general linear model. However, the power analysis showed that the power of this model was very low (power=0.07, effect size=0.18). Thus, potential for a type II error in this model was high and significant differences due to elevation or the interaction between mountain and elevation could have been missed. Three low-altitude sites (DUB, LAW and MNT) and one high-altitude site (LAW; Table 4-1) had higher post- than pre-freeze glucose levels. Between zero (LAWHIGH) and 100% (LOMHIGH) mortality was observed post-freezing across sites (Mean survival=0.57±0.33; Table 4-1). Out of 167 tadpoles tested for freeze survival, 57% of tadpoles survived overall. Both altitude and mountain, but not their interaction, were significant in the general linearized model, with individuals from low-altitude sites showing significantly higher survival than those from high-altitude sites (z=2.31, p=0.02).

4.4.2 Adult spawning behaviour in relation to altitude

Temperature on the day of egg mass collection was, on average, 7.5±2.1°C (Table 4-2) and did not vary significantly between high- and low-altitude sites (p=0.16). Likewise, no significant difference was found between the average temperature in the week prior to egg mass collection between high- and low-altitudes (Mean temperature=4.8±0.9°C; p=0.15). Degree days prior to egg mass collection was highly variable across sites (24.5±19.1; Table 4-2) but also did not

significantly differ by altitude (p=0.133). The date of egg mass collection was on average 30 days later at high- compared to low-altitude sites (Table 4-2) but the daily mean temperature at all sites had exceeded the threshold value of 5°C in the week prior to egg mass collection (Figure 4-2).

4.5 Discussion

4.5.1 Larval physiology in relation to altitude

4.5.1.1 Routine metabolic rate

Within each mountain, RMR was significantly different between individuals from high- and low-altitude sites from DUB, LOM and MNT (Table 4-1). However, the direction of the relationship between high- and low-altitude RMR differed for individuals from these three mountains, with individuals from DUB and MNT showing higher, and LOM lower, RMR at low-altitude (Figure 4-1). Due to the moderate power of the model (power=0.69), type II error cannot be conclusively ruled out. For the individuals from mountains that did not show a significant difference between high- and low-altitude RMR, there was a trend for higher RMR in individuals from low-altitude for LAW and from high-altitude for IME (Figure 4-1). The three mountains with individuals that showed lower RMR at high-altitude (DUB, MNT and LAW) have the highest high-altitude sites in the study system (high-altitude sites≥900m; Figure 2-2). It has been suggested that a lower resting metabolic rate can allow more energy to be allocated to growth in resource-limited environments (Lindgren & Laurila 2009) and a link between lower RMR and increased growth rate has been found in the southern toad (Beck & Congdon 2000), Sydney rock oyster (Bayne 2000) and snapping turtle (Steyermark 2002). Furthermore, Sears (2005) found that both increased growth rates and reduced RMR were positively correlated with altitude in sagebrush

lizards. The three mountains in my study system where RMR was lower in individuals from high-altitudes have been shown to be locally adapted to mean spring temperatures, with larval period decreasing and growth rate increasing at high-altitude (Chapter 3). Therefore, the lower RMR of individuals from high-altitude from DUB, MNT and LAW, is in line with the increased growth rates observed at these sites. Lindgren & Laurila (2009) did not find a link between growth rates and RMR in *R. temporaria* along a latitudinal gradient in Sweden. Therefore, this is the first potential evidence of reduced RMR being linked to increased growth rate as an adaptation to low-temperatures in an amphibian. However, the significantly higher RMR at high- than low-altitude in LOM (Figure 4-1), could suggest that local climatic conditions other than altitude are also important in driving divergence in RMR. Furthermore, RMR is influenced by temperature and there was up to a 3°C difference in temperature whilst measuring RMR of individuals from different sites, as well as a difference between the temperature at which the tadpoles were raised (15°C) and the temperature at which RMR was measured (19-22°C). Therefore, further research is needed to assess whether site-specific differences in RMR remain constant through time and whether other mountains with breeding sites of above 900m also show reduced metabolic rate at high- vs. low-altitude.

4.5.1.2 Freeze tolerance and glucose accumulation

Altitude did not significantly predict the level of glucose accumulated in the blood of larval *R. temporaria* during freezing (Table 4-1). However, the small effect size (0.117), coupled with a reduced sample size due to tadpole mortality (Table 4-1), meant that the model had very low power (0.067). Therefore, it is likely that not all significant relationships would have been seen with this sample size. However, just over half of the tadpoles that were frozen survived (57%; Table 4-1) and altitude was significant in predicting freeze survival; individuals sampled from low-altitude survived freezing significantly better than those from high-altitude ($z=2.31$, $p=0.02$). However, the large differences in observed mortality between sites (100% mortality in individuals from LOMHIGH

and 100% survival in individuals from LAWHIGH; Table 4-1) demonstrate that there is a large amount of variability in survival even between sites within the same altitudinal category. Voituron *et al.*(2005) suggested that *R. temporaria* adults were freeze intolerant, as 100% mortality was observed after eight hours of complete bodily fluid freezing. However, Pasanen & Karhapää (1997) found that *R. temporaria* adults could survive 24 hours in a sub-zero environment but died within three days (the actual period an individual was frozen was not measured in that study). My results suggest that tadpoles of *R. temporaria* are also capable of surviving short periods of freezing. This is the first time, to the best of my knowledge, that larval freeze tolerance has been demonstrated in any amphibian. Further studies, with a greater sample size, are needed to elucidate whether glucose accumulation is linked to this freeze tolerance.

Although the results presented here suggest that tadpoles are capable of surviving being frozen, the finding of a greater survival of low- compared to high-altitude individuals appears counterintuitive given the longer period of sub-zero temperatures at high- than low-altitude in Scotland (Trivedi *et al.* 2007) and the sub-zero average winter temperature of -2.2°C at high-altitude, compared to 1.0°C at low-altitude at the breeding sites used in this study (Chapter 3). Indeed an increase in freeze tolerance with altitude has been found in plants (*Arabidopsis thaliana,* Zhen & Ungerer 2008) and insects (Somme & Zachariassen 1981; Lencioni 2004), but freeze tolerance and altitude has not been explicitly linked in herptiles. However, evolution of freeze tolerance in frogs, lizards and turtles has been linked to ecological pressures relating to winter temperatures experienced (Claussen *et al.* 1990; Costanzo & Claussen 1990; Storey & Storey 1992). In general, freeze tolerant species are those that terrestrially overwinter in sub-zero temperatures, as opposed to avoiding freezing in deep water bodies (Voituron *et al.* 2009). Therefore, a higher freeze tolerance would suggest that low-altitude tadpoles are more often exposed to freezing temperatures, whereas high-altitude tadpoles may avoid such exposure altogether by inhabiting deep water bodies or metamorphosing prior to winter. Formation of deep water pools is inhibited at high-altitudes in Scotland due to the rocky, exposed landscape (Kernan *et al.* 2002). Therefore, freeze exposure is

more likely avoided in high-altitude tadpoles by metamorphosing within a single active season, facilitated by a faster growth rate in conjunction with a lower RMR (Chapter 3, this study). Therefore, my results potentially suggest that low-altitude individuals are more likely to overwinter as tadpoles than high-altitude individuals. However, it is possible that long periods of snow cover at high-altitude (Trivedi *et al*. 2007) could insulate tadpoles from freezing temperatures even in shallow water bodies. Therefore, further field research is needed to assess whether fewer, if any, high- than low-altitude individuals overwinter as tadpoles.

4.5.2 Adult spawning behaviour in relation to altitude

No significant difference was found between the temperatures recorded in the week prior to, and on the day of, egg mass collection (the date eggs were first observed) between high-and low-altitude sites (Table 4-2). Likewise, no significant differences in degree days prior to spawning were seen between high- and low-altitude sites (Table 4-2). However, degree days were highly variable across sites, suggesting that degree days are not an accurate predictor of spawning activity in *R. temporaria*. All sites had exceeded the 5°C temperature threshold generally thought to initiate activity and breeding in *R. temporaria* (Odin *et al*. 1983; Laurila *et al*. 2001) in the week prior to spawning, resulting in an average delay in spawning of 30 days at high- vs. low-altitude sites. My results therefore support 5°C as the activity threshold for *R. temporaria* regardless of altitude of breeding site, and demonstrate that high-altitude individuals experience a longer period of low-temperatures resulting in delayed spawning compared to low-altitude individuals. The longer period of low temperatures at high- compared to low-altitudes prior to spawning supports the hypothesis that high-altitude individuals experience a shorter annual activity period, which is line with my previous finding of an average 4.5°C lower mean annual temperature at high- compared to low-altitude breeding sites in this system (Chapter 2).

Breeding at a lower temperature allows individuals to spawn earlier in the year, thus providing their offspring with a longer period in which to develop prior to metamorphosis (Moore 1939). Although breeding at lower temperatures is an adaptation observed in amphibian species that live at higher latitudes compared to their lower latitude counterparts (Moore 1939), to the best of my knowledge, breeding at a lower temperature has never been observed to facilitate survival in low-temperature environments within species. However, phenological studies that quantify breeding temperature typically use local weather station data (e.g. Blaustein *et al*. 2001; Phillimore *et al*. 2010). The spatial scale of these temperature data are not fine enough to represent local conditions in mountain areas where temperatures can vary rapidly over short geographical distances (Briggs *et al*. 1997; Corn 2003; Körner 2007). Therefore, such studies are liable to miss within-species differences in spawning temperature in relation to altitude. Despite quantifying temperature at a local level in this study, my results do not support the hypothesis that breeding at lower temperatures facilitates survival at high-altitude within *R. temporaria*.

4.6 Conclusion

R. temporaria larvae appear to be physiologically adapted to surviving at high-altitude in terms of routine metabolic rate but not freeze tolerance. Furthermore, adults at high-altitude do not show behavioural adaptations in terms of breeding at lower temperatures. How individuals respond to environmental temperature at a local level is an important step in relating ecological and evolutionary pressures to phenotypes (Tanino & Storey 2012). Further research is needed to establish whether lack of physiological adaptation in tadpoles from high-altitude in terms of freeze tolerance is due to absence of individuals overwintering prior to metamorphosis.

Table 4- 1: Physiological trait variation by mountain and altitude measured in a common environment, showing the number of individuals per site measured (n) for each of the parameters mean routine metabolic rate (RMR), mean glucose accumulation (GA) and the proportion of survivors following freezing (Survival). Standard deviations are indicated, except for survival, which had only a single measurement per site.

Mountain	Altitude	RMR n	RMR (ml O_2 g^{-1} h^{-1})	Freeze n	GA (mmol l^{-1})	Survival
DUB	HIGH	8	0.07±0.02	0	NA*	NA*
DUB	LOW	19	0.10±0.02	20	0.4	0.60
IME	HIGH	20	0.06±0.02	20	-0.1	0.25
IME	LOW	19	0.05±0.01	20	-0.6	0.50
LAW	HIGH	10	0.08±0.02	10	0.1	1.00
LAW	LOW	19	0.09±0.02	20	0.1	0.65
LOM	HIGH	14	0.09±0.03	14	NA**	0.00
LOM	LOW	12	0.02±0.01	24	-1.2	0.79
MNT	HIGH	20	0.07±0.01	19	-0.2	0.95
MNT	LOW	20	0.09±0.02	20	0.4	0.40

*No freeze tolerance results are available for DUBHIGH due to complete tadpole mortality prior to the freeze tolerance experiment.

**No glucose accumulation data is available for LOMHIGH due to complete tadpole mortality during the freeze tolerance experiment.

Table 4- 2: Spawning date and temperature by mountain and altitude, shown as the date of egg mass collection (Collection date) and corresponding Julian day; alongside the Degree days prior to egg mass collection, the daily mean temperature on the day of egg mass collection (Collection day temp; ˚C), and the mean temperature of the week prior to egg mass collection (Week prior temp; ˚C). Mean values are accompanied by their standard deviation.

Mountain	Altitude	Collection date	Julian day	Degree days	Collection day temp	Week prior temp
DUB	HIGH	19-Apr	109	30.6	7.1±7.1	4.7±5.4
DUB	LOW	23-Mar	82	33.5	6.8±4.6	5.6±5.5
IME	HIGH	02-Apr	92	NA*	NA*	NA*
IME	LOW	24-Feb	55	1.5	5.8±0.3	4.3±0.9
LAW	HIGH	15-Apr	105	31.1	5.5±2.1	4.3±4.2
LAW	LOW	21-Mar	80	22.8	8.3±2.6	3.3±4.0
LOM	HIGH	09-Apr	99	62.9	10.8±4.5	6.0±3.9
LOM	LOW	01-Mar	60	5.5	4.5±0.6	5.3±1.1
MNT	HIGH	10-Apr	100	28.0	9.8±4.0	5.9±4.1
MNT	LOW	21-Mar	80	5.1	8.8±2.4	4.2±4.6

*data not available due to complete logger failure

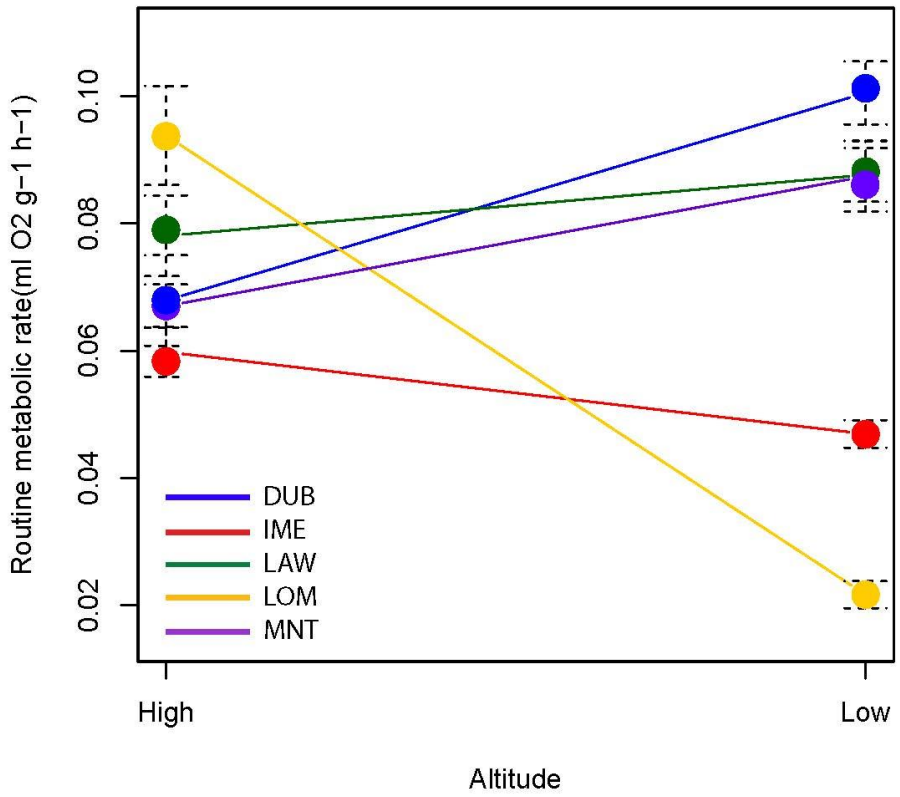

Figure 4- 1: Routine metabolic rate by mountain and altitude. The mean routine metabolic rate per site is shown by a circle, with the bars representing the standard deviation around the mean. Low- and high- altitude sites within each mountain are linked using a straight line.

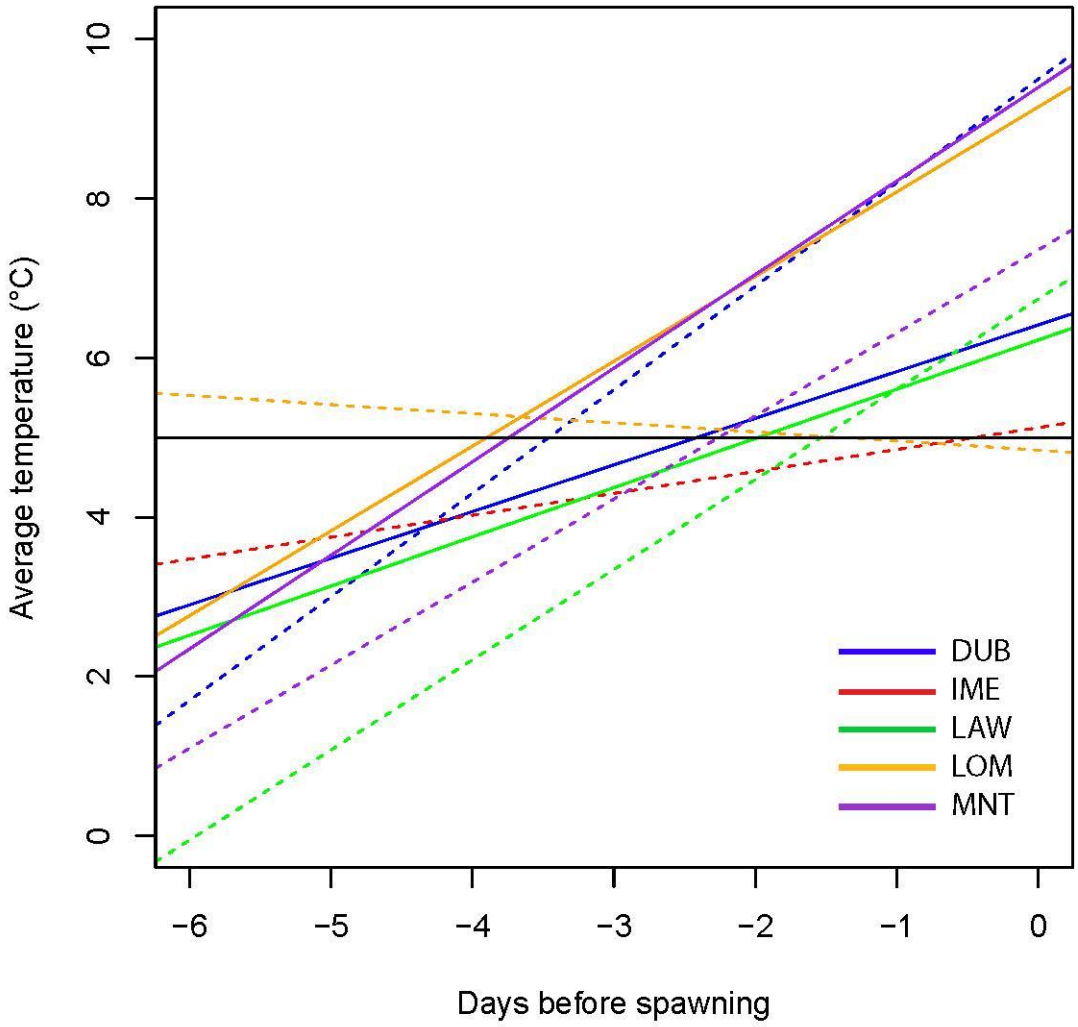

Figure 4- 2: The average daily temperature for the week prior to egg mass collection (Days before spawning) for each site, seen as a linear regression line of the points. Solid lines show high- and dashed lines show low-altitude sites per mountain. The black horizontal line shows the 5°C threshold generally considered to limit activity in *R. temporaria*.

Chapter 5 – Spatial variation in species composition of *Saprolegnia*, a parasitic oomycete of amphibian eggs

5.1 Abstract

Amphibians are experiencing unprecedented worldwide declines that have been increasingly linked to disease. Parasitic water moulds in the genus *Saprolegnia* are known to cause mortality of amphibian embryos and reduced size at metamorphosis. However, the range of species of *Saprolegnia* that infect amphibian eggs in the wild and how species composition varies spatially has not been studied. The overall aim of this study was to investigate the distribution of water moulds sampled from common frog (*Rana temporaria*) egg masses in Scotland. In particular, I asked the questions: 1) Does *Saprolegnia* species composition vary within- and between-sites?; and 2) Is presence of *Saprolegnia* species, or genetic distance between water mould samples, related to geographical or environmental parameters? *R. temporaria* eggs showing signs of *Saprolegnia* infection were sampled from ten sites, the water mould cultured, the 28S region of the rDNA array sequenced, and samples identified to species level by comparison with reference sequences in Genbank. Environmental measurements were taken at each site and Student's t-tests of presence/absence and Mantel tests using genetic distance between samples were used to assess *Saprolegniaceae* ecology. Thirteen samples isolated from four sites were identified as members of the *Saprolegniaceae*, with a further 33 non-*Saprolegnia* species identified from across all ten sites, the majority of which are plant matter associated saprobes, parasites and pathogens. Four putative species of *Saprolegnia* were isolated, but could not be identified to species level due to lack of a generally well resolved taxonomy based on molecular markers in this genus. Multiple *Saprolegnia* water moulds were isolated from within sites and species composition varied between sites. Acidity

was significantly lower at sites where *Saprolegniaceae* were present, but genetic distance between samples was not correlated with environmental or geographic distance. These findings question the previous focus on *S. ferax* as the primary agent of *Saprolegnia* infection and suggest that future studies of virulence need to consider the synergistic effect of multiple *Saprolegnia* species.

5.2 Introduction

Biodiversity is declining worldwide at an unprecedented rate (Butchart *et al*. 2010; Barnosky *et al*. 2011), with amphibians showing higher extinction rates than any other vertebrate taxa (Stuart *et al*. 2004; Allentoft & O'Brien 2010). Causes of the observed amphibian declines include direct anthropogenic effects such as habitat conversion and loss, overexploitation and introduction of invasive species (Blaustein & Kiesecker 2002; Collins & Storfer 2003; Stuart *et al*. 2004). However, up until the late 1990s there were also many documented cases of "enigmatic" declines, where suitable habitat remained and the causes of declines were not fully understood (Stuart *et al*. 2004; Fisher *et al*. 2009). Such declines have increasingly been linked to *Batrachochytrium dendrobatidis* (Bd), a chytrid fungus that causes chytridiomycosis in amphibians (Fisher *et al*. 2009), and Bd has become the focus of most epidemiological studies in amphibians since its discovery (Duffus 2009). However, increased mortality in amphibians has also been linked to other diseases including ranavirus (Cunningham *et al*. 1996; Pasmans *et al*. 2008), redleg disease (Bradford 1991), and water moulds in the genus *Saprolegnia* (Bragg 1962; Blaustein *et al*. 1994; Fernández-Benéitez *et al*. 2008).

Water moulds (genus *Saprolegnia*) are ubiquitous freshwater and soil oomycetes that show both saprobic and parasitic feeding strategies (Romansic *et al*. 2006; Hulvey *et al*. 2007). *Saprolegnia* species infect a range of hosts and cause saprolegniasis in fish, a disease of significant economic importance to the

aquaculture industry (Vanwest 2006). In amphibians, *Saprolegnia* infects eggs and larvae, passing from one individual to another via free-swimming zoospores or, more commonly, via direct contact with growing hyphae (Robinson *et al*. 2003; Romansic *et al*. 2006); making communally spawning amphibians particularly vulnerable to infection (Kiesecker & Blaustein 1997). Non-viable eggs are more readily colonised by *Saprolegnia* than viable eggs, but infections can rapidly spread to adjacent live eggs, causing embryo mortality (Robinson *et al*. 2003), or a reduced size at metamorphosis (Uller *et al*. 2009) and thus increased adult mortality (Altwegg & Reyer 2003).

Although extensive research has investigated the effects of parasitism by *Saprolegnia* on amphibian embryos (Romansic *et al*. 2009; Ruthig 2009; Uller *et al*. 2009), very little attention has been paid to the species of *Saprolegnia* that infect amphibian embryos in the wild and whether species composition varies by site. Furthermore, virulence studies have focussed predominantly on the effect of *S. ferax* (Romansic *et al*. 2009), or single unidentified *Saprolegnia* species (Sagvik *et al*. 2007; Uller *et al*. 2009), without knowledge of the *Saprolegnia* species causing amphibian infection in the wild. Molecular methods for species-level identification offer the opportunity to examine which species of *Saprolegnia* are found on amphibian eggs, a question that has previously been neglected due to the challenging nature of morphological species identification in this group (Hulvey *et al*. 2007; Petrisko *et al*. 2008; Ault *et al*. 2012).

The common frog (*Rana temporaria*) is found throughout Europe and inhabits a wide variety of habitats throughout its range (Laugen *et al*. 2003b). It is an explosive breeder and females lay their eggs communally in the shallow water around the edge of pools immediately after winter dormancy (Inns 2009). Multiple egg masses join to form communal egg mats, which are thought to regulate and maintain temperature conditions for growing embryos (Håkansson & Loman 2004). However, communal spawning puts *R. temporaria* eggs at increased risk of hyphal spread of *Saprolegnia* between individuals (Kiesecker & Blaustein 1997). Indeed *Saprolegnia* has been found to cause mortality in *R. temporaria* eggs by spreading from infected dead eggs to live eggs (Robinson *et*

al. 2003). In Scotland, *R. temporaria* breed in a wide variety of water bodies, thus experiencing a range of different environmental conditions (Inns 2009), making them ideal for studying the relationship between *Saprolegnia* species presence and environmental conditions. Furthermore, west central Scotland has relatively low levels of intensive agriculture (Swan *et al*. 1994), avoiding confounding interactions between water mould presence, amphibian susceptibility and pollutants that have been found elsewhere (Romansic *et al*. 2006).

The overall aim of this study was to investigate the distribution of water moulds sampled from *R. temporaria* egg masses in Scotland. In particular, I asked the questions: 1) Does *Saprolegnia* species composition vary within- and between-sites?; and 2) Is presence of *Saprolegnia* species, or genetic distance between water mould samples, related to geographical or environmental parameters?

5.3 Methods

5.3.1 Sampling

Common frog (*R. temporaria*) eggs that showed evidence of infection by water mould (identified as a white "cotton wool" covering the surface of the egg; Fernández-Benéitez *et al*. 2008) were collected from ten sites across Scotland during the 2012 breeding season (March-April; Figure 5-1). Three potentially infected eggs were collected from each of five egg masses per site. Eggs were stored in individual eppendorf tubes and transported to the laboratory in cool bags. At each site the water parameters pH (to 0.01 pH), conductivity (to 1 µS cm^{-1}) and total dissolved solids (to 1 ppm) were recorded using an HI 98129 Waterproof pH/EC/TDS/Temperature Tester (Hanna instruments, Leighton Buzzard).

In the laboratory, a section of white water mould (roughly $1mm^3$) was removed from each egg, ensuring that no egg tissue or jelly capsule remained attached, and rinsed with distilled water containing 100mg l^{-1} of penicillin C (Fernández-Benéitez *et al*. 2011). The water mould was then placed on a glucose-peptone-salts (GYPS) agar plate containing 5g l^{-1} glucose, 0.5g l^{-1} peptone, 0.5g l^{-1} KH2PO4, 0.05g l^{-1} yeast extract and 0.15g l^{-1} MgSO4.7H2O (Beakes & Ford 1983). Antibiotics (10ml l^{-1} of ampicillin and 5ml l^{-1} of chloramphenicol) were also added to agar plates to prevent bacterial growth (Fernández-Benéitez *et al*. 2011). Plates were sealed and maintained at room temperature (around 23°C) until hyphae growth covered half of the plate surface, at which point hyphae from the edge of the mycelium mat were transferred to a second plate for growth to continue. After ten days of growth, peripheral hyphae were transferred to a 1.5ml eppendorf tube containing 500µl of liquid GYPS media and maintained in a thermal cabinet at room temperature (23°C) for 72 hours (Cenis 1992).

5.3.2 DNA extraction and sequencing

DNA extraction was carried out following the protocol in Cenis (1992): eppendorf tubes containing mycelium mats and liquid media were centrifuged for 10 minutes at 13,000 rpm, excess liquid media was removed and 500µl of TE buffer added before centrifuging again for five minutes; from this point extractions followed a standard DIGSOL extraction method (Nicholls *et al*. 2000). DNA was resuspended in 30µl of TE buffer.

A 622 bp section of the 28S rRNA region, including the hypervariable stem and loop regions between helices C1 and D2, was amplified using the primers: C1 (5°-ACCCGCTGATTTAAGCAT-3°) and D2 (5° - TCCGTGTTTCAAGACGG-3°) (Leclerc 2000; Hulvey *et al*. 2007). Polymerase chain reactions (PCR) were performed in 20µl reaction volumes containing: 2.5 mM $MgCl_2$ (Invitrogen), 1 x PCR Buffer

(Invitrogen), 0.2 mM dNTP (Invitrogen), 0.1 μM forward primer, 0.1 μM reverse primer, 0.5 units of Taq polymerase (Invitrogen) and 1 μl of DNA template. Initial denaturisation took place at 94°C for 3 minutes; followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 90 seconds; with a final extension step of 72°C for 10 minutes. Amplified samples were cleaned with ExoSAP-IT (USB, Cleveland), according to the manufacturer's instructions, and sent to the GenePool core genomics facility at the University of Edinburgh, where they were sequenced on an ABI 3730 automated sequencer.

5.3.3 Within- and between-site species composition

Sequences were aligned and base-calling errors corrected using Sequencher v4.5 (Gene Codes Corporation, Ann Arbour), and matched to published sequences in the NCBI Genbank database using megaBLAST. Species names were assigned to samples based on the maximum percentage of identical nucleotides between the sample and reference sequences within the alignment length (Max ident), when the percentage of the sample sequence covered by the reference sequence (Query coverage) was at least 90%.

The reference *Saprolegnia* sequences that had the closest match with sample sequences, and that were identified to species level, were downloaded from Genbank (Accession numbers HQ665061, HQ665062, HQ665127, HQ665142, HQ665197, HQ665214, HQ665253, HQ665270: Robideau *et al*. 2011; AF119613, AF119616: Riethmüller *et al*. 2000; AF218166: Leclerc 2000). Reference sequences were aligned to the sample sequences identified as members of the *Saprolegniaceae* using Sequencher v4.5. To visualise differences among the sample and reference sequences, a maximum-likelihood phylogenetic tree was constructed using the Tamura-Nei model of evolution, with 500 bootstrap replications, as implemented in MEGA v5.05 (Tamura *et al*. 2011). *Albugo*

candida was used as an outgroup (Accession number HQ643110: Robideau *et al*. 2011).

5.3.4 Presence and genetic distance in relation to geographical and environmental parameters

Geographical distance between sites was measured as straight-line distance using ArcGIS v10. Pairwise regressions were carried out between each of the environmental parameters (geographic distance, altitude, pH, conductivity, total dissolved solids and temperature) in R v2.12.1 (R core development team). If significant relationships between environmental parameters were found, only one parameter per pair was used in the Mantel and Student's t-tests. Student's t-tests were used to assess whether there were significant differences in environmental parameters between sites where *Saprolegnia* species were and were not isolated. Mantel tests (Mantel & Valand 1970) were used to test for correlations of between-sample genetic distance (dependent variable) with between-sample geographical and environmental distances (independent variable), as implemented in Arlequin v3.5 (Excoffier & Lischer 2010) using 10,000 permutations. Pairwise genetic distances used in the Mantel tests were calculated in MEGA v5.05 using the Tajima-Nei model.

5.4 Results

5.4.1 Within- and between-site species composition

In total, 72 samples from across the ten sites showed colony growth, were sequenced and had a match identified using BLAST (Table C1, Appendix C).

Overall, 24 microbial families, consisting of 37 species were identified, including 33 non-*Saprolegnia* species (Table 5-1). The highest number of families recorded at a single site was QP (8), with AU, BI, CV and DM all showing the lowest diversity, with only three families isolated. The most commonly isolated species were *Didymella phacae* (*Pleosporomycetidae*) and *Microdochium phragmitis* (*Hyponectriaceae*), both isolated seven times from three sites, but never from the same site (Table C1, Appendix C). Nineteen of the species were only isolated from a single sample, but 11 species were isolated from more than one site (Table C1, Appendix C).

Thirteen samples from four sites were identified as belonging to the *Saprolegnia* family: three samples from BM, four samples from CV, four samples from DM and two samples from QP (Table 5-2; Figure 5-1). All samples had at least a 98% match and 90% coverage with Genbank reference sequences (Table 5-2). However, sequences from multiple species in Genbank showed equally high matches with each sample sequence, resulting in between two and seven species names being assigned to each sample (Table 5-2).

The bootstrap consensus tree with maximum-likelihood branch lengths showed that sequences from samples DM1-4 were identical to each other and to reference sequences identified as *S. mixta* and *S. ferax* (Cluster A; Figure 5-2). Samples CV1-4, BM2 and 3, and QP2 clustered together but did not cluster with any of the reference sequences (Cluster B; Figure 5-2). BM1, although most similar to *S. litoralis*, did not cluster with any reference or sample sequences (the only member of Cluster C; Figure 5-2). Finally, QP1 was identical to samples identified as *S. unispora*, *S. torulosa* and *S. monilifera*, but not with any other sequences from this study (Cluster D; Figure 5-2). Bootstrap resolution was not sufficient to draw conclusions about the relationships among clusters, but clusters A and B appear to be more closely related to one another than C and D. The sequence clusters isolated at each site were as follows: only Cluster A was isolated from DM; only Cluster B was isolated from CV; Clusters B and C were isolated from BM; and Clusters B and D were isolated from QP (Table 5-2, Figure 5-2).

5.4.2 Presence and genetic distance in relation to geographical and environmental parameters

The average conductivity at collection sites was 113±87μS, total dissolved solids were 68±59ppm, pH was 6.5±0.6, temperature at collection was 11±3.9°C and altitude of sites was 236±296m (Table 5-3). The distance between sites where *Saprolegnia* was identified varied between 5.86 and 29.38km (Table 5-4). Conductivity and total dissolved solids had a significant relationship in the regression analysis ($r=0.93$, $p<0.01$), as did altitude and temperature ($r=0.46$, $p=0.05$); thus only conductivity and altitude, along with pH and geographic distance, were used in the Mantel and Student's t-tests. There was a significant difference between sites where *Saprolegnia* species were and were not isolated in terms of pH ($p=0.02$), with a significantly higher pH at sites where *Saprolegnia* species were isolated (Figure 5-3); but not in terms of conductivity ($p=0.18$) or altitude ($p=0.08$) (Figure 5-3). However, a trend for higher altitude at sites where *Saprolegnia* was isolated was observed (Figure 5-3). Genetic distance among *Saprolegnia* samples was not found to be significantly correlated with geographic distance (Table 5-3; $r=0.01$, $p=0.46$), altitude ($r=0.11$, $p=0.24$), pH ($r=-0.03$, $p=0.58$), or conductivity ($r=-0.10$, $p=0.70$).

5.5 Discussion

5.5.1 Within- and between-site species composition

The majority of species isolated from the eggs of *R. temporaria* did not belong to the *Saprolegniaceae*, despite the targeted sampling of water mould infected

eggs. Eighty two percent of samples were non-*Saprolegnia* species (Table C1, Appendix C) and almost all of the 33 species found (Table 5-1) are typically associated with plant matter, either as pathogens, parasites or saprobes. An interesting species isolated was *Curreya pityophila,* a parasite associated with Scot's Pine (*Pinus sylvestris),* which is very rare throughout the UK and has previously only been identified in Easterness and Midlothian in Scotland (NBN Gateway; Kruys & Wedin 2009). In my study, *Curreya pityophila* was isolated from three samples from BM, a marsh site at the edge of a Scot's Pine woodland. The noteable exception to the plant-associated species was *Phialemonium curvatum*, a waterborne opportunistic pathogen that can cause serious infections in humans and animals, predominantly in immunosuppressed individuals (Rao *et al*. 2009), which was also isolated from BM (Table C1, Appendix C). These results highlight the microbial diversity found in freshwater systems, and the array of moulds that amphibian eggs come into contact with, either whilst still alive or post mortem.

Although thirteen samples isolated from four sites belonged to the *Saprolegniaceae* family, none could be unambiguously identified to species level. Reference sequences deposited in Genbank showed a 98-100% match with sample sequences in terms of identical nucleotides within the alignment length, and at least a 90% coverage of the sample sequence by reference sequences (Table 5-2). However, reference sequences from between two and seven different species were equally similar to each sample sequence (Table 5-2). When a phylogenetic tree was used to visualise sequence similarity, samples formed four distinct sequence clusters (Table 5-2, Figure 5-2). All samples from DM formed a single sequence cluster (Cluster A), along with reference sequences identified as *S. mixta* and *S.* ferax (Figure 5-2), suggesting that samples from DM are all the same species and potentially *S. mixta* or *S. ferax*. The majority of samples: CV1-4; BM1 and 2; and QP2, formed a sequence cluster (Cluster B; Figure 5-2) that was distinct from any of the reference sequences, suggesting a reference sequence has not yet been deposited for this species in Genbank. Similarly, BM1 (Cluster C) did not cluster with any reference sequences, but neither did it cluster with other sample sequences, although it was most closely

related to *S. litoralis* (Figure 5-2). QP1 clustered with the reference sequences deposited as *S. unispora*, *S. tortulosa* and *S. monilifera* (Cluster D), suggesting it is one of these three species. These results potentially suggest that four species of *Saprolegnia* were present within the sites sampled, but that sequences have not yet been deposited in Genbank for all the species isolated. Furthermore, the 28S region doesn't clearly distinguish between species and multiple identical sequences in Genbank have been assigned different species names. In a pilot study, I used both 28S and ITS molecular markers to identify samples to species level, but different species names were assigned to each sample depending on the marker used (Appendix D). Ault *et al.* (2012), the only other study to have attempted to assess *Saprolegnia* diversity using molecular methods, also reported an inability to identify members of the *Saprolegniaceae* family to species level using the ITS molecular marker, due to lack of morphological identification of reference samples to species level, with many Genbank sequences simply recorded as "*Saprolegnia sp*". At present, taxonomy of the *Saprolegniaceae* based on molecular markers is not generally well resolved and currently no markers are available that will allow molecular identification to species level.

Despite lack of conclusive species identification, it is still possible to draw conclusions about the *Saprolegnia* diversity within- and between-sites based on phylogenetic sequence clusters. Cluster B was the most commonly isolated *Saprolegnia*, found at three of the four sites where *Saprolegnia* was identified (Figure 5-2). Interestingly this most common cluster was highly distinct from the cluster containing *S. ferax*, which is typically thought to be the most common *Saprolegnia* species to infect amphibian eggs and thus used in studies of virulence (Romansic *et al.* 2009). However, all the samples from DM clustered with *S. ferax*, suggesting that *S. ferax* may infect amphibian eggs, at least at some sites in Scotland. Samples isolated from two sites belonged to more than one cluster, with Clusters B and C isolated from BM, and Clusters B and D isolated from QP, suggesting that multiple species can infect amphibian eggs within a single site, and that *Saprolegnia* species assemblages vary by site. Ault *et al.* (2012) also isolated multiple species of *Saprolegnia* from a lake in the USA,

and Fernández-Benéitez *et al*. (2011) identified *S. ferax* and *S. diclina* from a single site in Spain, but both these studies only assessed species richness at a single site. Therefore, this study is the first to assess species diversity across multiple sites and to report that *Saprolegnia* species presence and species assemblage varies by site. The observed *Saprolegnia* species diversity in this study suggests that the focus on the effects of *S. ferax* as the agent of amphibian mortality (e.g. Romansic *et al*. 2009), or on a single unidentified *Saprolegnia* species (e.g. Robinson *et al*. 2003; Sagvik *et al*. 2007; Ruthig 2009; Uller *et al*. 2009), has been too narrow. The synergistic effects of pathogens can be important in predicting mortality in amphibians (Romansic *et al*. 2011), but the effect of infection by multiple species of *Saprolegnia* on amphibian survival has not yet been addressed.

5.5.2 Presence and genetic distance in relation to geographical and environmental parameters

Sites where *Saprolegnia* species were isolated did not vary in terms of conductivity, altitude or spatial distribution (Figures 5-1 and 5-3), but sites where *Saprolegnia* was found showed a significantly higher pH than at those where it was not (Figure 5-3). Higher acidity has previously been linked to higher occurrence of *Saprolegnia* infections in a range of amphibian species, including *R. temporaria*, from lakes in the Netherlands (Leuven *et al*. 1986). However, the acidity of the lakes sampled by Leuven *et al*. (1986) were much greater than in my study, with extremely low acidity classed as below pH 4, moderately acid as pH 4-5, and neutral as above pH 5; all the sites sampled in Scotland would be classed as neutral using these categories (Scotland sites: pH 5.7-6.5; Table 5-3). Furthermore, *R. temporaria* show relatively higher mortality and lower occurrence in high acid environments and are less acid tolerant than their close relative *R. arvalis* (Leuven *et al*. 1986; Andren *et al*. 1988). Therefore, this study has potentially identified a finer scale of pH related *Saprolegnia* presence and

absence in neutral environments, in relation to a less acid tolerant amphibian species, with a relatively higher pH favouring *Saprolegnia* growth.

Despite the results showing pH based *Saprolegnia* presence, the conclusion that *Saprolegnia* infection was absent from sites where *Saprolegnia* species were not isolated must be treated with caution, due to the inherent problems of water mould culture methods prior to DNA extraction (Ault *et al*. 2012), such as the risk of contamination (Appendix D). During a pilot study, despite collecting samples from sites where *Saprolegnia* had been isolated in 2010, none of the samples collected in 2011 were identified as *Saprolegniaceae* (Appendix D). Ault *et al*. (2012) recently introduced a new method for molecular identification of water moulds, avoiding the culture step, by extracting total embryo associated DNA, amplifying DNA with primers capable of amplifying a broad array of eukaryotic microorganisms, and constructing clone libraries, each clone of which was then sequenced. A repeat study in Scotland using the new molecular identification method would be interesting to elucidate the biases associated with water mould culture.

Although the sites in this study varied in terms of environmental parameters (Table 5-1) and geographical distance from one another (Figure 5-1; Table 5-4), no significant correlation was observed between genetic distance, and geographical or environmental distances. This is surprising, as sampled sites differed in the type and number of putative water mould species found (Figure 5-2). Kiesecker *et al*. (2001) found that different isolates of *S. ferax* caused different levels of mortality in western toad embryos, suggesting variability in virulence between strains. Furthermore, Sagvik *et al*.(2008) identified a genetic component to resistance to infection by an unidentified *Saprolegnia* species in the moor frog. Therefore, the potential differences observed between sites in terms of species diversity and presence could be due to variability in levels of virulence of different members of the *Saprolegniaceae* and/or host resistance to water moulds.

The findings of this study are particularly important in light of a changing climate, where disease dynamics and species ranges are predicted to change (Marcogliese 2001; Pounds 2001; Parmesan 2006). Therefore, amphibian populations may come into contact with novel *Saprolegnia* species that are more virulent or to which they have lower resistance. Further research is needed to determine whether host resistance to water moulds varies by breeding site.

5.6 Conclusion

Multiple species of *Saprolegnia* infect *R. temporaria* eggs in Scotland, species composition varies within- and between-sites, and presence of *Saprolegnia* appears to be dependent on the acidity of the site. These findings question the previous focus on *S. ferax* as the primary agent of *Saprolegnia* infection in amphibians and suggest that future studies of virulence need to consider the synergistic effect of multiple *Saprolegnia* species. Studies are needed to resolve *Saprolegniaceae* taxonomy based on molecular markers, in order for species-level ecology to be further elucidated.

Table 5- 1: Microbial sequences isolated per site, showing the total number of families isolated from each site (Total families); the number of species isolated excluding those identified as *Saprolegnia* sp. (Non-*Saprolegnia* sp.); and the number of species identified as *Saprolegnia* (*Saprolegnia* sp.).

Site	Total families	Non-*Saprolegnia* sp.	*Saprolegnia* sp.
AU	3	3	0
BI	3	3	0
BM	5	5	2
BW	4	5	0
CR	4	4	0
CV	3	2	1
DM	3	2	1
GL	4	6	0
MU	4	6	0
QP	8	7	2
Total	**24**	**33**	**4**

Table 5- 2: Identification of species from each site where *Saprolegnia* was isolated, including: site of sample collection (Site) and sample ID (Sample); alongside the species identified using Genbank (Species), the percentage of identical nucleotides between the sample and reference sequences within the alignment length (% match)/the percentage of the sample sequence covered by the reference sequence (% coverage); and sequence clusters assigned based on phylogeny (Cluster; Figure 2).

Site	Sample	Species	% match	% coverage	Cluster
BM	BM1	*S. parasitica/litoralis*	98	100/100	C
	BM2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	100/90/100/100/100/100/93	B
	BM3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	100/90/100/100/100/100/93	B
CV	CV1	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	100/90/100/100/100/100/93	B
	CV2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	100/90/100/100/100/100/93	B
	CV3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	100/90/100/100/100/100/93	B
	CV4	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	100/90/100/100/100/100/93	B
DM	DM1	*S. ferax/unispora*	100	100/100	A
	DM2	*S. ferax/ unispora*	100	100/100	A
	DM3	*S. ferax/unispora*	100	100/100	A
	DM4	*S. ferax/ unispora*	100	100/100	A
QP	QP1	*S. monilifera/ unispora/ torulosa/ terrestris*	99	100/100/99/99	D
	QP2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/hypogyna*	99	100/90/100/100/100/100/93	B

Table 5- 3: Environmental parameter measurements taken at sample collection, including the water parameters: conductivity, total dissolved solids, pH and temperature; and the geographical parameter: altitude.

Site	Conductivity (µS)	Dissolved solids (ppm)	pH	Temperature (°C)	Altitude (m)
AU	111	97	6.3	12.1	19
BI	189	107	6.4	21.0	93
BM	102	50	6.5	10.4	72
BW	333	233	5.7	7.7	179
CV	56	29	6.1	12.1	228
DM	250	133	6.4	13.1	149
GL	45	23	5.8	15.1	50
MU	149	78	6.1	12.9	51
QP	127	65	6.4	12.5	163
RE	75	39	6.3	11.7	53

Table 5- 4: Comparison of pairwise between-sample genetic distances (upper triangle) with geographic distance between sites of sample origin (lower triangle; km).

	QP1	DM1	DM2	DM3	BM1	QP2	BM2	CV1	CV2	CV3	CV4	DM4	BM3
QP1	-	0.027	0.027	0.027	0.037	0.024	0.024	0.024	0.024	0.024	0.024	0.027	0.024
DM1	5.86	-	0.000	0.000	0.032	0.003	0.003	0.003	0.003	0.003	0.003	0.000	0.003
DM2	5.86	0.00	-	0.000	0.032	0.003	0.003	0.003	0.003	0.003	0.003	0.000	0.003
DM3	5.86	0.00	0.00	-	0.032	0.003	0.003	0.003	0.003	0.003	0.003	0.000	0.003
BM1	23.61	29.38	29.38	29.38	-	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032
QP2	0.00	5.86	5.86	5.86	23.61	-	0.000	0.000	0.000	0.000	0.000	0.003	0.000
BM2	23.61	29.38	29.38	29.38	0.00	23.61	-	0.000	0.000	0.000	0.000	0.003	0.000
CV1	23.73	28.89	28.89	28.89	8.29	23.73	8.29	-	0.000	0.000	0.000	0.003	0.000
CV2	23.73	28.89	28.89	28.89	8.29	23.73	8.29	0.00	-	0.000	0.000	0.003	0.000
CV3	23.73	28.89	28.89	28.89	8.29	23.73	8.29	0.00	0.00	-	0.000	0.003	0.000
CV4	5.86	28.89	28.89	28.89	8.29	5.86	8.29	0.00	0.00	0.00	-	0.003	0.000
DM4	5.86	0.00	0.00	0.00	29.38	5.86	29.38	28.89	28.89	28.89	28.89	-	0.003
BM3	23.61	29.38	29.38	29.38	0.00	23.61	0.00	8.29	8.29	8.29	8.29	29.38	-

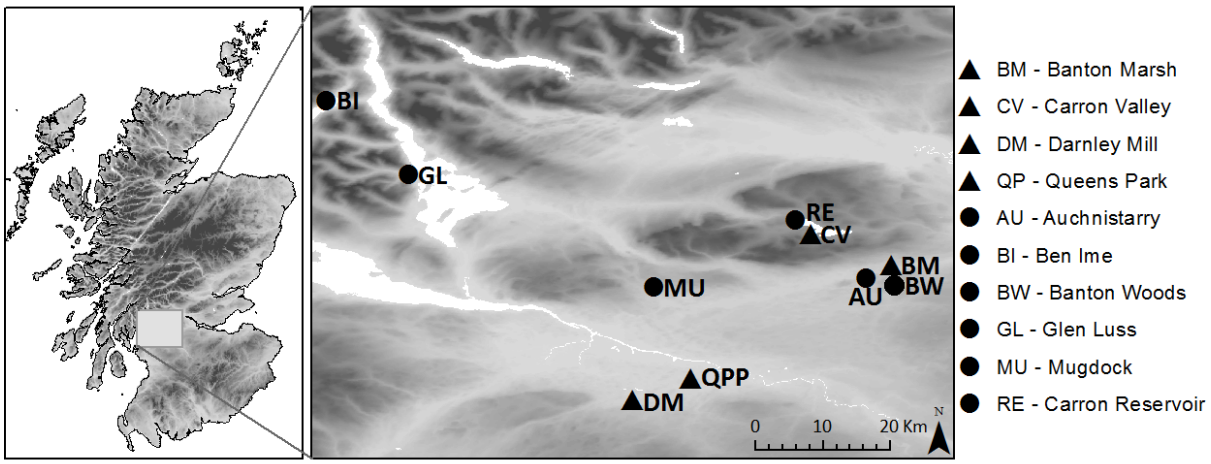

Figure 5- 1: Sites of sample collection within central Scotland; triangles show sites where *Saprolegnia* species were identified and circles show sites where *Saprolegnia* species were not identified.

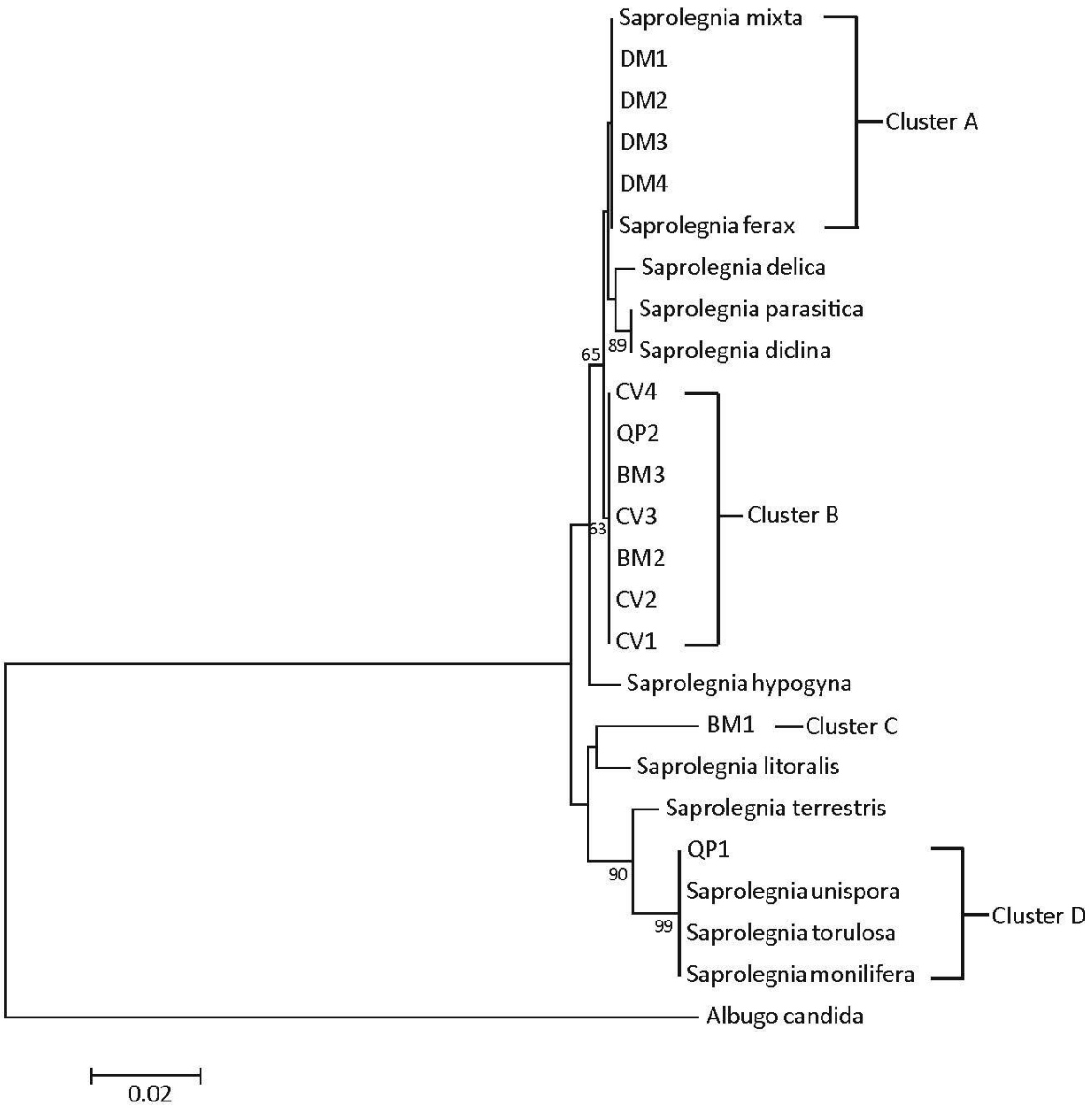

Figure 5- 2: Bootstrap consensus tree with maximum-likelihood branch lengths showing reference sequences identified as *Saprolegnia* species in Genbank, alongside the sample sequences from this study. Sequences from this study have been grouped into sequence clusters with those identified in Genbank. Bootstrap values above 60% are indicated but only those above 70% should be interpreted as resolved (Hillis & Bull 1993).

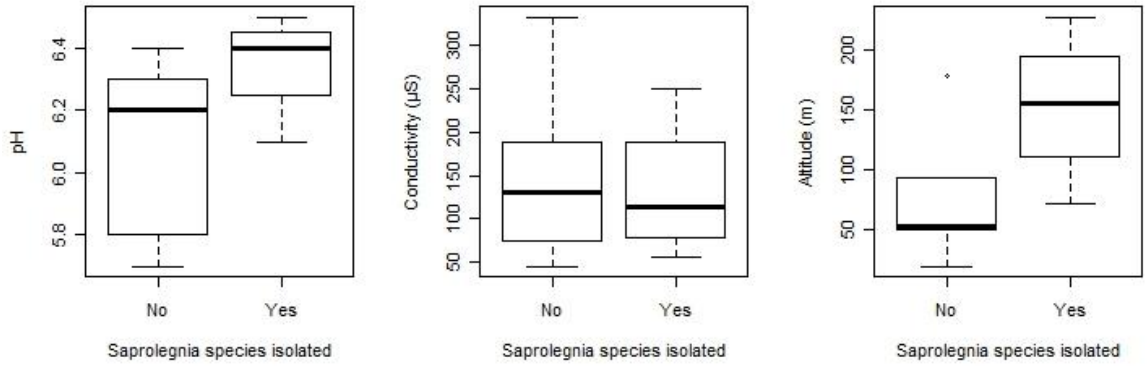

Figure 5- 3: Boxplots of environmental parameters recorded at sites where the *Saprolegnia* species were isolated and were not isolated. Thick bars show the median trait value with interquartile ranges either side; whiskers show the range of values observed. A significant difference was found in terms of pH (p=0.02) but not conductivity (p=0.18) or altitude (p=0.08).

Chapter 6 – General Discussion

6.1 Population genetics of the common frog in relation to climate

The overall aim of this thesis was to assess population-level relationships with climate in *Rana temporaria* in Scotland, in order to make predictions about susceptibility to environmental change. In Chapter 2, I placed current population structure in the context of phylogeographic background, in order to rule out genetic variation due to historical patterns of colonisation. I found that all Scottish amphibians belonged to the mitochondrial clade common to western Europe, a clade lacking in genetic variation. From this baseline, I was able to assess fine-scale population structure in relation to current climatic conditions along altitudinal gradients. No population structure was found using microsatellites within or between altitudinal gradients at any scale (3-50km), despite a mean annual temperature difference of 4.5°C between low- and high-altitude sites. Levels of genetic diversity and heterozygosity were considerable but did not vary by site, altitude or temperature. These results suggested a greater dispersal ability and lower site philopatry of *R. temporaria* than has been found for other amphibians and that movement of individuals was not limited by different thermal environments.

Although neutral genetic divergence is useful for interpreting recent demography, it is not a predictor of adaptive genetic divergence, as local adaptation has been observed to take place even with gene flow (Richter-Boix *et al.* 2010). Therefore, in Chapter 3, I assessed whether local adaptation to altitude had taken place in the face of high gene flow and examined the environmental drivers of this local adaptation. I found that *R. temporaria* showed evidence of local adaptation in all larval fitness traits measured:

metamorphic weight, SVL gain, larval period and growth rate. However, only variation in larval period and growth rate was consistent with adaptation to altitude. Moreover, this was only evident in the three highest mountains (high-altitude sites at least 900m), suggesting the possibility of a threshold for local adaptation. This variation was correlated with spring temperature, suggesting that temperature acts as a strong environmental selection pressure influencing local adaptation along altitudinal gradients, even in the face of high gene flow. Furthermore, by using multiple common temperature treatments to assess local adaptation, I was able to look at genotype-by-environment interactions and discovered that individuals were phenotypically plastic in terms of all larval traits studied except SVL gain, but that phenotypic plasticity did not vary by altitude.

Having established that *R. temporaria* are locally adapted to temperature, in Chapter 4 I went on to investigate the physiological and behavioural adaptations that allow survival at high-altitude in low-temperature environments. Larval *R. temporaria* showed reduced routine metabolic rate at high-altitude, but only in the three highest mountains, where increased growth rate had been observed in Chapter 3. These results suggest that there is a resource-limited trade-off between growth rate and routine metabolic rate in these mountains. High-altitude individuals were not more freeze tolerant than their low-altitude neighbours, and adult *R. temporaria* did not breed at a lower temperature than low-altitude individuals, suggesting these are not responses linked to survival in low-temperature environments.

Finally, in Chapter 5, I assessed whether the amphibian egg pathogen, *Saprolegnia*, varied spatially in terms of presence and species composition. Four species of *Saprolegnia* were isolated overall, multiple *Saprolegnia* water moulds were isolated from within sites, and species composition varied between sites. A lower acidity was linked to *Saprolegnia* presence, but genetic distance between samples was not correlated with environmental or geographic distance. These findings question the previous focus on *S. ferax* as the primary agent of

Saprolegnia infection and suggest that future studies of virulence need to consider the synergistic effect of multiple *Saprolegnia* species.

6.2 Susceptibility of the common frog to a changing climate

Three responses are predicted to facilitate survival in a changing climate: dispersing to evade changing climatic conditions and remain within climatic tolerance limits; evolving to stay adapted to local environmental conditions as they change; and plastically responding to changes in climate without underlying genetic changes (Reusch & Wood 2007; Gienapp *et al*. 2008; Sork *et al*. 2010). The potential to disperse to remain within species-specific tolerance levels is dependent on dispersal ability and availability of habitat to move into (Perry *et al*. 2005; Parmesan 2006). *R. temporaria* throughout western Europe show very low levels of genetic diversity, consistent with a leptokurtic model of range expansion, suggesting that post-glacial colonisation during the last period of global warming was rapid and consisted of low numbers of long-distance dispersers causing multiple founder events. Furthermore, high levels of gene flow were observed between *R. temporaria* breeding pools on the scale of 3-50km (Chapter 2). Therefore, my results suggest that *R. temporaria* have the potential to disperse long distances and that, given historical dispersal in response to global warming, evasion will be a likely response of *R. temporaria* to a changing climate. Although Scotland is relatively sparsely populated by humans and urban barriers to range movements will be few (Thompson & Brown 1992; Swan *et al*. 1994; Werritty *et al*. 1994), habitat availability is still limited by the height of Scotland's mountains and the North Sea when considering movements to higher latitudes and altitudes, as have been observed in a range of other species (Parmesan & Yohe 2003). Therefore, *R. temporaria* have the potential to disperse, but only up to the limit of terrestrial habitat in Scotland. As some individuals already live at the top of mountains and on the north coast of Scotland, a dispersal response will not be an option for these populations.

The second potential ecological response to a changing climate, evolving as the environment changes in order to retain fitness in the new conditions, will depend on whether individuals are locally adapted to current climatic conditions and their evolutionary potential in terms of genetic variability (Gienapp *et al.* 2008). The local adaptation observed in my study only took place in the highest mountains, where high-altitude sites were at least 900m; high-altitude sites on the mountains where local adaptation was not observed were 720m or lower. These results suggest that a threshold exists in terms of the environmental conditions necessary to cause local adaptation; this could be a result of the absolute environmental conditions at the higher altitudes, or the relative difference between environmental conditions at high- vs. low-altitude among mountains. My results, identifying spring temperature as the ecological driver of local adaptation to altitude in terms of growth rate and larval period, would imply that the local adaptation threshold seen between mountains is in terms of spring temperature. The average spring temperature difference between high- and low-altitude on the three mountains that showed local adaptation to altitude was 4.67°C, and on the mountains that did not show local adaptation was 3.87°C. Therefore, potentially, a change in temperature somewhere between 3.87°C and 4.67°C is sufficient to elicit an evolutionary response in *R. temporaria* in Scotland. Temperature is set to rise within the west of Scotland between 0.8°C and 4.4°C in the next 50 years, depending on emissions scenario and uncertainty range (UKCP09 2011). Therefore, environmental selection pressures could change sufficiently in the next 50 years that *R. temporaria* will be required to evolve to remain locally adapted to the environment experienced. My findings of considerable genetic diversity and heterogeneity across all sites, in conjunction with high levels of gene flow between sites, suggest that *R. temporaria* have evolutionary potential to respond to changing environmental conditions through evolution.

Finally, the third response that will facilitate survival in a changing climate is phenotypic plasticity (Reusch & Wood 2007; Sork *et al.* 2010). Phenotypic plasticity is a more rapid response than evolution, as it has the potential to occur within a lifetime or generation (Nussey *et al.* 2005; Charmantier *et al.*

2008), and a more robust response than dispersal, as individuals can remain within their current geographical range (Jump & Penuelas 2005). Organisms that show relatively higher levels of phenotypic plasticity are thus predicted to be less susceptible to a changing climate (Crozier *et al*. 2008; Hallsson & Björklund 2012). My results have shown that *R. temporaria* are phenotypically plastic in terms of multiple larval fitness traits (metamorphic weight, larval period and growth rate) in response to multiple thermal environments and that this plasticity does not vary with altitude. Furthermore, I found that adult frogs were plastic in terms of breeding date, responding to a 5°C thermal threshold to commence breeding. The occurrence of phenotypic plasticity in *R. temporaria* is a potentially positive sign for persistence in a changing climate. However, further research is needed to assess the limits to this phenotypic plasticity, whether plasticity limits will be exceeded by predicted climate change, and whether phenotypic plasticity itself can evolve.

6.3 Conservation implications

Current conservation methods revolve around habitat preservation within fixed geographical boundaries, designed to protect all species inhabiting a particular ecosystem (Araújo *et al*. 2004). However, protected areas alone may no longer successfully conserve the species they house when changes in climate are causing dynamic alterations to the environment, species ranges and community interactions (Hannah *et al*. 2002). My results, showing that *R. temporaria* have high dispersal ability, suggest that the creation of climate paths: areas of suitable habitat that *R. temporaria* can move through as the climate changes (Early & Sax 2011), will potentially be the most successful conservation measure available to ensure common frog survival in a changing climate. The observed high gene flow and genetic diversity within Scotland is a positive sign in terms of evolutionary potential of *R. temporaria*. Therefore, conservation strategies must also include maintenance and protection of current habitat connectivity in order to preserve dispersal routes between breeding sites. However, the local

adaptation seen in this study suggests that translocation of individuals could result in maladaptation in a new environment and that such conservation measures should be approached with caution. The phenotypic plasticity of *R. temporaria* will potentially provide time for the relatively slower evolutionary and dispersal responses to take place. Therefore, the outlook is positive for *R. temporaria* in Scotland and I would recommend conservation strategies with a focus on creation and maintenance of dispersal corridors and climate pathways to support survival in a changing climate.

Dispersal-corridor based conservation strategies are also likely to benefit the other relatively widespread native amphibians in Scotland: the smooth newt (*Lissotriton vulgaris*), palmate newt (*L. helveticus*) and common toad (*Bufo bufo*). However, the great crested newt (*Triturus cristatus*) is rare in Scotland and the natterjack toad (*Epidalea calamita*) is restricted to one location on the Solway Firth in Scotland (Inns 2009). Therefore, further research is needed to determine whether these vulnerable species show local adaptation, what is driving this local adaptation, and whether translocation to new areas as the environment changes is a conservation option to promote survival.

6.4 Global amphibian decline research

In recent years, amphibian decline research has focussed on the effects of the chytrid fungus (*Batrachochytrium dendrobatidis*), almost to the exclusion of all other possible causes of mortality (Duffus 2009). My research highlights that even the ubiquitous pathogenic water moulds of the *Saprolegniceae*, which have been linked to embryo mortality (Robinson *et al.* 2003), are still widely unknown in terms of species that cause infection, species ecology, species virulence and host immunity. I have identified that multiple species of *Saprolegnia* are associated with amphibian eggs in Scotland and that environmental conditions are linked to *Saprolegnia* presence, and are thus subject to change in a changing climate. Therefore, it is important that amphibian epidemiology researchers and

funders acknowledge that the chytrid fungus is not the only disease risk to amphibians in a changing climate and that amphibian decline research is broadened accordingly.

Despite amphibians being a high risk taxa in a changing climate (Blaustein *et al*. 2010), *R. temporaria* appear to have a good chance of survival. Therefore, my results demonstrate that even within threatened groups there is the potential for individual species to be of lower conservation concern. Therefore, it is important that all species are not treated under the same blanket conservation measures, but that susceptibility is determined on a case by case basis and that local conservation measures reflect this. Potentially other species of amphibian, as well as *R. temporaria*, are robust to changing environmental conditions and it is important that these species are identified, as well as those at high risk of extinction that tend to be the focus of research efforts. Acting to conserve species with the potential for survival in a changing climate may be the only way to slow the global amphibian extinctions already in progress.

6.5 A framework for assessing susceptibility to a changing climate

The framework used in this study is based on inferring relationships with current climate in order to make predictions about the three potential responses of organisms to climate change: evasion, evolution and plasticity. These three potential responses apply not just to amphibians, but to all animals and plants in the face of a changing climate (Reusch & Wood 2007). Thus, this framework has the potential to be broadly applied in climate change susceptibility studies (Jump & Penuelas 2005; Sork *et al*. 2010). The methods for assessing evolutionary potential, dispersal potential and phenotypic plasticity will vary depending on the species or taxa of interest. For instance, in many plant species range shifts will occur via seed dispersal and germination success, whereas for animal species range shifts will be subject to the movement of individuals (Parmesan 1996; Savolainen *et al*. 2007); for many large and/or rare species,

experimental manipulation is not a logistical and/or ethical option (Antoniazza *et al*. 2010); and terrestrial and marine species will be subject to different selection pressures and constraints (Perry *et al*. 2005). However, following the evasion/evolution/plasticity (EEP) framework using a variety of methods will still function to allow robust predictions of species survival in a changing climate.

Given the species-specific susceptibility predictions required, molecular methods provide an opportunity to infer demography, genetic diversity and local adaptation and can be broadly applied (Allendorf & Luikart 2007). Furthermore, many non-invasive methods of genetic data collection are available including using hair, scat and feathers (Taberlet & Luikart 1999). Therefore, molecular methods can be used on even the most endangered species without causing disturbance or reducing population sizes. Molecular ecology also allows relationships with recent climate conditions to be assessed in a shorter timescale than observational studies, an important consideration given the rapid pace of current environmental change (McKay & Latta 2002; Reusch & Wood 2007). Therefore, using molecular methods in line with the EEP framework will allow species susceptibility to be assessed within a timescale that will permit conservation strategy formation and application based on the results.

The next step to progress the EEP framework would be to incorporate this research-based knowledge into a geographical model, so that not only the potential for responses to climate change is predicted, but also the direction, speed and success of these responses, in line with different climate change scenarios. Such integration of approaches is key to forming successful conservation strategies, as current models are limited by lack of species-specific knowledge of relationships with climate (McCarty 2001; Araújo *et al*. 2006). Such accurate predictions will allow conservation planners to plot and protect important dispersal routes, plan successful translocation strategies, and focus conservation attention on species that are at risk but have the potential for survival given conservation intervention. For species to survive human-induced climate change, conservation management also needs to rapidly respond in order to keep pace with the changing environment we've created.

Appendix A: Supplementary Information for Chapter 2

Table A1: Pairwise Jost's D estimates (lower triangle) and altitudinal difference between sites (upper triangle). The abbreviation MNT is used for Meall nan Tarmachan.

	Dubhchraig High	Dubhchraig Low	Ime High	Ime Low	Lawers High	Lawers Low	Lomond High	MNT High	MNT Low
Dubhchraig High	--	703	197	155	90	685	180	0	677
Dubhchraig Low	-0.005	--	506	42	793	18	523	703	26
Ime High	0.022	0.011	--	548	287	488	17	197	480
Ime Low	0.006	-0.001	0.006	--	835	60	565	745	68
Lawers High	-0.001	0.003	0.02	0.011	--	775	270	90	767
Lawers Low	0	0.005	0.017	0	0	--	505	685	8
Lomond High	0.001	0.001	0.013	0.013	0.003	0.007	--	180	497
MNT High	0.055	0.067	0.052	0.06	0.046	0.041	0.067	--	677
MNT Low	0.019	0.03	0.058	0.027	0.024	-0.002	0.031	0.059	--

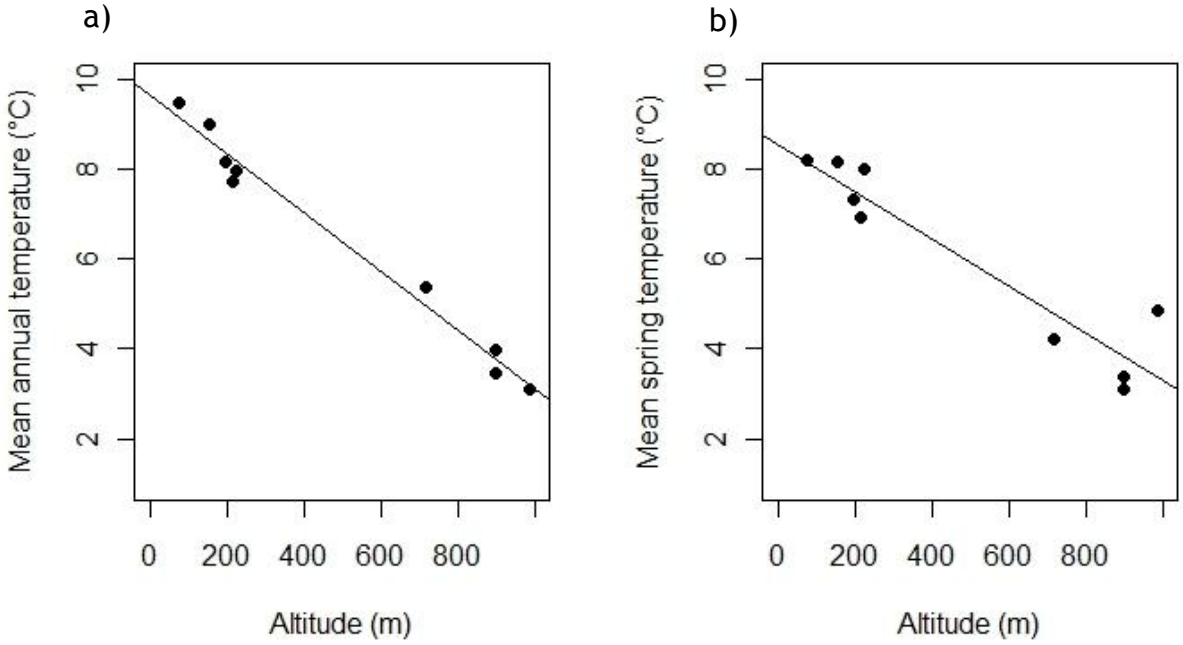

Figure A1: Plot of a) mean annual temperature and b) mean spring temperature, by altitude, fitted with the linear regression lines: a) Mean annual temperature = (-0.0065 x Altitude) + 9.66, (r^2=0.98, p<0.001); b) Mean spring temperature = (-0.0053 x Altitude) + 8.57, (r^2=0.87, p<0.001).

Appendix B: Supplementary Information for Chapter 3

Table B1: Comparison of pairwise genetic distances based on F_{ST} from microsatellite markers (lower triangle) with Q_{ST} of metamorphic weight (upper triangle).

	DUBHIGH	DUBLOW	IMEHIGH	IMELOW	LAWHIGH	LAWLOW	LOMHIGH	MNTHIGH	MNTLOW
DUBHIGH	--	0.010	0.160	0.105	0.218	0.006	0.067	0.290	0.037
DUBLOW	-0.007	--	0.176	0.104	0.263	0.055	0.064	0.386	0.021
IMEHIGH	0.027	0.025	--	0.080	0.001	0.295	0.006	0.100	0.078
IMELOW	0.005	0.000	0.020	--	0.197	0.268	0.007	0.224	0.014
LAWHIGH	0.003	0.001	0.052	0.021	--	0.408	0.010	0.008	0.121
LAWLOW	0.002	0.007	0.037	0.020	-0.012	--	0.132	0.495	0.107
LOMHIGH	0.005	0.019	0.018	0.031	0.012	0.005	--	0.030	0.019
MNTHIGH	0.020	0.025	0.038	0.028	0.022	0.023	0.029	--	0.200
MNTLOW	0.036	0.046	0.070	0.041	0.004	0.004	0.033	0.025	--

Table B2: Comparison of pairwise genetic distances based on F_{ST} from microsatellite markers (lower triangle) with Q_{ST} of larval period (upper triangle).

	DUBHIGH	DUBLOW	IMEHIGH	IMELOW	LAWHIGH	LAWLOW	LOMHIGH	MNTHIGH	MNTLOW
DUBHIGH	--	0.725	0.148	0.112	0.323	0.705	0.057	0.121	0.642
DUBLOW	-0.007	--	0.150	0.712	0	0.028	0.026	0	0.300
IMEHIGH	0.027	0.025	--	0.234	0.685	0	0.004	0.616	0.003
IMELOW	0.005	0	0.020	--	0.234	0.685	0	0.054	0.616
LAWHIGH	0.003	0.001	0.052	0.021	--	0.003	0.133	0.027	0.046
LAWLOW	0.002	0.007	0.037	0.020	-0.012	--	0.308	0.080	0.113
LOMHIGH	0.005	0.019	0.018	0.031	0.012	0.005	--	0.037	0.366
MNTHIGH	0.020	0.025	0.038	0.028	0.022	0.023	0.029	--	0.154
MNTLOW	0.036	0.046	0.070	0.041	0.004	0.004	0.033	0.025	--

Table B3: Comparison of pairwise genetic distances based on F_{ST} from microsatellite markers (lower triangle) with Q_{ST} of survival (upper triangle).

	DUBHIGH	DUBLOW	IMEHIGH	IMELOW	LAWHIGH	LAWLOW	LOMHIGH	MNTHIGH	MNTLOW
DUBHIGH	--	0.165	0.003	0.054	0.020	0.145	0.011	0.190	0.067
DUBLOW	-0.007	--	0.220	0.082	0.040	0.002	0.082	0.025	0.058
IMEHIGH	0.027	0.025	--	0.090	0.037	0.194	0.026	0.236	0.106
IMELOW	0.005	0	0.02	--	0.001	0.077	0.011	0.115	0.002
LAWHIGH	0.003	0.001	0.052	0.021	--	0.042	0.002	0.065	0.004
LAWLOW	0.002	0.007	0.037	0.020	-0.012	--	0.076	0.010	0.058
LOMHIGH	0.005	0.019	0.018	0.031	0.012	0.005	--	0.116	0.018
MNTHIGH	0.020	0.025	0.038	0.028	0.022	0.023	0.029	--	0.091
MNTLOW	0.036	0.046	0.070	0.041	0.004	0.004	0.033	0.025	--

Table B4: Comparison of pairwise genetic distances based on F_{ST} from microsatellite markers (lower triangle) with Q_{ST} of SVL gain (upper triangle).

	DUBHIGH	DUBLOW	IMEHIGH	IMELOW	LAWHIGH	LAWLOW	LOMHIGH	MNTHIGH	MNTLOW
DUBHIGH	--	0.029	0.248	0.209	0.208	0.005	0.118	0.082	0.064
DUBLOW	-0.007	--	0.281	0.259	0.234	0	0.182	0.101	0.068
IMEHIGH	0.027	0.025	--	0.008	0.100	0.327	0.364	0.057	0.075
IMELOW	0.005	0	0.020	--	0	0.258	0.398	0.042	0.051
LAWHIGH	0.003	0.001	0.052	0.021	--	0.267	0.342	0.035	0.049
LAWLOW	0.002	0.007	0.037	0.020	-0.012	--	0.181	0.085	0.06
LOMHIGH	0.005	0.019	0.018	0.031	0.012	0.005	--	0.308	0.262
MNTHIGH	0.020	0.025	0.038	0.028	0.022	0.023	0.029	--	0.001
MNTLOW	0.036	0.046	0.070	0.041	0.004	0.004	0.033	0.025	--

Appendix C: Supplementary information for Chapter 5

Table C1: All species identified at each site, including: site of sample collection (Site) and Sample ID in the form: site reference (e.g. R1), spawn clump reference per site (e.g. A), and egg number per clump (e.g. 1); alongside the species identified using Genbank (Species), the percentage of identical nucleotides between the sample and reference sequences within the alignment length (% match), and the Family of each species identified.

Site	Sample ID	Species	% match	Family
Auchinstarry	R3A2	*Pleurophoma pleurospora*	98	*Incertae sedis*
Auchinstarry	R3B1	*Microdochium phragmitis*	100	*Hyponectriaceae*
Auchinstarry	R3B3	*Hanseniaspora clermontiae*	100	*Saccharomycodaceae*
Banton Marsh	R2A1	*Didymella phacae*	100	*Pleosporomycetidae*
Banton Marsh	R2A3	*Trametes versicolor*	100	*Polyporaceae*
Banton Marsh	R2B2	*Phialemonium curvatum*	97	*Cephalothecaceae*
Banton Marsh	R2B3	*Didymella phacae*	100	*Pleosporomycetidae*
Banton Marsh	R2C1	*Didymella phacae*	100	*Pleosporomycetidae*

Table C1 continued

Banton Marsh	R2C2	*Curreya pityophila*	100	*Pleosporomycetidae*
Banton Marsh	R2C3	*Curreya pityophila*	100	*Pleosporomycetidae*
Banton Marsh	R2D1	*S. parasitica/litoralis*	98	*Saprolegniaceae*
Banton Marsh	R2D2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Banton Marsh	R2D3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Banton Marsh	R2E1	*Curreya pityophila*	100	*Pleosporomycetidae*
Banton Marsh	R2E3	*Monographella lycopodina*	100	*Hyponectriaceae*
Banton Wood	R1A1	*Microdochium phragmitis*	99	*Hyponectriaceae*
Banton Wood	R1A2	*Microdochium phragmitis*	99	*Hyponectriaceae*
Banton Wood	R1B1	*Microdochium phragmitis*	99	*Hyponectriaceae*
Banton Wood	R1B2	*Guehomyces pullulans*	80	*Cyfstofilobasidiaceae*
Banton Wood	R1B3	*Guehomyces pullulans*	98	*Cyfstofilobasidiaceae*
Banton Wood	R1C1	*Microdochium phragmitis*	84	*Hyponectriaceae*
Banton Wood	R1D1	*Monographella lycopodina*	100	*Hyponectriaceae*
Banton Wood	R1D3	*Phoma herbarum*	99	*Pleosporomycetidae*
Banton Wood	R1E1	*Neottiosporina paspali*	99	*Massarinaceae*

Table C1 continued

Banton Wood	R1E3	*Microdochium phragmitis*	99	*Hyponectriaceae*
Ben Ime	R4B1	*Trichoderma viride*	82	*Hypocreaceae*
Ben Ime	R4D3	*Varicosporium delicatum*	99	*Helotiaceae*
Ben Ime	R4E2	*Mortierella fimbricystis*	95	*Mortierellaceae*
Carron Reservoir	R7B1	*Bionectria ochroleuca*	100	*Bionectriaceae*
Carron Reservoir	R7B2	*Hypholoma fasciculare*	99	*Strophariaceae*
Carron Reservoir	R7D1	*Mortierella elongata*	99	*Mortierellaceae*
Carron Reservoir	R7E1	*Microdochium phragmitis*	98	*Hyponectriaceae*
Carron Valley	R8B3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8C1	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8C2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8C3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8D1	*Mortierella elongata*	99	*Mortierellaceae*
Carron Valley	R8D3	*Ceratobasidium cornigerum*	98	*Ceratobasidiaceae*
Carron Valley	R8E1	*Mortierella elongata*	99	*Mortierellaceae*
Darnley Mill	U2A2	*S. ferax/ unispora*	100	*Saprolegniaceae*

Table C1 continued

Darnley Mill	U2B3	*S. ferax/ unispora*	100	*Saprolegniaceae*
Darnley Mill	U2C1	*S. ferax/unispora*	100	*Saprolegniaceae*
Darnley Mill	U2C2	*Westerdykella multispora*	100	*Sporormiaceae*
Darnley Mill	U2D3	*Paraphoma chrysanthemicola*	99	*Pleosporomycetidae*
Darnley Mill	U2E2	*S. ferax/unispora*	100	*Saprolegniaceae*
Glen Luss	R5A1	*Diaporthe angelicae*	99	*Diaporthaceae*
Glen Luss	R5A2	*Phaeocytostroma ambiguum*	99	*Diaporthaceae*
Glen Luss	R5B1	*Phoma complanata*	100	*Pleosporomycetidae*
Glen Luss	R5B3	*Articulospora tetracladia*	99	*Hypogastruroidea*
Glen Luss	R5C1	*Trametes versicolor*	99	*Polyporaceae*
Glen Luss	R5C3	*Didymella phacae*	99	*Pleosporomycetidae*
Glen Luss	R5E1	*Didymella phacae*	99	*Pleosporomycetidae*
Glen Luss	R5E2	*Didymella phacae*	99	*Pleosporomycetidae*
Mugdock	R6A2	*Pseudeurotium zonatum*	99	*Pseudeurotiaceae*
Mugdock	R6B1	*Pseudeurotium zonatum*	99	*Pseudeurotiaceae*
Mugdock	R6B3A	*Varicosporium scoparium*	96	*Helotiaceae*

Table C1 continued

Mugdock	R6B3B	*Sarcoleotia turficola*	96	*Helotiaceae*
Mugdock	R6D1	*Didymella phacae*	99	*Pleosporomycetidae*
Mugdock	R6D2	*Pythium paddicum*	99	*Pythiaceae*
Mugdock	R6D3	*Plectosphaerella plurivora*	100	*Plectosphaerellaceae*
Queens Park	U1A1	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/hypogyna*	99	*Saprolegniaceae*
Queens Park	U1A3	*Trametes versicolor*	99	*Polyporaceae*
Queens Park	U1C1A	*Trichoderma viride*	99	*Hypocreaceae*
Queens Park	U1C1B	*Phoma exigua*	100	*Pleosporomycetidae*
Queens Park	U1C2A	*Penicillium solitum*	100	*Trichocomaceae*
Queens Park	U1C2B	*Westerdykella multispora*	100	*Sporormiaceae*
Queens Park	U1C3A	*Mucor hiemalis*	100	*Mucoraceae*
Queens Park	U1C3B	*Penicillium solitum*	100	*Trichocomaceae*
Queens Park	U1D1	*Mucor hiemalis*	99	*Mucoraceae*
Queens Park	U1D1	*S. monilifera/ unispora/ torulosa/ terrestris*	99	*Saprolegniaceae*
Queens Park	U1E1	*Trametes versicolor*	99	*Polyporaceae*
Queens Park	U1E2	*Neobulgaria pura*	96	*Leotiaceae*

Appendix D: Molecular identification of Saprolegnia species

Methods

Sampling and DNA extraction

Common frog (*Rana temporaria*) eggs that showed evidence of infection by water mould (identified as a white "cotton wool" covering the surface of the egg; Fernández-Benéitez *et al*. 2008) were collected from seven sites across west-central Scotland during the 2010 breeding season (March-April; Table D1) and ten sites across west-central Scotland during the 2011 breeding season (March-April; Figure 2-2). In 2010, a single potentially infected egg was collected from each site and in 2011 five eggs were collected per site. In both sampling years, eggs were stored in individual plastic containers and transported to the laboratory in cool bags.

In the laboratory, a section of white water mould (roughly $1mm^3$) was removed from each egg, ensuring that no egg tissue or jelly capsule remained attached, and placed on a glucose-peptone-salts (GYPS) agar plate containing 5g l^{-1} glucose, 0.5g l^{-1} peptone, 0.5g l^{-1} KH2PO4, 0.05g l^{-1} yeast extract and 0.15g l^{-1} MgSO4.7H2O (Beakes & Ford 1983). Plates were sealed and maintained at room temperature (around 23°C) until hyphae growth covered two thirds of the plate surface, at which point hyphae from the edge of the mycelium mat were transferred to a second plate for growth to continue. After two weeks of growth, peripheral hyphae were transferred to a 1.5ml eppendorf tube containing 500 µl of liquid GYPS media and maintained in a thermal cabinet at room temperature (23°C) for 72 hours (Cenis 1992).

DNA extraction and sequencing

DNA extraction was carried out following the protocol in Cenis (1992): eppendorf tubes containing mycelium mats and liquid media were centrifuged for 10 minutes at 13000rpm, excess liquid media was removed and 500µl of TE buffer added before centrifuging again for five minutes; from this point extractions followed a standard DIGSOL extraction method (Nicholls *et al*. 2000). DNA was resuspended in 30µl of TE buffer.

A 622 bp section of the 28S rRNA region, including the hypervariable stem and loop regions between helices C1 and D2, was amplified using the primers: C1 (5°-ACCCGCTGATTTAAGCAT-3°) and D2 (5° - TCCGTGTTTCAAGACGG-3°) (Leclerc 2000; Hulvey *et al*. 2007) and the ITS region was amplified using the primers ITS1 (forward 5'-3' TCCGTAGGTGAACCTGCGG) and ITS2 (reverse 5'-3' TCCTCCGCTTATTGATATGC) (Hulvey *et al*. 2007). Polymerase chain reactions (PCR) were performed in 20µl reaction volumes containing: 2.5 mM $MgCl_2$ (Invitrogen), 1 x PCR Buffer (Invitrogen), 0.2 mM dNTP (Invitrogen), 0.1 µM forward primer, 0.1 µM reverse primer, 0.5 units of Taq polymerase (Invitrogen) and 1 µl of DNA template. Initial denaturisation took place at 94°C for 3 minutes; followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 90 seconds; with a final extension step of 72°C for 10 minutes. Amplified samples were cleaned with ExoSAP-IT (USB, Cleveland), according to the manufacturer's instructions, and sent to the GenePool core genomics facility at the University of Edinburgh, where they were sequenced on an ABI 3730 automated sequencer.

Species identification

Sequences were aligned and base-calling errors corrected using Sequencher v4.5 (Gene Codes Corporation, Ann Arbour), and matched to published sequences in the NCBI Genbank database using megaBLAST. Species names were assigned to

samples based on the maximum percentage of identical nucleotides between the sample and reference sequences within the alignment length (Max ident), when the percentage of the sample sequence covered by the reference sequence was at least 90% (Query coverage).

Results and Discussion

Sequences from six of the samples collected during 2010 were able to be identified to species level, but the sequences from both the ITS and 28S region of the IML sample were heterozygous and thus could not be identified. Four samples were identified as members of the *Saprolegniaceae* using the ITS region and five using the 28S region (Table D2). All *Saprolegnia* samples showed at least a 96% match (maximum percentage of identical nucleotides between the sample and reference sequences within the alignment length) with Genbank reference sequences (Table D2). However, three of the samples were identified as different *Saprolegnia* species depending on the marker used: *S. diclina* using the ITS region and *S. ferax* using the 28S region; and one sample was identified as *S. ferax* using 28S and a *Chalara* species using ITS. These results suggest that taxonomy based on molecular markers is generally not well resolved for the *Saprolegniaceae*. Furthermore, the ITS region was found to have extensive length variation among species, which meant that sequences could not be aligned, making them unsuitable for phylogenetic analyses. Therefore, the 28S region is potentially more informative for *Saprolegnia* species diversity studies.

None of the 2011 samples showed the characteristic white cotton-wool appearance of *Saprolegnia* during culture. A subset of 46 samples from across the ten sites sampled were sequenced at the 28S region, none of which were identified as members of the *Saprolegniaceae*. The majority of samples were identified as *Penicillium*, a ubiquitous fungus that can colonise organic matter rapidly via aerial dispersion (Elmholt & Hestbjerg 1999). The 2011 samples were grown in the laboratory at the Scottish Centre for Ecology and the Natural

Environment (SCENE), but at the time of this study the laboratory was rarely used, was not sterile and did not have a laminar flow workstation. Therefore, the potential for contamination of plates during preparation and culture was high. Furthermore, during a two day electrical failure of the fridge where agar plates were being stored prior to use, mould growth was visible despite samples not having yet been added. Therefore, samples from this data collection period were not used for further analysis given the high likelihood of contamination. Therefore, the culture stage of molecular identification of *Saprolegnia* species hinders species isolation due to the risk of contamination by air-borne microbes.

Suggestions for future studies

Saprolegnia culture success could be increased by reducing the probability of microbial infection by adding antibiotics to agar plates to prevent bacterial growth, and by preparing plates and adding samples to plates within a laminar flow workstation to avoid contact with air-borne contaminants. Furthermore, decreasing the growth time on each plate, and placing a larger section of water mould on each plate, would potentially reduce the risk of *Saprolegnia* being out-competed by other water moulds. At the sequencing stage, the non-alignable nature of ITS sequences suggests that 28S molecular markers would be more informative for phylogenetic studies of the *Saprolegniaceae*.

Table D1: Location of sites sampled during 2010 including site name, site abbreviation, latitude and longitude.

Site	Abbreviation	Latitude	Longitude
Ben Ime Low	IML	56.20	-4.76
Ben Ime Mid	IMM	56.21	-4.79
Ben Lawers High	LAH	56.54	-4.23
Ben Lawers Low	LAL	56.50	-4.24
Meall nan Tarmachan Low	MTL	56.50	-4.25
Meall nanTarmachan High	MTH	56.52	-4.30
Ben Heasgarnich Low	HEL	56.49	-4.50

Table D2: Identification of species isolated from each site sampled in 2010, including: site of sample collection (Site); the species identified using Genbank using the ITS and 28S region; and the percentage of identical nucleotides between the sample and reference sequences within the alignment length (% match) for each molecular marker.

Site	ITS	% match	28S	% match
IML*	NA	-	NA	-
IMM	*S. litoralis*	98	*S. litoralis*	99
HEL	*Trichoderma viride*	100	*Trichoderma viride*	100
LAL	*S. diclina*	98	*S. ferax*	99
LAH	*S. diclina*	99	*S. ferax*	99
MTL	*Chalara sp.*	94	*S. ferax*	99
MTH	*S. diclina*	96	*S. ferax*	99

*Sample not identified due to sequence heterozygosity

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