The effects of climate and habitat on breeding blue tits (Cyanistes caeruleus) and their prey

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Declaration

The material contained within this thesis has not previously been submitted for a degree at Durham University or any other university. The research reported within this thesis has been conducted by the author unless indicated otherwise.

Claire Jane Branston
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Abstract

Ephemeral resource and productivity peaks are characteristic of temperate woodland ecosystems. An ecological model system, which both exhibits and exploits these ephemeral peaks, and is the focus of this thesis, is the deciduous tree-herbivorous caterpillar-insectivorous bird food chain. Phenological synchrony within food chains, displaying ephemeral peaks, is crucial to maximise fitness of higher trophic levels. In the well-studied oak-winter moth-insectivore system, the phenology of all levels are highly temperature sensitive, and given atmospheric warming since the mid-20th century has been, and is predicted to continue, occurring at an unprecedented rate, this tri-trophic system is at risk of phenological mismatch. The effects of climate change on this system have been well studied in oak (*Quercus spp.*) dominated woodlands across Europe, as oak supports a wide variety of invertebrates. However, little is known about the importance of other deciduous tree species, which also support a variety of invertebrate species that are likely to be important to nesting blue tits (*Cyanistes caeruleaus*). Understanding the predictors of phenology and productivity across multiple trophic levels is required to understand the pressures on this food chain with a changing climate.

The overarching aim of this thesis is to explore the effects of both climate and habitat on the phenology and productivity of both blue tit and herbivorous caterpillars. The effect of tree leafing phenology and air temperature on herbivorous caterpillar phenology was investigated at a local scale, across an extensive woodland site in Durham (UK) through collecting fallen frass. Frass fall phenology was not predicted by temperature or tree phenology, and did not differ across four common deciduous tree species. However, frass fall was most abundant under oak trees, corroborating the importance of oak for Lepidoptera. Across the same Durham study site, nestling blue tits faecal sacs were collected, with a view of using next-generation sequencing to elucidate nestling diet and resource usage, to expand the food chain into a more complex food web. Due to difficulties extracting and amplifying DNA from blue tit nestling faecal sacs, sequencing was unable to be undertaken. However, I provide a method for extracting DNA from difficult faecal samples, which are likely inhibitor-rich and contain highly degraded DNA. The effects of climate and habitat on blue tit phenology and productivity were then considered, by combining bird nesting data from 34 sites across the UK with local temperature and habitat variables. Overall, climatic factors were more important predictors of blue tit phenology and productivity than habitat. Decreased clutch size and earlier breeding phenology, but decreased risk of nest failure, is predicted at higher temperatures. These results, combined, depict a mixed picture for how blue tits may fare with changing climate. To further work presented in this thesis, the effects of climate and habitat on recruitment need to be explored to understand the full implications of the results presented here on blue tit population dynamics.

Chapter 1: General introduction

1.1 Motivation

Climate change is occurring at an unprecedented rate, with global surface temperature having increased, on average, by 0.12°C per decade between 1951 and 2012 (IPCC, 2014). Temperatures are forecast to have warmed by an additional 0.3 to 4.8°C by 2100 compared to 1900 temperatures (IPCC, 2014). Additionally, changes in precipitation are occurring with the frequency of intense rainfall now more probable, though spatially variable in the likelihood of occurrence (IPCC, 2014). Changes to annual mean precipitation are also forecast to increase at high latitudes and decrease in mid-latitude regions (IPCC, 2014). Due to rapid recent climate change, ecological responses to climate change are now one of the most widely researched areas in ecology. There is now a large body of evidence indicating recent changes in global climate are affecting a wide variety of organisms from all major ecosystems (Walther et al., 2002). Changes have been documented, and further changes predicted, in relation to a wide variety of biological processes, including: phenology (e.g. Crick et al., 1997; Dell et al., 2005; Jonsson and Jonsson, 2009), physiology (e.g. Bozinovic and Pörtner, 2015; Portner and Farrell, 2008), life history (e.g. Coulson et al., 2001; Pinceel et al., 2016; Wegge and Rolstad, 2017), community structure (Walther, 2010; Walther et al., 2002), morphology (e.g. Guerin et al., 2012; McCauley et al., 2018), population dynamics (e.g. Clark et al., 2003; Sillett et al., 2000; Thompson and Ollason, 2001) and spatial distributions (e.g. Chen et al., 2011; Parmesan, 2006; Wilson et al., 2007).

In addition to climate change, other anthropogenic factors are also exerting pressures on ecological systems. For example, land-use changes, through the destruction, fragmentation and disturbance of habitats, are one of the most important drivers of current species extinctions (Hoffmann et al., 2010). However, climate change is predicted to become equally, or potentially even more, important for species losses over the next few decades (Newbold, 2018).

Investigating the impacts of anthropogenic activities on ecological systems has become particularly pertinent in light of recent biodiversity loss (Butchart et al., 2010). Sixty percent of total biodiversity decline (biodiversity decline was calculated as a sum of all the decline fractions from IUCN red list status of birds and mammals between 1996 and 2008), has occurred in only seven countries (Indonesia, Malaysia, Papua New Guinea, China, India, Australia and the USA (mostly Hawaii); Waldron et al., 2017). Most biodiversity loss has been attributed to local-scale processes such as habitat loss and/or degradation, overexploitation or effects of invasive species (Pearce-Higgins and Green, 2014). However, climate and land-use occur at a wider, less local, scale and are now believed to be the two largest threats to global biodiversity (Bellard et al., 2012).

To date, research into the impacts of climate on biological systems has mostly focussed on species' spatial distributions or phenology (Chen et al., 2011; Walther et al., 2002). However, in order to understand how climate change will impact ecosystems at local scales, a better understanding of the processes and changes in community structure are needed, in order to make informed conservation decisions (Mace et al., 2008; McLean et al., 2016).

Common species provide a rich opportunity to investigate the effects of environmental changes (Gaston, 2010; Gaston and Fuller, 2008) and such species are integral to the provision of many ecosystem services (Gaston, 2010). The effects on ecosystems of loss, or decline, of previously common species are often the most pronounced, due to the cascade of reductions and loss of other species reliant on common species, altering many biotic interactions (Gaston, 2010). In European temperate woodland ecosystems, blue tits (*Cyanistes caeruleus)* are one such common species, with a distribution across the Western Palearctic (Stenning, 2018) and are the focus of the research presented in this thesis. In the UK blue tits occupy approximately 3.4 million territories during the breeding season, making them the most abundant UK woodland bird (Musgrove et al., 2013). Consequently, the blue tit has been widely used as a model organism for research in woodland systems, with several long term studies across Europe investigating aspects of the species life history and behaviour, as well as population responses to changing climate (e.g. UK - Wytham Woods, Oxfordshire and Nagshead, Gloucestershire; the Netherlands - Hoge Veluwe and Wageningen).

1.2 Outline of the introduction

The remainder of this introduction is split into four sections. In the first section I will provide a brief overview of the influence of climate change on ecological systems, focussing on phenology, range shifts and biotic interactions. In the second section I will describe the model system deciduous tree-herbivorous caterpillar-blue tit, which forms the basis of the research presented in this thesis. I will then discuss previous research, concerning both life history and the impacts of climate change, on the tree-caterpillar-insectivorous bird model system. In the third section, I will explore the current limitations and knowledge gaps in the tree-caterpillar-insectivorous bird system. Finally, I will outline the aims of this thesis.

1.3 Influence of climate change on ecological systems

1.3.1 Phenological changes

Phenology, the timing of seasonal processes in animals and plants, has been widely studied particularly in temperate systems with marked seasonality, possibly due to it being one of the easiest ecological processes to track (Walther et al., 2002). Generally, the phenology of spring activities across Europe, such as breeding (e.g. Beebee, 1995; Both et al., 2004; Crick et al., 1997),

the return of migratory species to breeding grounds (e.g. Hüppop and Hüppop, 2003; Jonsson and Jonsson, 2009) and the emergence of butterflies (Dell et al., 2005) have advanced in recent decades. There is some evidence that autumn phenological events, such as leaf colour change across Europe has delayed by approximately 4.8 days during 1959 to 1993 (Menzel and Fabian, 1999). However, the timing of autumn migration in birds, shows a less uniform response, for example, long-distance migrants are leaving breeding grounds earlier, whereas short-distance migrants are delaying their departure (Jenni and Kéry, 2003). Phenological changes are not limited to terrestrial systems, with seasonal peaks of meroplankton, some classes of holozoplankton and dinoflagellates in the North Sea having advanced significantly between 1958 and 2002 (Edwards et al., 2004). In addition to advancements and delays to phenology, there is also evidence for changes in the duration of events. For example, a lengthening of the vegetative growing season in the Northern Hemisphere of between 1.1 – 4.9 days per decade has been observed, since the 1950s (Menzel, 2000; Menzel et al., 2003; Menzel and Fabian, 1999). Extended flight periods have also been documented in invertebrate taxa, such as aphids and butterflies in the UK (Bell et al., 2015; Roy and Sparks, 2000). Of 55 aphid species in the UK, 85% have extended their flight periods (Bell et al., 2015). Across Europe, butterflies have not only extended their flight periods, but have also altered the number of generations they have in a year, with increased frequency of second and subsequent generations in warmer years (Altermatt, 2010). All of these processes have been shown to exhibit strong correlates with climatic conditions (Altermatt, 2010; Bell et al., 2015; Both et al., 2004; Parmesan, 2006; Walther et al., 2002).

Phenological changes, such as those previously discussed, can occur through two mechanisms, evolution or plasticity. Evolutionary adaptation occurs through climate-induced selection pressure on traits with genetic variance (Hoffmann and Sgrò, 2011). For example, there are adaptive optima for breeding phenology in systems with ephemeral peaks in resources, such as laying date in passerine birds, leading to differences in selection pressure between early and late breeders (Marrot et al., 2018). In contrast, phenotypic plasticity is when an organism changes its phenotype e.g. breeding phenology, in response to changes in their environment. Unlike evolutionary responses plastic responses are not genetically based, although plasticity itself is a selectable, heritable and variable trait (Nussey et al., 2005), and allows populations to respond rapidly to environmental change (West-Eberhard, 1989). In practice, responses are unlikely to be reliant on just one mechanism alone and are likely a combination of both local-adaption and plasticity (Phillimore et al., 2010).

1.3.2 Range shifts

Species distributions are heavily influenced by climatic suitability, with suitability based upon species-specific environmental requirements determined by physiology, as well as abiotic and

biotic requirements of other species in the food chain. Changing climate alters the location of these climatically suitable regions, which, in temperate regions are generally shifting poleward or to higher altitudes (Walther et al., 2002). Many species across a number of taxa have exhibited such range shifts (Hickling et al., 2005; Moiseev and Shiyatov, 2003; Parmesan, 1996; Thomas and Lennon, 1999) with evidence that these range changes are due to changing climate. Shifts to higher latitudes and elevations have occurred at a median rate of 16.9 kilometres and 11 metres per decade, respectively across Europe, North America, Chile, Malaysia and Marion Islands, across a number of taxonomic groups (Chen et al., 2011). For example, in the UK, between 1960 and 2000, 275 species range margins have shifted northwards, 52 species southwards and 2 species range margins have not changed, out of 329 species from 16 different taxa (Hickling et al., 2006). In the same study, when changes in elevation were considered, 227 species have moved to higher altitudes, and 102 species to lower altitudes over the same time period (Hickling et al., 2006). Unless species are highly mobile, or able to migrate, they may lag behind the rapidly changing climate (Devictor et al., 2008; La Sorte and Jetz, 2012). This could be as a result of poor dispersal ability (Schloss et al., 2012) or long generation times but could ultimately result in reduced ranges and population sizes, which could lead to extinction (Pearce-Higgins and Green, 2014). This highlights the need for a good understanding of life history parameters, as well as distributions, to elucidate the effects of climate change on species ranges.

1.3.3 Biotic interactions

Responses to climate change by individuals and species will have implications for communities as a whole, due to intra- and inter-species interactions (Harrington et al., 1999; Van Der Putten et al., 2010; Walther, 2010). Ecosystem functioning requires biotic interactions, which are reliant on spatial and temporal overlap of two or more species, with many such interactions being highly influenced by climate (Parmesan, 2006; Walther et al., 2002). To be able to accurately predict species responses to climate change, it is imperative that species interactions are incorporated into predictions (Gilman et al., 2010).

Biotic interactions can be disrupted when nodes in a network respond to climate change differently, and therefore put strain upon existing linkages, create new species combinations or change dominance within communities (Walther, 2010). One example of where strains on existing linkages occur has been termed the match-mismatch hypothesis. The match-mismatch hypothesis is where resource users time key events, such as breeding, to coincide with ephemeral resource peaks (Cushing, 1969), with consumer fitness being reliant on resource and consumer phenology matching temporally (Cushing, 1990). If resource and consumer phenology mismatch (resource phenology is earlier or later than consumer demand), consumer fitness will be reduced as a result (Durant et al., 2007). Many species' phenology's are changing as a result of climate change, and

the changes are not uniform within and between species (Parmesan and Yohe, 2003), which can lead to an uncoupling of once synchronous trophic interactions (Both et al., 2009; Winder and Schindler, 2004). Evidence of such uncoupling has already been documented in marine systems, where primary, secondary and tertiary producers in the North Sea, which many fish species are reliant on, have demonstrated marked differences temporally in phenological responses (Edwards et al., 2004). The primary producers, diatoms and dinoflagellates, have shifted their summer phenology by 0 and 23 days respectively, whereas the secondary and tertiary producers, copepods, non-copepod holozooplankton and meroplankton, have shifted their phenology by 10, 10 and 27 days, respectively. Phenological changes in plankton, as well as range changes and overfishing, have been linked to declines in cod stocks in the North Sea (Beaugrand et al., 2002). Phenological mismatch has also been reported In terrestrial systems, with evidence of phenological mismatch in predator prey interactions (e.g. insectivorous woodland birds and caterpillar prey (Burgess et al., 2018); marmots emerging earlier, before snow has melted and plant growth has resumed, in the Colorado Rocky Mountains (Inouye et al., 2000)) and plant-pollinator interactions (Parmesan, 2007). Generally, in terrestrial, marine and freshwater systems, there is evidence that secondary consumers are less sensitive and responsive to climate change than primary consumers or producers (Thackeray, 2016) and these differing responses to temperature are likely driving observed mismatch.

In addition to disruption between predator and prey phenology, there is evidence that host-parasite interactions may also be disrupted due to climate change. For example, across Europe the common cuckoo (*Cuculus canorus*), a migratory brood parasite, is becoming increasingly mismatched with some of its host species (Saino et al., 2009). Across Europe, cuckoos are arriving back to their breeding grounds, on average, 5.3 days earlier (between 1947 and 2007), whereas their host species are returning 14.6 days earlier, if they are short distance migrants, and 6.0 days earlier if they are long-distance migrants (Saino et al., 2009). This apparent mismatch between the cuckoo and some of its host species has been suggested as a contributing factor to the recent observed cuckoo declines (Saino et al., 2009).

1.4 A simple food chain as a model system

The simplified food chain of deciduous tree-herbivorous caterpillar-insectivorous bird has become a model system in temperate woodlands for exploring life history, predictors of phenology and impacts of climate change on trophic interactions (Both et al., 2009; Charmantier et al., 2008; Visser et al., 1998), and is the focus of the research presented in this thesis. This highly synchronous system relies upon correctly timed phenology across all trophic levels to maximise fitness, with all levels exploiting ephemeral peaks resources. The primary producers in this system, deciduous trees, produce new leaves annually after lying dormant over winter to avoid

frost damage, whilst simultaneously exploiting increasing photoperiod to maximise growth (Linkosalo et al., 2000). Lepidopteran larvae, primary consumers in this food chain, utilise these newly emerged leaves to support their own growth. The optimal foraging period for Lepidopteran larvae is when leaves are young, where protein content is maximal and tannin build-up, which inhibits larval growth, is minimal (Feeny, 1970). Finally, secondary consumers, passerine birds such as tit (*Paridae*) and flycatcher (*Muscicapidae*) species time their breeding such that peak nestling demand coincides with the ephemeral peak in Lepidopteran larval availability. Most tit and flycatcher species are single brooded (Lundberg and Alatalo, 2010; Perrins, 1979; Stenning, 2018), therefore correctly timed breeding phenology is essential to maximise productivity (Burger et al., 2012; Wilkin et al., 2009). Many woodland tit and flycatcher species are hole-nesting, relying on pre-excavated cavities to breed in and readily utilised man-made nestboxes (Stenning, 2018). This, along with their abundance, make them model species for studying phenology and the consequences of varying environmental parameters on life history and fitness.

The environmental cues, or conversely physiological constraints, underpinning trophic interactions in this system, along with a knowledge of life histories, need to be well understood to make informed predictions about how this food chain will respond to climate change.

For the primary producers, leaf development is temperature sensitive and temperature is responsible for inter-annual variation in the timing of first leafing, referred to as leaf-out date (Linkosalo et al., 2006). Most temperate trees require a period of winter chilling followed by warming to facilitate bud development (Hunter and Lechowicz, 1992; Tansey et al., 2017), with specific thermal requirements varying by species (Morin et al., 2009). Some species also use seasonal changes in photoperiod as a cue to initiate leaf development, but not all populations have the same requirements, with photoperiod ecotypes across latitudinal gradients in some species (Partanen, 2004). The addition of photoperiod in the regulation of leaf-out phenology prevents species from leafing early in response to an unusually warm period in early spring when a frost risk remains (Caffarra and Donnelly, 2011), but limits their ability to respond to early springs. Although all species in a given area likely experience similar environmental conditions, interspecific differences in leaf-out phenology remain, explained in part by differences in stem anatomy (Lechowicz, 1984). However, individual trees are highly repeatable in their phenology among years e.g. early leafing trees are always early leafing (Cole and Sheldon, 2017; Crawley and Akhteruzzaman, 1988; Hinks et al., 2015; Wesołowski and Rowiński, 2006a), which could be attributable to either genetics or consistent environmental and developmental conditions. There is evidence that trees have responded to recent climatic change, with earlier bud-burst occurring in warmer springs (Parmesan and Yohe, 2003), but with interspecies variability in the mechanisms and ability to track optimal phenological timing (Tansey et al., 2017). In the UK, it is predicted that

many tree species will be able to advance their phenology to track optimal climatic conditions (Tansey et al., 2017). However, not all species will advance at the same rate, which could lead to a change in the order species leaf-up in temperate woodland systems (Roberts et al., 2015). In turn, this could alter woodland composition, due to new combinations of species competing for light (Roberts et al., 2015).

The primary consumers, Lepidopteran larvae, developmental requirements are less well understood in comparison to the tree primary producers. Temperature exhibits a positive relationship with caterpillar growth and feeding rates, until a thermal limit is reached after which both growth and feeding rate reduces (Kingsolver, 2000). Ephemeral Lepidopteran larval peaks occur during spring, in temperate deciduous woodlands, and are typically dominated by a few species (Hunter and Lechowicz, 1992; Shutt, 2017). In Europe and America, green oak tortrix (*Tortrix viridana*) and winter moth (*Operophtera brumata*) larvae contribute substantially to such peaks (Hunter and Lechowicz, 1992; Shutt, 2017; Wesołowski and Rowiński, 2006b). In an oak dominated UK woodland, green oak tortrix and winter moth contributed 46% and 26% to the ephemeral peak (Hunter and Lechowicz, 1992). However, in mixed woodlands across Scotland, green oak tortrix was not detected, but winter moth still accounted for 33% of the caterpillar peak and three species (winter moth, *Operophtera fagata* and *Agriopos aurantiaria)* accounted for over 50% of the peak (Shutt, 2017). Winter moth has been found to be responsible for high levels of defoliation on host trees, *Quercus spp.,* across Europe and in Nova Scotia (Crawley, 1985; Cuming, 1961; Wesołowski and Rowiński, 2006b) and can even outcompete sympatric species, such as the green oak tortrix, in some instances (Hunter and Willmer, 1989). Due to winter moth's abundance and its ability to reach pest status (Embree, 1971; Speight, 1979), more is known about the effects of environmental conditions on its lifecycle than many other Lepidopteran species. Winter moth is polyphagous (Cuming, 1961; Tikkanen et al., 2000; Vanbergen et al., 2003; Wint, 1983) but is most likely to use oak (*Quercus spp.)* or willow (*Salix spp.)* as host species (Shutt, 2017). Temperature is important during all life stages, exerting different effects during each stage (Holliday, 1985). During the larval stage, caterpillars emerge earlier and grow faster when reared under warmer temperatures (Buse et al., 1999). However, humidity also influences larval phenology with eggs hatching three days later when reared under 50%, as opposed to 70%, relative humidity (Embree, 1970). Larvae need to synchronise their hatching phenology with leaf-out of their host tree, as young leaves are most nutritious and once the leaves mature they become unpalatable to caterpillars (Feeny, 1970). Peak caterpillar abundance and oak leafing are highly correlated in the field (Burgess et al., 2018; Hinks et al., 2015). Both caterpillars and trees respond at a similar rate to warming spring temperatures, with oak (*Quercus robur)* bud-burst and winter moth egg hatching predicted to remain synchronous in the future (van Asch et al., 2013; Buse and Good, 1996). In the UK, caterpillar phenology and the duration of availability is later and

longer, respectively, in the north and west (Smith et al., 2011). However, little is known about whether their development period in the field i.e. time between hatching and pupating, has varied with changing climate. Experimentally, development time is shorter when caterpillars are reared at warmer temperatures (Holliday, 1985).

Passerine birds, secondary consumers in this food chain, are thought to use a combination of cues to initiate breeding, including both temperature and photoperiod (Visser and Lambrechts, 1999), to ensure nestling demand coincides with the period when caterpillars are available. Lengthening photoperiod initiates photo-stimulation and secretion of gonadotropin-releasing hormone, which controls gonadal growth and maturation (Dawson et al., 2001; Sharp, 2005). However, photoperiod alone does not explain inter-annual variation in breeding phenology, as photoperiod is consistent inter-annually. A combination of other factors are believed to fine tune breeding phenology, once transition into a reproductive state has been induced through photoperiod (Visser and Lambrechts, 1999). Spring temperature is one such factor, altering the rate of gonadal growth, with more rapid growth at higher temperatures (Engels and Jenner, 1956). However, the sensitivity to temperature may differ within and between species, with an experimental study in house finches (*Haemorhous mexicanus*) and some field populations of great tits (*Parus major*) reporting no effect on the timing of reproduction (Visser and Lambrechts, 1999; Watts et al., 2018). Close correlation between bud-burst, in oak and silver birch, and first egg dates of tits have been reported in the field (Bourgault et al., 2010; Burgess et al., 2018; Cole et al., 2015; Hinks et al., 2015; Slagsvold, 1976), with local scale oak phenology predicting breeding phenology of great tits (Hinks et al., 2015). However, experimentally the same result has not been proven (Schaper et al., 2011; Visser et al., 2002). Tree phenology and avian food availability at the time of breeding may influence avian breeding phenology, by providing information as to when food may be abundant for nestlings (Visser and Lambrechts, 1999). However, the causal mechanisms for how these cues may be used by the birds are unclear. Chemical cues from the trees, which indicate bud-burst is imminent, have been hypothesised as one mechanism for how birds may process local phenological cues (Bourgault et al., 2006). This would require scale buds to be ingested for the birds to receive the cue, however scale bud consumption is low, and therefore phenological cues from trees are unlikely to operate through this pathway (Bourgault et al., 2006).

Generally, birds in the UK have been exhibiting advancements in breeding phenology since 1970s (Crick et al., 1997), which has been attributed to warming springs (McCleery and Perrins, 1998). Across Europe, passerine woodland birds are no exception (Visser et al., 2003), and have advanced breeding by approximately 3.5-5 days for each 1°C rise in spring temperature (Phillimore et al., 2016; Thorley and Lord, 2015; Vedder et al., 2013; Visser et al., 1998). However, the responses have not been uniform across populations, and are not fully explained by

temperature alone (Visser et al., 2003). The advancement of tit breeding phenology with warming temperature is likely to have occurred through phenotypic plasticity (Charmantier et al., 2008; Phillimore et al., 2016; Thorley and Lord, 2015), and with selection strongly favouring early breeding individuals (Marrot et al., 2018). Phenological advancement has also been linked to the North Atlantic Oscillation index, suggesting winter temperature and precipitation may also influence breeding phenology (Sanz, 2002). In the UK, tit species have been continuously monitored in Wytham Woods (Oxfordshire, UK) since 1947 (Perrins, 1979), and much of the understanding of finer scale predictors of breeding phenology comes from this site. Topographical variables, such as aspect and altitude, as well as nesting habitat have been shown to further fine tune breeding phenology (Wilkin et al., 2007). Altitudinal differences are likely to be due to bioclimatic factors, with temperature decreasing as height above sea level increases and therefore breeding being delayed at higher altitudes (Wilkin et al., 2007). Phenology has been shown to vary in a counterintuitive way with nest site orientation, with north facing slopes having earlier breeding phenology than warmer south facing slopes, possibly due to increased invertebrate availability in damp and humid conditions allowing females to reach breeding condition quicker (Wilkin et al., 2007).

Factors unrelated to climatic conditions also influence breeding phenology. For example, birds breeding at high densities exhibit delayed first egg dates, with the causal mechanism of this unproven, but postulated to be due to increased competition for resources (Wilkin et al., 2006). Phenology also varies due to habitat, for example birds nesting in areas with high oak density breed earlier than those with lower oak density, at a single site in the UK (Wytham Woods, Oxfordshire; Wilkin et al., 2007). At this site, birds nesting at woodland edges, in 20th century woodland plantations or grassland, exhibit delayed breeding phenology in comparison to birds nesting in the centre of woodlands and in older woodland habitats (such as ancient, semi-natural woodlands), independent of the number of oak trees (Wilkin et al., 2007). Due to phenological variation existing between habitats, independent of oak, the presence of other (unidentified) species or woodland structure may be important in determining breeding phenology (Wilkin et al., 2007).

Birds must not only correctly time the initiation of breeding, so nestling demand coincides with food availability, but also subsequent events such as hatching. Hatching phenology can be modulated through a number of mechanisms such as egg pauses, variable incubation length and/or clutch size (Cresswell and Mccleery, 2003). All of these adjustments, first egg date included, have to occur prior to the individual experiencing the optimal conditions and therefore are reliant on cues to indicate when these optimal conditions may occur. The cues used to elicit modulations post egg laying phenology are, in comparison to first egg date, relatively poorly

understood. Temperature during egg laying, as opposed to pre-egg laying for first egg date, influences decisions such as when to commence incubation (Cresswell and Mccleery, 2003). However, experimental increases in nest temperature does not decrease the duration of incubation in great tits (Vaugoyeau et al., 2017), which given embryonic development is more rapid at higher incubation temperatures (DuRant et al., 2013) is counterintuitive. Clutch size decreases as the breeding season progresses, in typically single-brooded species such as tits (Crick et al., 1993; Perrins, 1979; Stenning, 2018), but there is no evidence seasonal declines are temperature mediated with no relationship between temperature and clutch size being reported (Dolenec, 2007). Instead, seasonal declines in clutch size could be due to reduced resource availability later on in the breeding season (Perrins and McCleery, 1989), or due to later breeding individuals being of lower quality or inexperienced. There has been a suggestion that the focus of phenological studies should shift from first egg date to hatching date, which may be more biologically relevant when considering the effects of climate change (Tomás, 2015). Similarly to first egg date, local oak tree phenology predicts the timing of hatching (Hinks et al., 2015), suggesting birds are using oak as a reliable predictor of food availability for nestlings, or that both trees and birds are using similar cues. Experimentally it has also been shown that hatching date advances with warmer temperatures (Vedder, 2012). However, to my knowledge, there is no indication whether this stands in field studies, although hatching date of blue tit, great tit and pied flycatcher have shown similar temporal advancement to first egg date (Both et al., 2009).

It is clear phenology has advanced in the oak-herbivorous caterpillar-insectivorous bird tri-trophic system, and the changes, at least in part, have been temperature driven. For food chains to remain synchronous, the advancements in phenology at each trophic level need to occur at the same rate. Thackeray et al., (2016) showed that consumers, across many taxa and species, advanced their phenology less than the producers they are reliant on, and work on this tri-trophic system corroborates these findings (Both et al., 2009). Bud-burst and peak caterpillar availability have remained synchronous with elevated spring temperatures (Both et al., 2009; Burgess et al., 2018). However, whether bud-burst and moth egg hatching has remained synchronous is less clear, with both synchrony (Buse et al., 1999) and disruptions to synchrony (van Asch and Visser, 2007; Visser and Holleman, 2001) being reported. Secondary avian consumer, and primary caterpillar consumer phenology have not remained as synchronous, with secondary consumers responses being weaker than those of primary consumers (Both et al., 2009; Buse et al., 1999). In the UK, resident tit species, both blue and great tit, appear to be remaining more synchronous (Burgess et al., 2018; Vatka et al., 2014) than the migratory pied flycatcher (Burgess et al., 2018). Asynchrony is greater in warmer springs and, therefore, mismatch is predicted to increase between secondary consumers and caterpillars with continued spring warming, even those secondary consumer species that are currently synchronising with caterpillar peaks (Burgess et al.,

2018). Data from a single UK study site corroborates the national findings, suggesting that tit breeding phenology is currently synchronised with tree phenology, and most synchronised in areas dominated by tree species used for foraging (Cole et al., 2015). In addition, passerine phenology was best predicted when vegetation phenology was considered at a local scale (Hinks et al., 2015). Insectivorous birds mismatching with their prey has consequences for their individual fitness, but does not necessarily have negative effects on demography (Reed et al., 2013), despite producing fewer fledglings per female. Demography has been buffered by the number of fledglings recruiting into the population only being weakly affected by mismatch (Reed et al., 2013).

1.5 Limitations and knowledge gaps in the simple deciduous tree-herbivorous caterpillar-insectivorous bird food chain

A wealth of research has been conducted on the simple oak-herbivorous caterpillar-tit food chain, across Europe, leading to a good understanding of natural history and the effects of climate change on phenology. However, a number of unanswered questions remain.

To date, the majority of research on the tree-tit food chain has been conducted in woodlands dominated by mature oak, due to its dominance across much of Europe (Ozenda and Borel, 2000), despite being a relatively uncommon habitat type in the UK (Forestry Commission, 2011, 2013). Blue and great tits are not exclusively oak woodland specialists; they thrive in many different habitat types, from mixed deciduous woodland to urban and wetland areas (Stenning, 2018). To date, there has been limited research into the implications of climate change on tits breeding in habitats not dominated by oak, despite previous work showing blue tits nesting in urban and suburban areas have lower breeding success than woodland counterparts (Pollock et al., 2017). Habitat variables, such as woodland age and local oak density have been shown to influence the breeding phenology of some woodland insectivores (Wilkin et al., 2007), but such habitat effects have been largely ignored in wider scale studies, and, to date have only been investigated at a few single sites. Therefore, little is known as to whether habitat may be able to buffer the negative effects of climate change on breeding parameters detected in oak dominated woodlands.

In addition to the research focus on oak woodland, there is also a disproportionate balance in knowledge concerning different levels of this woodland food chain. Insectivorous secondary consumers, such as blue and great tits, have received more research attention than primary consumers or primary producers, for example. This is possibly due to the ease with which each level can be studied. Relative to birds, little is known about the response of insects to climate change, and most research to date (in this system) has focussed upon winter moth larvae, due to their abundance in oak woodlands (Kennedy and Southwood, 1984). Most insights into the ephemeral peak in caterpillar abundance comes from indirect sampling methods such as

collecting fallen frass (e.g. Smith et al., 2011). Caterpillar frass cannot be attributed to a specific species, providing a representation of more general invertebrate availability but only under a single tree. To date, studies of frass fall have been heavily focussed upon oak trees, despite Lepidopteran larvae, and other invertebrates, being found in good numbers on other tree species (Kennedy and Southwood, 1984).

Much of the knowledge on the tree-tit food chain has arisen from research conducted at single sites, especially Wytham Woods, UK and Hoge Veluwe, the Netherlands. Both sites have been monitored over long-time periods (in ecological research terms), however the results are difficult to extrapolate out to other populations, as effects of climate change differ at small geographical scales (Visser et al., 2003).

Examining a tri-trophic food chain (deciduous tree-herbivorous caterpillar-insectivorous passerine), further simplified to oak-caterpillar (winter moth)-passerine (tit or flycatcher) has allowed an understanding of the drivers of change within and between different trophic levels. However, tri-trophic interactions are, of course, an oversimplification of a more complex ecosystem and its associated food webs. Both tits and flycatchers are predominantly insectivorous during the breeding season (Betts, 1955; Lundberg and Alatalo, 2010; Perrins, 1979; Stenning, 2018), with adults and nestlings consuming large numbers of Lepidoptera but also Araneae, Hymenoptera, Coleoptera, Diptera and Hemiptera (Betts, 1955; Cowie and Hinsley, 1988; Shutt, 2017). Each species within these invertebrate orders have their own suite of host species and are found on a wide variety of deciduous trees (Kennedy and Southwood, 1984). Further knowledge of the trophic interactions insectivorous birds rely on would offer potential insights into, for example, whether mismatch can be buffered through habitat or prey switching. This thesis provides an opportunity to expand knowledge of the oak-caterpillar-blue tit system, by investigating the broader role of non-oak trees and to explore the effects of climate change on this tri-trophic system.

1.6 Thesis aims

The overarching aims of this thesis are to explore:

1) The effects of both climate and habitat on the phenology of two trophic levels, invertebrates and blue tit, of the deciduous tree-herbivorous caterpillar-blue tit tri-trophic system.
2) To expand the impacts from 1) into the productivity of the secondary consumers, blue tits.

I have four data chapters that I outline below.

In Chapter 2, I will explore the variation in caterpillar frass fall phenology, at a single site in Northern England, over three years and under four tree species that are common in UK woodlands. This is with the view to helping identify tree species that are likely to be important sources of Lepidopteran prey for nestling blue tits, and expanding our knowledge of the must understudied level of the tree-tit system. The novelty here lies in the comparison between the relative role (in terms of abundance and phenology of frass fall) of oak and other common tree species for insectivorous woodland birds.

In Chapter 3, I aimed to explore the potential of using next generation sequencing to elucidate the diet of nestling blue tits. Previous research has highlighted Lepidopteran abundance within nestling diets, however taxonomic resolution is poor through standard methods, and through using molecular techniques this could be addressed. In this chapter I test DNA extraction methods, and primer pairings to efficiently amplify prey DNA from blue tit nestling faecal sacs. However, due to difficulties discussed in the chapter, I was unable to sequence the extracted DNA during the duration of this thesis, and so this chapter focuses on comparing different DNA extraction methods, within and between species, to try and elucidate why blue tit samples are difficult to obtain high levels of DNA amplification from.

Next, in Chapter 4, I investigate the predictors of blue tit phenology across the UK expanding previous findings, from single study sites, to a multi-site (34 site) scale and include the effect of local tree species composition, through utilising a long-term citizen science dataset. This work enables trends between different populations to be explored, to find general patterns for the role of different tree species on blue tit phenology and investigate whether the detailed studies from individual sites (e.g. Wytham Woods) are generalizable.

Finally, in Chapter 5, I continued to examine the effects of climate and habitat, across the UK, using the same long-term dataset (from Chapter 4), on blue tit productivity and whether environmental variables influence the risk of nest failure.

Chapter 2: Caterpillar phenology: variation and drivers

2.1 Abstract

In temperate systems many insectivorous passerines have a lifecycle that tracks ephemeral peaks in insect availability. Caterpillars of Lepidoptera are vital prey for insectivorous woodland birds, and as such birds time their breeding to coincide with peaks in caterpillar availability. Broadleaved woodland covers 1.3 million hectares in the UK, with 18% being covered by oak trees, which host a large number of larval Lepidopteran species. Previous phenological research of caterpillars has been oak-focussed, despite non-oak species hosting Lepidopteran larvae. Environmental factors, such as temperature and leafing phenology have previously been shown to impact caterpillar development and, in light of warming springs is a key area for field research.

Here I investigate the drivers of phenology, duration and magnitude of caterpillar frass (faecal pellet) fall, as a proxy for caterpillar availability, under four common UK tree species in a woodland in Northern England. Seventy percent of total frass fall was produced under oak trees and therefore reinforces oaks importance for Lepidopteran larvae, and insectivorous woodland birds.

Frass fall phenology was not predicted by temperature (either within site or between year), or leafing phenology, which could have effects on bird populations reliant on this resource. No interspecies variation in peak frass fall phenology was observed, providing no evidence of the potential for non-oak species to provide Lepidopteran food resources to breeding birds that have mistimed breeding events. These findings further strengthen the importance of oak within UK woodlands, and the need to ensure oak trees are not disadvantaged in future woodland management.

2.2 Introduction

In temperate systems many insectivorous passerines have a lifecycle that tracks insects annually, either through migration (e.g.swifts (*Apus spp.*, swallows and martins Hirundininae) or through timing breeding, to exploit ephemeral insect peaks (e.g. tit specie, Paridae). Birds are a major predator of insects, and caterpillars are an important part of the diet of many breeding woodland birds, especially in temperate regions (Perrins, 1991). For example, both tit and flycatcher species (Paridae, resident and Muscicapidae, migrant species, respectively) in the UK, breed predominantly in woodlands, and are reliant on invertebrates (mostly Lepidoptera (Betts, 1955) and Araneae (Grzędzicka, 2018)) to feed their chicks during their short breeding seasons. Birds and caterpillars often form part of a tri-trophic food chain (trees-caterpillars-birds), which is a well-studied biological system, and the subject of several long-term research projects (e.g. Wytham Woods, UK (e.g. Betts, 1955; McCleery and Perrins, 1998; Perrins, 1991) and Hoge Veluwe, Netherlands (Visser et al., 1998, 2006; Visser and Holleman, 2001). A key area of research in such tri-trophic systems is focused upon understanding the phenology of the three levels in relation to each other. The resource peaks of caterpillars usually occur as a narrow peak during spring, just after leaves have fully opened on deciduous trees. Due to this, birds aim to time their breeding, so peak demand from nestlings corresponds with peak availability of their food resource. In recent decades, the phenology of many species has been changing in relation to climate change (Walther et al., 2002), and this tri-trophic system has also been experiencing advancement (Buse et al., 1999; Crick et al., 1997). However, not all levels have advanced at the same rate, with secondary consumers advancing more slowly than producers (Burgess et al., 2018; Thackeray et al., 2010). This highlights the need for further research, to fully understand the effects of warming springs on each trophic level and the system as a whole.

Typically, European tit species feed their chicks mainly on larvae of moths of the families Noctuidae, Totricidae and Geometridae (Grzędzicka, 2018; Perrins, 1979, 1991). In temperate European woodland, winter moth *(Operophtera brumata,* family Geometridae*)* larvae make up a large proportion of the diet of nestlings and are a key source of important nutrients for nestlings (Arnold et al., 2010; Perrins, 1991).

Winter moth imagos typically emerge in November and December in the United Kingdom, laying their eggs (which then overwinter) in bark crevices in the tree canopy. The eggs hatch , into their larval stage, when leaves first emerge on their host trees (Holliday, 1985). The time it takes for the larvae to fully develop, before dropping to the ground and pupating in the soil depends on the air temperature, with development time being shorter when the temperature is warmer (Buse et al., 1999; Holliday, 1985).

Broadleaved woodland covers 1.3 million hectares in the UK, with wide ranges in woodland age and species composition (Forestry Commission, 2013). The five most dominant species in the UK, by area, are oak (*Quercus spp.),* birch (*Betula spp.* and *Carpinus betulus),* ash (*Fraxinus excelsior),* sycamore (*Acer pseudoplatanus)* and beech (*Fagus spp.)* (Forestry Commission, 2013). Despite UK woodlands comprising a mixture of species, and oak and birch combined comprising 36% (approximately 18% each; Forestry Commission 2012) of total woodland area, typically oak has been the focus for bird and caterpillar research (Perrins, 1979; Smith et al., 2011). Partially due to invertebrate, especially Lepidoptera, abundance being highest on oak trees (Kennedy and Southwood, 1984). The phenology and abundance of winter moth, a key resource for nestlings, is typically only investigated in relation to oak (e.g. Burgess et al., 2018; Buse et al., 1999; Perrins, 1991; Smith et al., 2011). However, the larvae are polyphagous found on a wide range of other tree species (Cuming, 1961; Shutt, 2017; Tikkanen et al., 2000; Vanbergen et al., 2003; Wint, 1983), and little is known about larval phenology and abundance across non-oak species. Although, it is worth noting that individual larvae will typically be monophagous, consuming the first host species it can become established on after hatching (Wint, 1983). Female winter moths are flightless, and are thought to have little control in selecting the host plant, or oviposition site, and this decision will depend more on pupation site and orientation at emergence, as well as what trees are locally available (Wint, 1983).

Many sampling techniques for canopy dwelling caterpillars are time intensive (e.g. branch beating), logistically challenging, and require excellent identification skills of larvae, which can be difficult. Branch beating is a common direct invertebrate sampling technique, however only gives an indication of the species present lower down in the canopy and samples are often removed off-site for identification at a later date. If samples are removed, and not replaced, and the study is at a large scale, this could bias observations being undertaken of higher trophic levels by artificially depleting the food resource. An indirect sampling approach to record phenology of arboreal caterpillars is frass sampling (Burgess et al., 2018; Glądalski et al., 2017; Sisask et al., 2010; Smith et al., 2011; e.g. Veen et al., 2009; Wesołowski and Rowiński, 2014), which cannot be used to identify Lepidopteran larvae present, but can provide insights into their phenology and abundance. To date, studies employing frass sampling have tended to focus only on oak species (Burgess et al., 2018; Smith et al., 2011), a mixture of species (Sisask et al., 2010; Wesołowski and Rowiński, 2014), or not explicitly specified the species sampled (Glądalski et al., 2017). However, Veen et al. (2009) investigated a suite of species (ash, birch, hazel, oak, pine, spruce) and found strong peaks under all deciduous species.

This study aims to assess the importance of four common tree species in UK woodlands (beech, *Fagus sylvatica;* silver birch, *Betula pendula;* sycamore, *Acer pseudoplatanus* and oak, *Quercus robur)* for Lepidopteran larvae, and therefore indirectly insectivorous woodland birds. I will assess

the potential importance of each species by comparing the total frass fall produced under each species, as a proxy for caterpillar availability. I will also investigate whether temperature (both within and between years) and/or tree phenology can be used to predict frass fall phenology (timing and duration of frass peaks), and to establish whether phenology varies spatially and/or temporally. I will also explore whether increased oak density surrounding the tree being monitored influences frass fall detection, and whether this is likely due to sampling contamination. I hypothesise peak frass fall phenology will be driven by both temperature and leafing phenology (Buse et al., 1999), and differ between species due to interspecies differences in leafing phenology (Roberts et al., 2015). Previous research has focussed heavily on oak, therefore I cannot predict the differences that will be found between species. However, due to oak likely hosting the greatest variety of Lepidopteran species, I predict oak will be important for caterpillars, and inter-annual variation in phenology will likely be driven by temperature and tree phenology.

2.3 Methods

2.3.1 Study site

This study was conducted in a near contiguous area of mixed semi-natural woodland, and suburbia, to the south of Durham City (covering 1 km grid squares NZ2740, NZ2741, NZ2840, NZ2841, NZ2641; Figure 2.1), owned by Durham University. The woodlands receive little management, apart from the removal of dangerous trees overhanging public right of ways. The woodlands are mostly comprised of sycamore (*Acer pseudoplatanus*), beech (*Fagus sylvatica*), english oak (*Quercus robur*) and silver birch (*Betula pendula*) with an understory of hazel (*Corylus avellana*) and hawthorn (*Crataegus monogyna*) in places. At ground level the woodlands are dominated by bluebells (*Hyacinthoides non-scripta*), alongside a mixture of other species typical of semi-natural woodland. The suburban parts of the study sites, mostly surrounding university accommodation and college buildings, are a mixture of native and non-native species. These receive more management, and the understory is generally grass species, with little or no dead wood or leaf litter, as opposed to woodland floor.

Figure 2.1: Map depicting woodland and suburban sampling area (red shaded area) in Durham, United Kingdom.

2.3.2 Frass sampling

Frass (caterpillar faecal pellets) sampling is a common method used for phenological studies of caterpillar availability, especially when coupled with observations of other trophic levels phenology e.g. birds (Glądalski et al., 2017; Sisask et al., 2010; Tremblay et al., 2003; Veen et al., 2009). Frass fall has been shown to highly correlate with results obtained through branch beating, giving reliable estimates of caterpillar availability (Fischbacher et al., 1998). The sampling method used here was based upon a method from Smith et al. (2011), described in brief below. Traps to

collect frass (caterpillar faecal pellets) were placed under beech, english oak, silver birch and sycamore trees, from 26th April until 1st July in 2015, 2016 and 2017, with the number of traps under each species detailed in Table 2.1.

Table 2.1: The number of each tree species monitored for frass fall in each year of the study

Species	Number of traps in given year		
	2015	2016	2017
Beech	13	15	15
Oak	8	15	15
Silver Birch	5	15	15
Sycamore	9	15	15

Frass traps consisted of single black plastic seed tray (approximately 37 x 22 cm, with drainage holes) lined with permeable white horticultural fleece (17 gsm thickness), which was fitted to line the tray so it sat flat on the base of the tray and up the sides to prevent frass being lost on collection. Trays were placed level on the ground (levelling those on sloping ground), directly under the canopy of the tree being sampled. Traps were held in place by pegged black plastic anti-butterfly netting (LBS Horticultural Supplies, mesh size: 7 x 6 mm), stretched tightly over the top of the tray. As well as preventing the trap from being dislodged, netting also prevented larger debris being caught in the liner. Liners were collected and replaced every five days. However, on a few occasions liners were left for slightly longer, or shorter, periods before collection (min: 4 days, max 6 days between collection, 32 (out of 2075) traps were not collected at the 5 day interval). On collection, liners were temporarily stored in labelled polythene bags prior to drying. Liners were air-dried after collection, for a minimum of 24 hours, and then, after sorting, the frass contents weighed. To separate the frass from debris, each liner was brushed (using a 50 mm paintbrush) to remove all frass and debris. Particles were sieved through metal 1 mm mesh to remove larger, non-frass debris. As frass is cylindrical and uniform in shape, when placed onto folded paper it can be 'rolled' off, as frass will tend to roll faster than non-frass debris. Resultant collected frass was placed under a low power dissection microscope (at 2x magnification; Nikon), and any non-frass material was removed using fine pointed tweezers. The total frass sample was then weighed (Kern EG 300-3M, ±1 mg).

Frass mass was converted into frass fall, defined as mg frass per m^2 per day. For analyses, the date associated with the frass fall mass was the mid-point between the start and end date of the collection period.

2.3.3 Smoothing raw frass fall data

Raw frass fall data (Figure 2.2) were smoothed using methods adapted from Soulsby and Thomas (2012). Raw data were smoothed to clearly define single smooth peaks, reducing the noise in the

data. This method was originally used to describe butterfly eclosion and flight periods. However, it can be applied to any curvilinear phenomenon that starts and ends with zero data.

The curve is a function of four parameters: N (maximum detectable frass fall), t_0 (the start of peak frass fall), T_E (duration of frass fall) and T (mean life span of caterpillar) whose values are fitted, to maximise log-likelihood, to each trap in each year:

$$n(t) = 0, for\ t' < 0$$

$$n(t) = \frac{3N}{4(a^2+9)}\left[a\ sin^3X - 3sin^2X.cosX + \frac{6}{a^2+1}(a\ sinX - cosX + e^{-aX})\right], for\ 0 \leq t' \leq T_E$$

$$n(t) = \frac{9N\,e^{-aX}(1 + e^{a\pi})}{2(a^2+9)(a^2+1)}, for\ t' > T_E$$

Where:

$n(t)$ is the frass fall at time, t

N is the maximum detectable frass fall

T is the mean life span of a caterpillar – fixed at the mean time from hatching to pupation for Winter Moth, 17 days (Holliday, 1985)

t_0 is the date of the beginning of the frass peak

$t' = T - t_0$, where t' is a time parameter, which is negative prior to the start of the frass peak

$a = \frac{T_E}{(\pi.T)}$, and $X = \pi\frac{t'}{T_E}$ are dimensionless parameters

T_E is the duration of the frass peak (days)

These models were fitted in 'R' v.3.5.1 using box-constrained optimisation ('L-BFGS-B', using 'optim' from the 'stats' package (R Core Team, 2017)), which constrained each of the variables to a minimum and maximum value. As many of these variables cannot be negative, box constraining improves model performance and keeps variables within plausible ranges. The following constraints were used: N, min: 1, max: 75; t_0, min: 1 (1st April), max: 50 (20th May); T_E, min: 5, max: 70.

Model performance was assessed using relative root error (RRE), to give model fit on a percentage scale, calculated as:

RRE = (root mean square error) / (root mean square count) * 100

Traps where model fitting failed were classed as not having a frass peak (coded 0), all others were defined as having a frass peak (coded 1). A summary of the RRE and number of trees being classed as having a frass peak are shown in Table 2.3.

Peak frass fall was taken as the date where modelled frass fall was maximal. The duration of the frass peak was taken as the fitted T_E value (following Soulsby and Thomas (2012)).

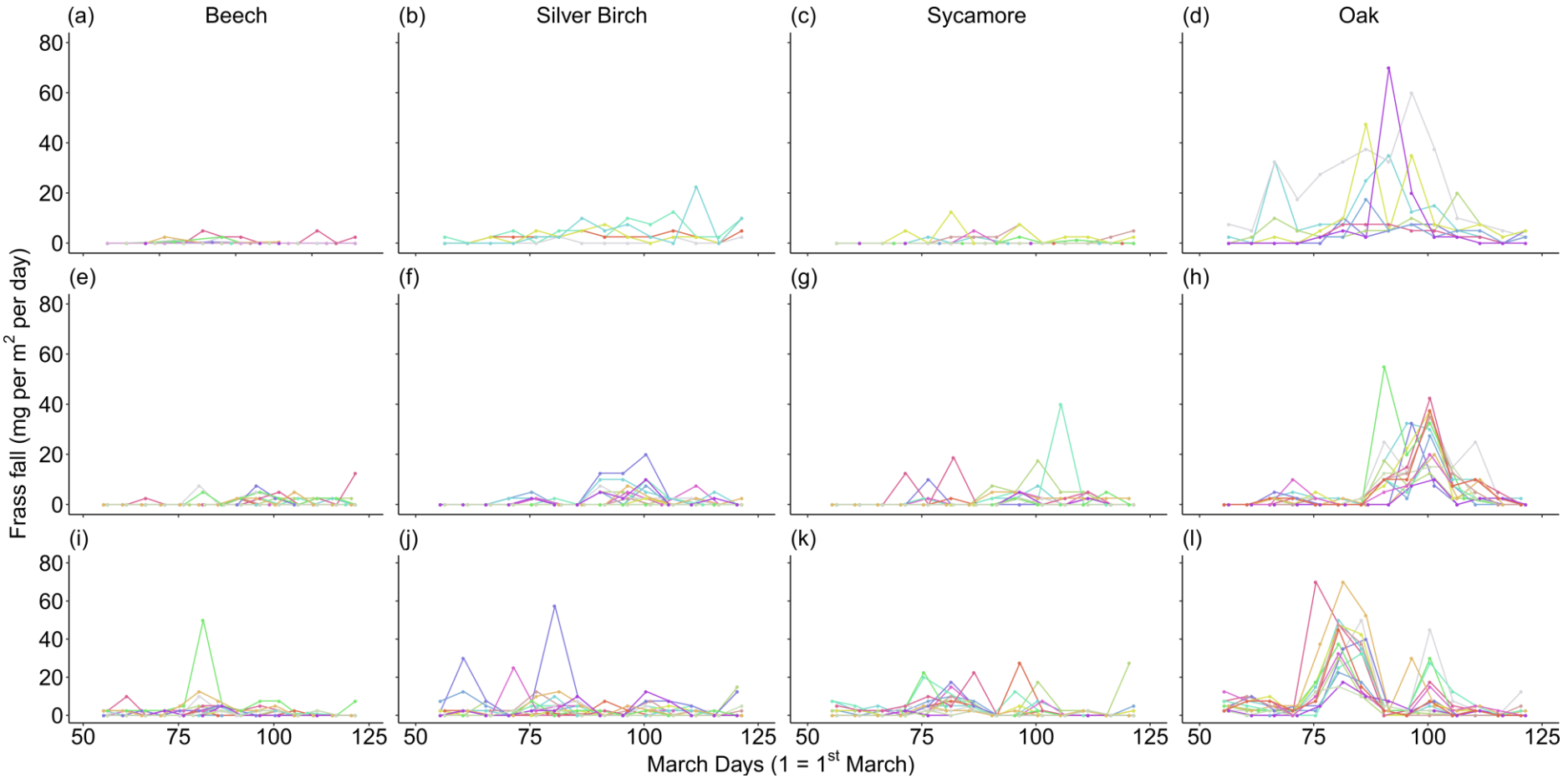

Figure 2.2: Temporal patterns of raw frass fall in 2015 (a, b, c, d), 2016 (e, f, g, h) and 2017 (i, j, k, l) under four tree species, beech (a, e, i), silver birch, (b, f, j), sycamore (c, g, k) and oak (d, h, l). Colours are consistent in columns for trees monitored in multiple years. Lines between points are to aid interpretation only, and do not represent any statistical relationship between points.

2.3.4 Temperature data

Temperature data were collected using iButtons (DS1921G-F5 thermochrons; HomeChip), which recorded ambient air temperature, at hourly or two hourly intervals; the latter schedule was adopted outside of the blue tit breeding season. iButtons were affixed, below nest boxes (to provide shading), using butterfly netting to create a ventilated hammock. Nest boxes were located on a tree close to each frass monitoring site. The mean temperature during March and April was then calculated for each logger. This time period was selected due to it being shown to best predict tree phenology for three out of the four tree species investigated here (Tansey et al., 2017), and tree phenology is typically highly correlated with caterpillar phenology (Both et al., 2009; Burgess et al., 2018). Two temperature variables were then calculated, allowing both within and between year effects of temperature to be explored, following van de Pol and Wright, (2009). The first temperature variable was the mean temperature across the site for each year, and the second a measure of how much each frass traps microclimate varied from the site mean by subtracting the local mean March/April temperature from the site mean.

2.3.5 Tree phenology

The trees monitored for frass fall, were also monitored to record tree phenology. Phenology data collected were: date of bud-burst - the first record of a leaf bursting through the bud; first leaf date - when the first fully formed leaf was observed and full leaf – when all the leaves were fully formed. Bud-burst was used for tree phenology as oak bud-burst and peak caterpillar abundance has been shown to be highly correlated previously (Both et al., 2009; Burgess et al., 2018)

2.3.6 Habitat

The total number of oak trees within 400 m^2 of each temperature logger were counted (with each logger at the centre of a 20 x 20 m quadrat).

2.3.7 Statistical Analyses

Temporal and species differences in mean phenological values or total frass fall per year were investigated through constructing mixed models with the phenological or total frass fall variable as the dependent variable, either year or species as a categorical fixed effect and trap ID as a random (intercept) effect to account for any trap variation not accounted for in the fixed effects. When investigating whether temperature or tree phenology could predict frass fall phenology, both a within year (across site) and between year measure of temperature and bud-burst were included as fixed effects, along with trap ID and year as random (intercept) effects to allow for variation between traps and years which were not accounted for in the fixed effects. In all analyses investigating frass fall phenology or total frass fall, derived variables (from model fitting, such as date of peak frass fall, or duration of frass fall) were only used when smoothed curves

could be successfully fitted. When frass peak detection was investigated in relation to the number of oak trees in the vicinity of the trap, frass traps from under oak trees were excluded from the dataset. For all models, normality and heteroscedasticity of residuals were checked, and continuous variables were scaled (through dividing by standard deviation) and mean centred (column mean subtracted). Individual model structures are described in Table 2.2, with all implemented as linear mixed models, using the package 'lme4' (Bates et al., 2015), in 'R' v.3.5.1 (R Core Team, 2017). Full models were constructed, and no model simplification was undertaken. P values were generated through 'lmerTest' (Kuznetsova et al., 2017), which are calculated using Satterthwaite's degrees of freedom method (Satterthwaite, 1946).

Table 2.2: Model responses, predictor variables and error distributions used in analyses.

Response	Fixed	Random	Observation	Family and link function
Date of peak T_E Log(total frass fall)	Year (factor, 3 levels)	Trap ID (random intercept, factor, 40 levels)	86	Gaussian, identity
Date of peak T_E Log(total frass fall)	Species (factor, 4 levels)	Trap ID (random intercept, factor, 40 levels)	86	Gaussian, identity
Date of peak T_E	Bud burst date (continuous) Mean site March April temperature (per year) (continuous) Observations deviation from mean March April site temperature (continuous)	Trap ID (random intercept, factor, 40 levels) Year (random intercept, factor, 3 levels)	86	Gaussian, identity
Binomial response, 1 = defined peak, 0 = no peak - peaks under oak trees excluded	Number of oak trees (continuous)	Trap ID (random intercept, factor: 39 levels	117	Binomial, logit

2.4 Results

In total 155 traps, from under 60 individual trees, were monitored over the three-year period of this study, with a slightly smaller sample size in the first year compared with subsequent years ($N_{2015} = 35$, $N_{2016} = 60$, $N_{2017} = 60$). Equal numbers of each of the four tree species were monitored in 2016 and 2017 (15 of each species), but differing numbers of each species in 2015 ($N_{Beech} = 13$, $N_{Silver\ Birch} = 5$, $N_{Sycamore} = 9$, $N_{Oak} = 8$). Over the three years, 86 out of the total 155 traps (55.5%) produced an identifiable frass peak with 35% of beech, 54% silver birch, 36% sycamore and 100% of oak trees producing a frass peak (Figure 2.3, Table 2.3). The smoothed data are shown in Figure 2.3, predicted on a daily basis for each trap for the duration of sampling period to produce smoothed curves.

Table 2.3: Model fits (relative root error) of the smoothed frass fall data, along with the number of trees where frass peaks could be identified or not.

Species	Peak	No peak	Min RRE (%)	Max RRE (%)	Mean RRE (%)
Beech	15	28	24.39	84.55	67.27
Silver birch	19	16	52.98	87.98	68.34
Sycamore	14	25	47.19	98.47	71.09
Oak	38	0	18.43	86.09	58.47
All species	86	69	18.43	98.47	64.24

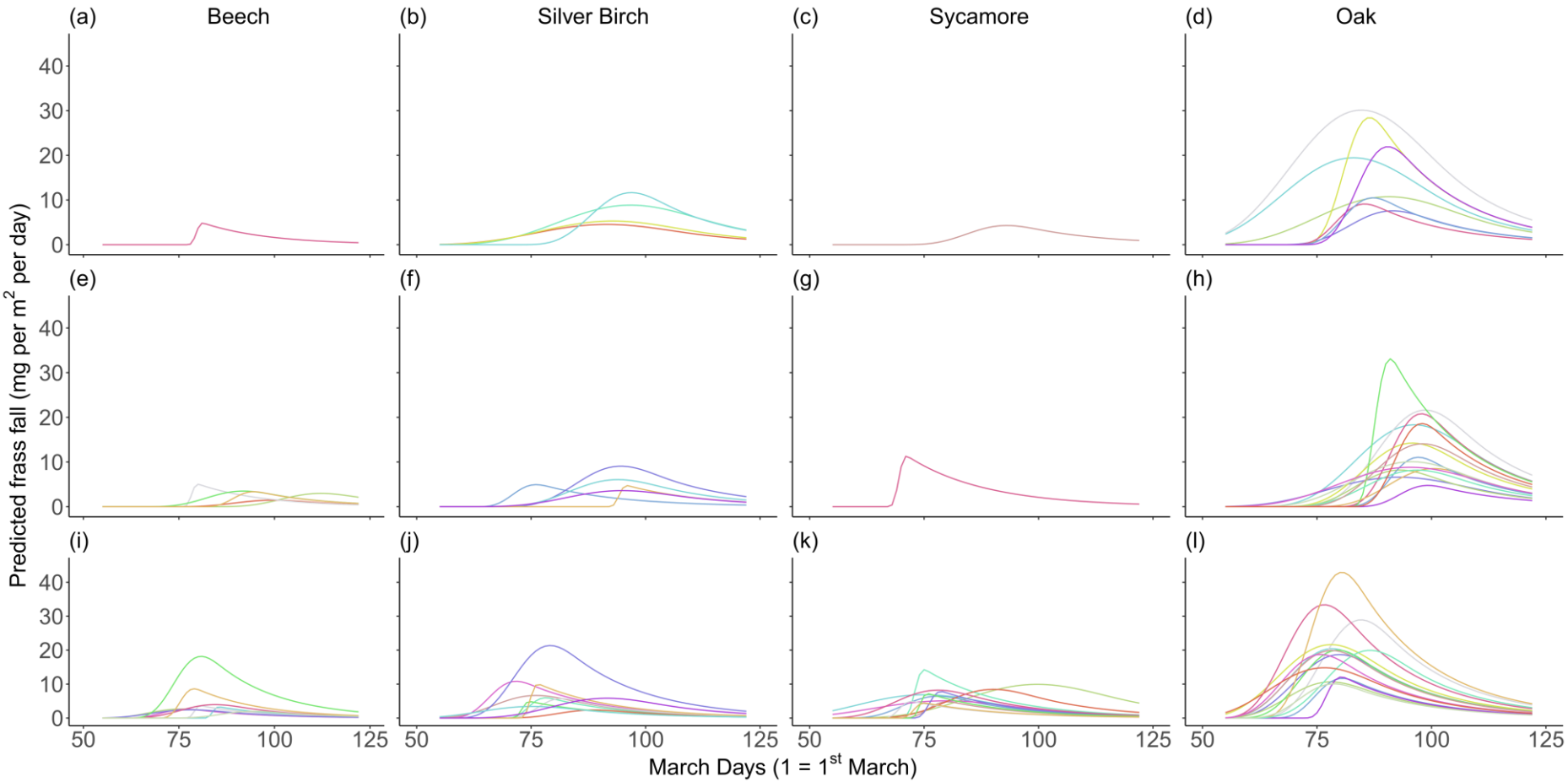

Figure 2.3: Smoothed temporal frass fall in 2015 (a, b, c, d), 2016 (e, f, g, h) and 2017 (i, j, k, l) under four species, beech (a, e, i), silver birch, (b, f, j), sycamore (c, g, k) and oak (d, h, l). Colours are consistent in columns for trees monitored in multiple years. Data are only shown from traps where a frass peak could successfully be detected.

2.4.1 Frass fall phenology

2.4.1.1 Timing of peak frass fall

The timing of peak frass fall differed among years, being earliest in 2017 and latest in 2016 (Figure 2.4, Table 2.4). After controlling for inter-annual differences, there was no difference in the timing of peaks among species (Figure 2.5, Table 2.5; ANOVA – analysis of deviance, Species: $\chi^2 = 1.85$, DF = 3, p = 0.61). Peak frass fall phenology was not predicted by temperature, neither within nor between years (Table 2.6, Figure 2.6). Peak frass fall phenology was also not predicted by host tree phenology (Table 2.6).

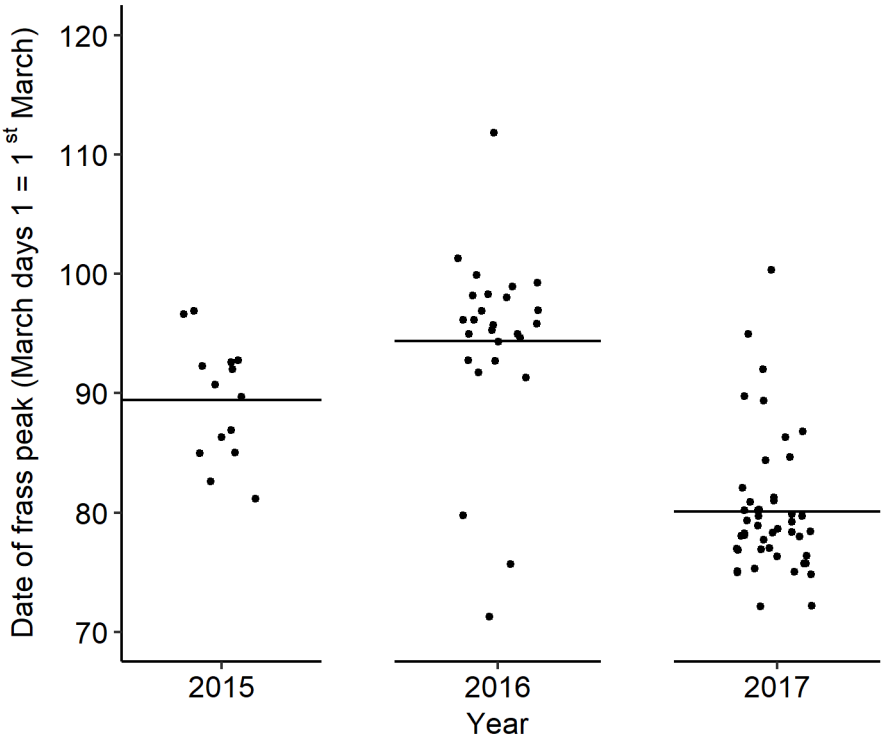

Figure 2.4: Timing of peak frass fall, between years. Each point represents a single tree (either beech, silver birch, oak or sycamore) in a given year. The points have been jittered on the horizontal axis to aid interpretation, as many overlay each other. The horizontal line represents the fitted intercepts for each year, after controlling for individual trap effects.

Table 2.4: Variable estimates for the fixed and random effects, from a general linear mixed model exploring whether the timing of peak frass fall differs between years. Rows in bold denote significant effects.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept (2015)	**89.43**	**1.71**	**83**	**52.26**	**$< 2 \times 10^{-16}$**
2016	**4.92**	**2.12**	**83**	**2.32**	**0.02**
2017	**-9.34**	**1.95**	**83**	**-4.78**	**7.5×10^{-6}**

Random effects	Variance	Standard deviation
Trap ID	0.00	0.00
Residual	41.71	6.46

Number of observations: 86
Number of groups (traps): 49

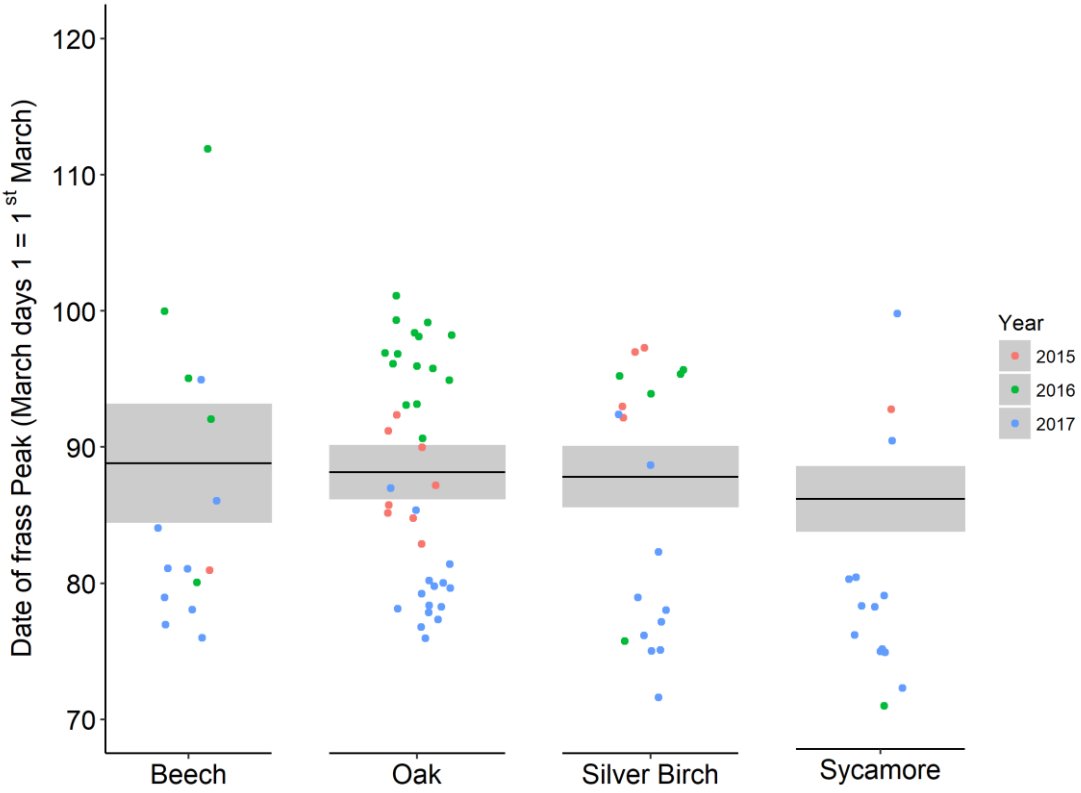

Figure 2.5: The timing of peak frass fall between species. Each point represents a single tree, coloured by sampling year. All points have been jittered on the horizontal axis to aid interpretation. The horizontal black line represents the fitted intercept for each species, after inter-annual and inter-trap differences have been accounted for, and the shaded area surrounding the fitted intercept denotes the standard error. Note that the number of peaks recorded for each species varied (see Table 2.2).

Table 2.5: Variable estimates for the fixed and random effects, from a general linear mixed model exploring whether the timing of peak frass fall differs between species.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept (Beech)	88.82	4.38	3	20.27	5.3×10^{-4}
Oak	-0.66	2.00	80	-0.33	0.74
Silver Birch	-1.00	2.25	80	-0.45	0.66
Sycamore	-2.85	2.44	80	-1.17	0.25

Random effects	Variance	Standard deviation
Year	48.38	6.96
Trap ID	< 0.0001	< 0.0001
Residual	41.81	6.47

Number of observations: 86
Number of groups (traps): 49
Number of years: 3

Table 2.6: Variable estimates for fixed and random effects, from the general linear mixed model, exploring the effect of both spatial and temporal variation in temperature and tree phenology on the timing of peak frass fall.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept	128.51	23.25	1	5.23	0.12
Bud burst	0.68	0.84	1	-1.77	0.33
Between year temperature	-5.84	0.85	81	0.47	0.64
Within year temperature	0.72	1.55	81	0.67	0.50

Random Effects	Variance	Standard deviation
Year	22.21	4.71
Trap ID	< 0.0001	< 0.0001
Residual	41.71	6.46

Number of observations: 86
Number of groups (traps): 49
Number of years: 3

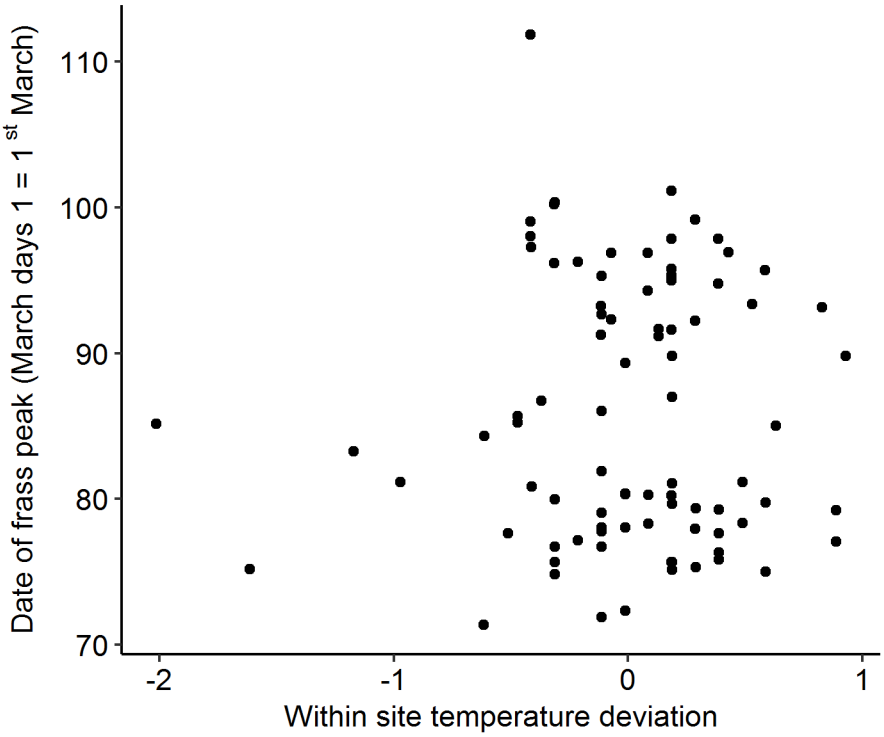

Figure 2.6: The relationship between within site temperature and peak frass fall phenology. Temperature is representative of microclimate deviations (°C) from the mean temperature across the site at each trap location. A negative value represents cooler temperatures and positive, warmer.

2.4.1.2 Duration of frass fall

The duration of frass fall differed significantly between years, with 2015 and 2017 being significantly different from one another (Figure 2.7; Analysis of Deviance: $\chi^2 = 7.34$, DF = 2, p = 0.03). On average, the duration of frass fall was shorter in 2017, than in 2015 (Figure 2.7, Figure 2.7). Frass fall duration was not different between any of the four tree species (Table 2.8, Figure 2.8). Leafing phenology of the host tree, temperature between years, nor local temperature predicted the duration of frass fall (Table 2.9).

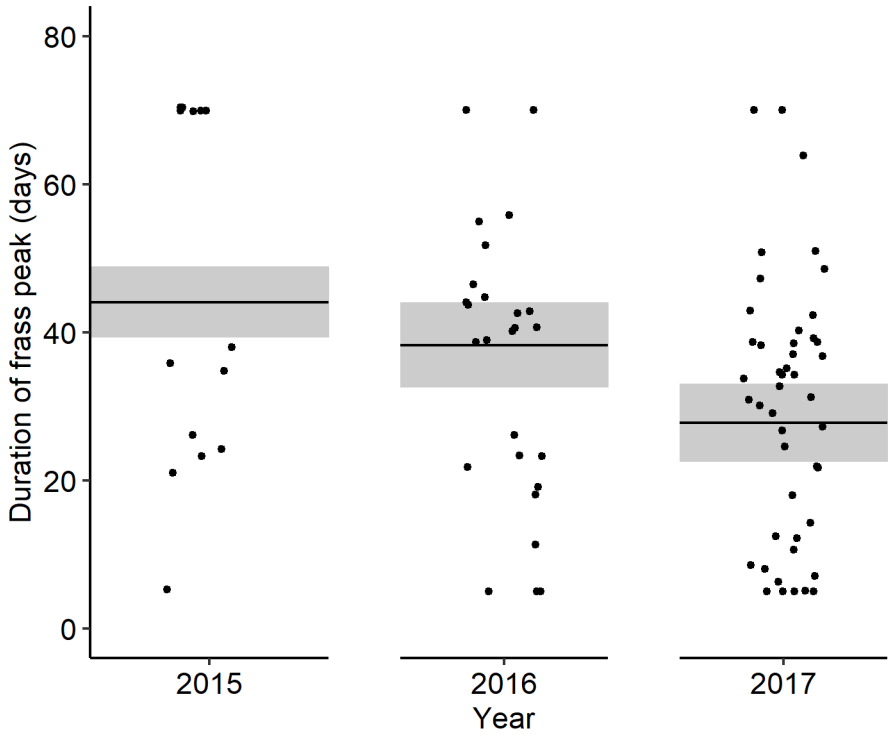

Figure 2.7: The difference in the duration of the frass fall between years. The horizontal black line represents the fitted intercept for each year, after controlling for inter-trap variation, with the shaded grey area depicting the standard error. Each point represents an individual tree; the points have been jittered on the horizontal axis to aid interpretation.

Table 2.7: Variable estimates for the fixed and random effects, from a general linear mixed model exploring whether the duration of frass fall differs between years. Rows in bold denote significant effects.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept (2015)	**43.35**	**4.84**	**77**	**8.96**	**$< 1.37 \times 10^{-13}$**
2016	-8.12	5.59	50	-1.45	0.15
2017	**-13.59**	**5.16**	**51**	**-2.63**	**0.01**

Random Effects	Variance	Standard deviation
Trap ID	113.1	10.63
Residual	246.4	15.70

Number of observations: 86
Number of groups (traps): 49

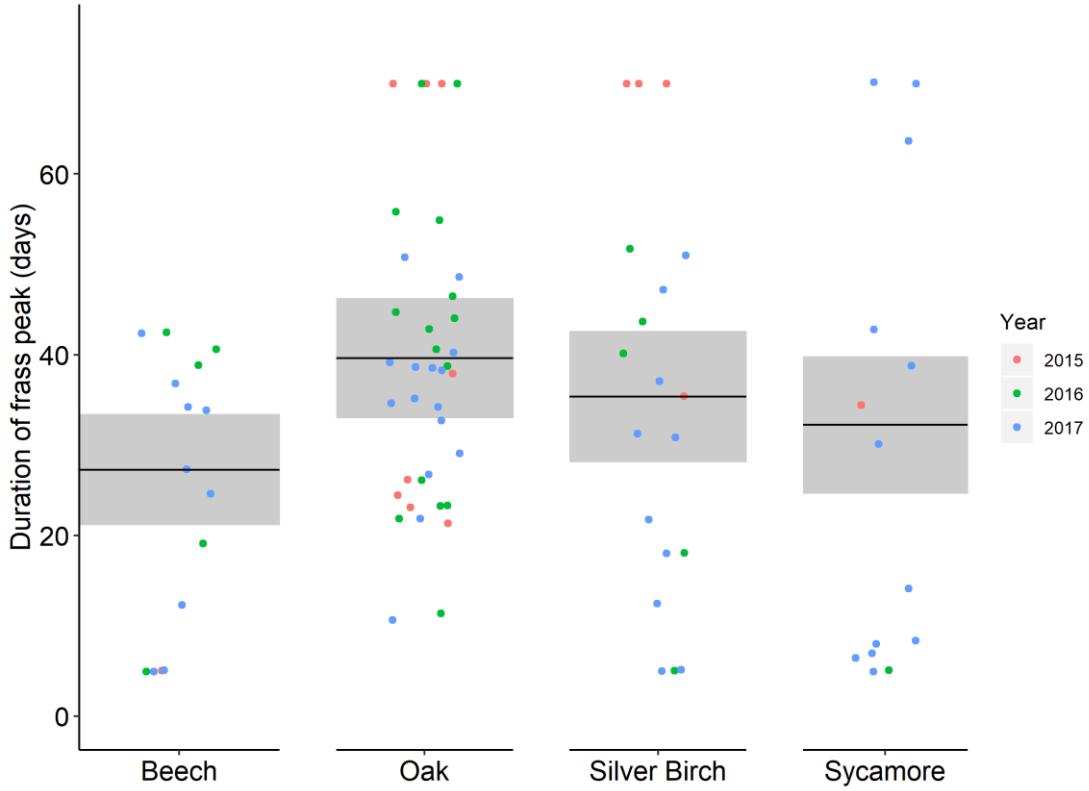

Figure 2.8: The difference in the duration of the frass fall between species. The horizontal lines represent the fitted intercepts, after controlling for inter-annual variation and inter-trap variation, with the shaded grey areas showing the standard error of these fitted values. Each point represents a single tree, coloured by year. All points have been jittered on the horizontal axis, to aid interpretation.

Table 2.8: Variable estimates for the fixed and random effects, from a general linear mixed model exploring whether the duration of the frass fall differs between species.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept (Beech)	27.27	6.05	14	4.51	<0.001
Oak	12.34	6.53	36	1.89	0.07
Silver Birch	8.09	8.09	42	1.13	0.27
Sycamore	4.96	4.96	52	0.66	0.51

Random effects	Variance	Standard deviation
Year	21.36	4.62
Trap ID	108.17	10.40
Residual	249.28	15.79

Number of observations: 86
Number of traps: 49
Number of years: 3

Table 2.9: Variable estimates for fixed and random effects, from a general linear mixed model exploring the effect of various predictors on the duration of frass fall.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept	55.98	46.64	1	1.2	0.45
Bud burst	-4.30	2.51	52	-1.71	0.09
Between year temperature	-2.59	5.98	1	74	0.64
Within year temperature	-2.83	4.48	74	-0.63	0.53

Random effects	Variance	Standard deviation
Year	83.57	9.14
Trap ID	118.32	10.88
Residual	237.95	15.43

Number of observations: 86
Number of groups (traps): 49
Number of years: 3

2.4.2 Total frass fall

Total frass fall varied inter-annually (Table 2.10; Analysis of deviance: year $\chi^2 = 11.70$, DF = 2, p = 0.003) and was significantly lower in 2016 than 2015 and 2017 (Table 2.10 and Figure 2.9). Oak produced significantly more frass fall (69.72% of total frass fall) than any of the other three tree species (Table 2.11 and Figure 2.10), with beech producing the least (6.19% of total frass fall) and silver birch and sycamore producing, on average, similar quantities (14.34 and 9.75% of total frass fall, respectively; Figure 2.10).

Table 2.10: Variable estimates for the fixed and random effects, from a general linear mixed model exploring whether the total amount of frass fall differs between years, controlling for species and trap ID.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept (2015)	3.91	0.37	4	10.59	5.38×10^{-4}
2016	**-0.42**	**0.16**	**54**	**-2.62**	**0.01**
2017	-0.04	0.15	53	-0.30	0.77

Random effects	Variance	Standard deviation
Species	0.46	0.68
Trap ID	0.10	0.32
Residual	0.20	0.45

Number of observations: 86
Number of traps: 49
Number of species: 4

Table 2.11: Variable estimates for the fixed and random effects, from a general linear mixed model exploring whether the total amount of frass fall differs between species, controlling for year and trap ID. Rows in bold denote significant effects.

Fixed effects	Estimate	Standard error	Degrees of freedom	T value	P value
Intercept (Oak)	4.66	0.16	4	28.59	6.53×10^{-6}
Beech	**-1.67**	**0.19**	**44**	**-8.76**	**3.14×10^{-11}**
Silver Birch	**-0.99**	**0.18**	**41**	**-5.56**	**1.75×10^{-6}**
Sycamore	**-1.03**	**0.19**	**55**	**-5.37**	**1.64×10^{-6}**

Random effects	Variance	Standard deviation
Year	0.46	0.68
Trap ID	0.10	0.32
Residual	0.20	0.45

Number of observations: 86
Number of traps: 49
Number of species: 4

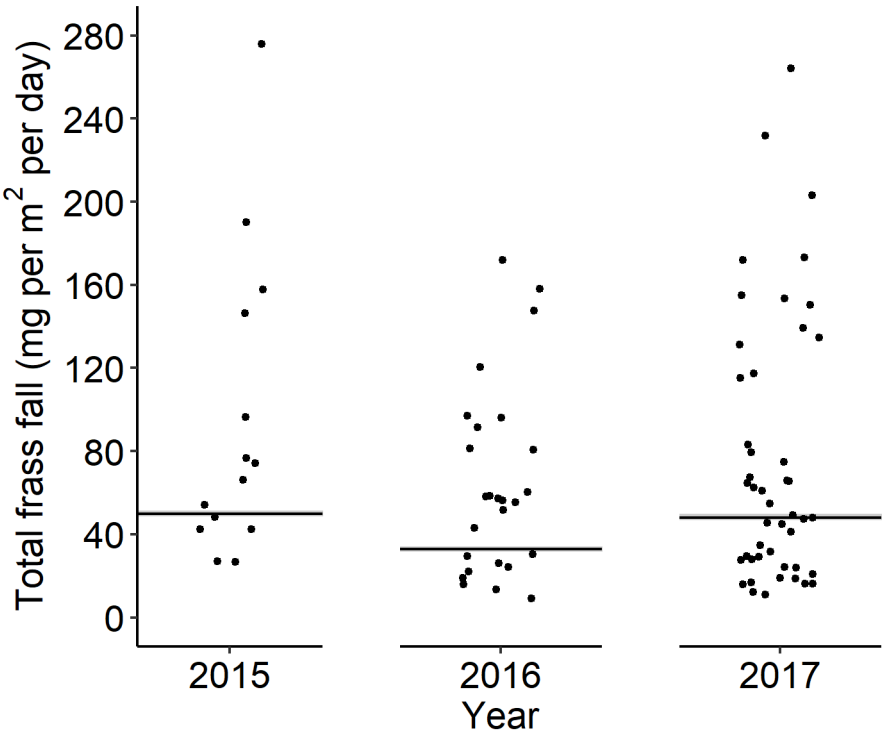

Figure 2.9: The differences in the total amount of frass fall between years. The horizontal black line represents the fitted intercept for each year, after controlling for species and trap variation, with the shaded grey area depicting the standard error. Each point represents an individual tree. The points have been jittered on the horizontal axis to aid interpretation. Note that the model was fitted on the log scale, and transformed for plotting.

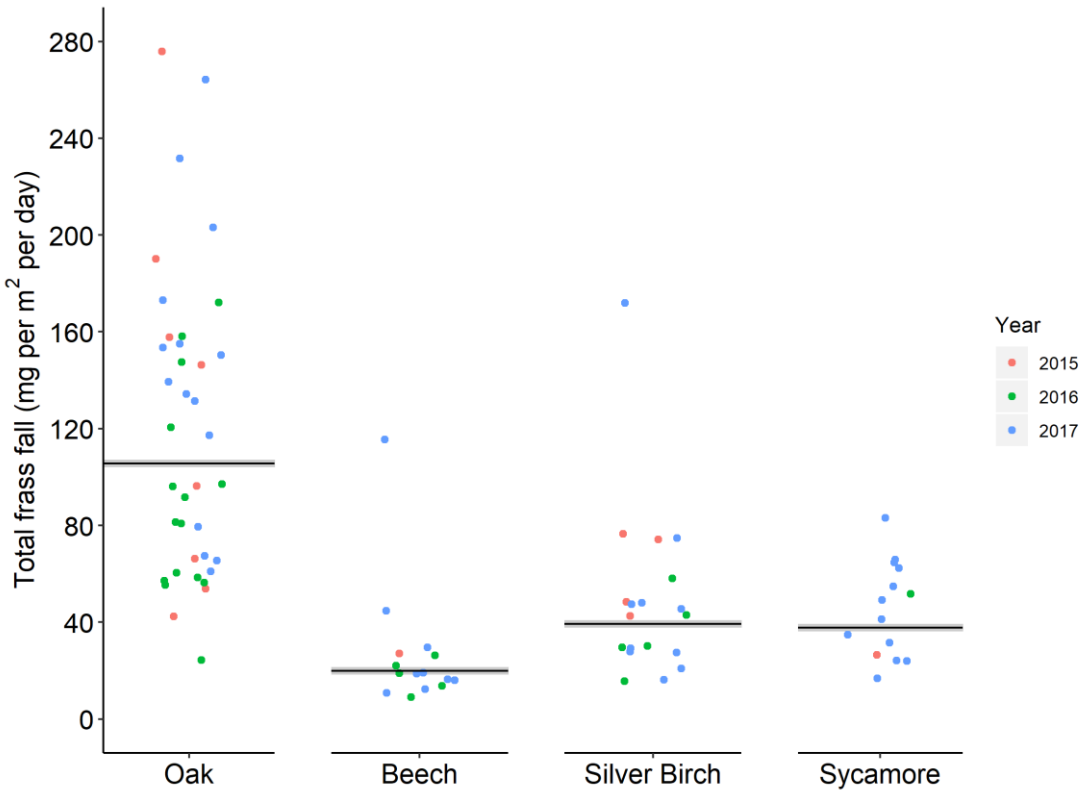

Figure 2.10: The differences in the total frass fall between species. The horizontal lines represent the fitted intercepts, after controlling for inter-annual and inter-trap variation, with the shaded grey areas showing the standard error of these fitted values. Each point represents a single tree, coloured by year. All points have been jittered on the horizontal axis, to aid interpretation. Note that the model was fitted on the log scale, and transformed for plotting.

2.4.3 Probability of a frass peak occurring

The probability of a frass peak occurring increased as the density of oak trees in the immediate vicinity of the host tree increased (Figure 2.11, Table 2.12).

Table 2.12: Variable estimates from a generalised mixed model (with a binomial, logit link function) exploring the effect of the density of oak trees in the immediate area surrounding the tree being sampled on the likelihood of a frass peak occurring. In this model all oak traps have been removed and only traps from under the other three tree species (beech, silver birch, and sycamore) are included.

Fixed effects	Estimate	Standard error	Z value	P value
Intercept	-0.23	0.21	-1.13	0.26
Number of Oak trees	**0.82**	**0.29**	**2.83**	**0.005**
Random effects	**Variance**	**Standard deviation**	Number of observations: 117 Number of groups (traps): 46	
Trap ID	<0.001	<0.001		

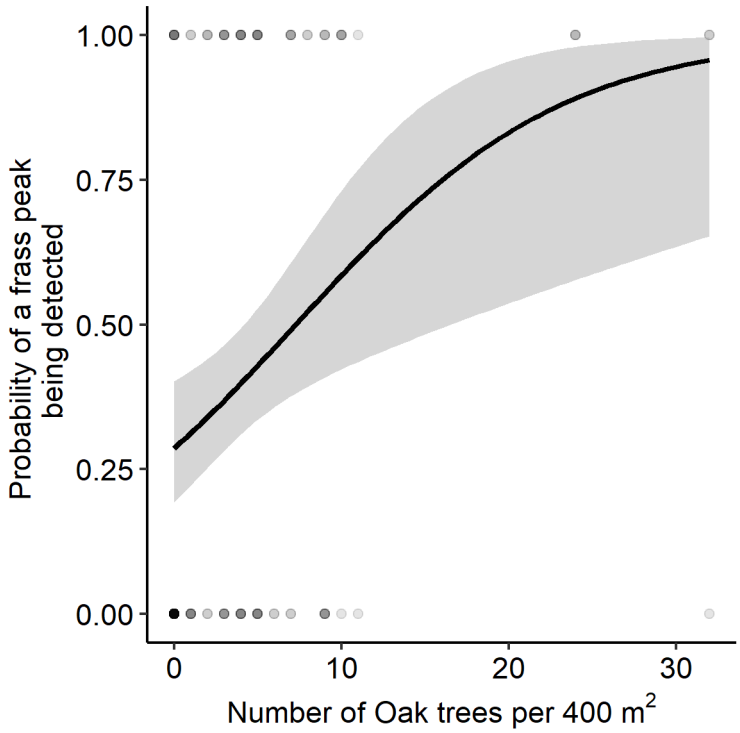

Figure 2.11: The probability of a frass peak (1) occurring or not (0) given the number of Oak trees. Each point represents a single tree, with the oak records removed, of either Beech, Silver Birch or Sycamore, with darker points having more points at the same position than lighter points. The black line represents the fitted line from the generalised mixed model, and the shaded grey area the 95% confidence interval.

2.5 Discussion

This study is among the first to investigate in detail the timing and duration of caterpillar availability, at an intra-site level, and to focus on multiple potential host tree species. Here, I aimed to assess the importance of four common tree species in UK woodlands (beech, silver birch, sycamore, and oak*)* for Lepidopteran larvae, and therefore indirectly insectivorous woodland birds. All species produced detectable frass peaks, in all years, although not all individual trees produced peaks. Of the four species, oak is the most important species for Lepidopteran larvae, due to producing significantly more frass fall. All species produced detectable frass peaks under at least one tree in each year. However, the probability of frass fall being detected under non-oak species significantly increased as surrounding oak density increased, suggesting wind-blown contamination could be an issue. Contrary to my hypotheses, neither temperature nor tree phenology predicted frass fall phenology.

2.5.1 Frass fall phenology

2.5.1.1 Timing of peak frass fall

Caterpillar phenology was monitored for three consecutive years and varied significantly inter-annually, with the timing of peak frass fall being earliest in 2017 and latest in 2016 (Figure 2.4, Table 2.6). Previous research suggested caterpillar phenology is driven by temperature with moth egg hatch being delayed when temperatures are cooler (Buse et al., 1999), and peak frass fall exhibiting the same trends (Smith et al., 2011). However, the results presented here are not consistent with these findings, as caterpillar phenology is not predicted by temperature between years or within the site (Table 2.6, Figure 2.6). Due to sampling only being undertaken in three years, there will have been a lack of power to detect between year relationships with temperature. There is more power to detect a within site relationship between peak frass fall phenology and temperature, however, temperature did not explain differences in frass fall phenology within the site. This may be due to the temperature window used, which was selected based upon being informative for tree bud-burst (Tansey et al., 2017), which is typically highly correlated with caterpillar phenology, however may not be informative when used as a predictor for frass fall. The temperature window used, mean March-April temperature, will not have been indicative of overwintering temperatures or any extreme variation in temperature experienced during spring. Overwintering temperature may be important as caterpillars spend a large proportion of their life cycle in the egg stage. For example, winter moth caterpillars are in the egg stage for approximately six months of the year as opposed to only two months in the larval stage (Holliday, 1985). However, the relationship between temperature and development during egg stage is relatively complex, as eggs pass through up to three distinct stages, making hatching

phenology difficult to predict using field measured temperature (Holliday, 1985). A combination of temperature and humidity influence the timing of egg hatching (Embree, 1970), and it is unclear whether temperature or humidity is more important, but eggs reared under lower humidity have been shown to hatch out later (Embree, 1970). It was not possible to measure humidity in this study, but may serve as an explanation as to why no relationship with temperature was found, as humidity may be more important for controlling hatching, and may not have varied across this site. Peak frass fall phenology did not differ among species (Figure 2.5, Table 2.5) or with tree phenology (Table 2.6), contrary to my hypotheses. This is surprising as the presence of young leaves are imperative for larval survival and, experimentally, hatching is typically highly correlated with host tree phenology (Both et al., 2009; Buse et al., 1999; Embree, 1965). In addition, peak frass fall has been shown to be highly correlated with first leaf date in oaks across the UK, in a nationwide study (Burgess et al., 2018). The lack of correlation between host tree phenology and peak frass fall, detected here, could suggest an uncoupling of a reportedly synchronous relationship, at this site, with predicted peak frass fall typically occurring approximately 35 days after bud-burst of the host tree. However, the measure of tree phenology used here may have been too sensitive, as bud-burst date represented the date at which new leaves were seen protruding from the bud on one or more buds. Caterpillars may not be highly synchronised with the first bud-burst, but more synchronised to when the majority of buds are bursting. At the broad UK scale caterpillars are tracking earlier, warmer, springs (Burgess et al., 2018) and this is consistent with findings from the Netherlands (Both et al., 2009). The caterpillar-oak relationship is predicted to remain synchronous, as both levels are believed to use the same cues and will therefore continue to track changing climate at the same rate (Buse et al., 1999). Due to peak frass fall phenology not differing amongst host tree species, this suggests that non-oak tree species would be unable to offer Lepidopteran resources to insectivorous birds that may have mistimed breeding with Lepidopteran resources on oak trees.

All raw frass fall data were smoothed, following Soulsby and Thomas (2012), to account for any extreme weather events, such as rainfall, that happened during sampling that weren't accounted for in the models. For example, raw frass fall data from under oak trees in 2017 appear to display a bimodal distribution (two peaks in frass fall; Figure 2.2), however this coincides with a heavy rainfall event. Rainfall can cause frass to disintegrate and become difficult to detect (Fischbacher et al., 1998). Smoothing data also accounts for the five day interval between sampling, allowing for phenological variables to be estimated at a finer resolution.

This study highlights the need for long term, more in-depth, local scale monitoring to establish whether there are finer scale processes, which are not apparent when generalised to large spatial scales, and to validate nationwide extrapolation.

2.5.1.2 Duration of frass fall

Similarly to peak frass fall phenology, duration of frass fall was also significantly different among years, however only between 2015 and 2017 (Figure 2.7, Figure 2.7). I hypothesised this would be due to temperature as caterpillars develop quicker when exposed to warmer temperatures (Buse et al., 1999; Holliday, 1985), taking less time to reach pupation. Therefore, a decrease in the duration of frass fall would be expected under warmer temperatures, due to shorter development times. However, when tested no relationship with between year temperature and the duration of frass fall was found (Table 2.9), which is surprising given caterpillars develop more rapidly at warmer temperatures (Buse et al., 1999; Holliday, 1985). The lack of relationship is likely due to the small sample size in this study, due to only three years of monitoring. Although, temperature differences across the site, within a year, also did not predict the duration of frass fall (Table 2.9), despite increased sample size. The duration of the peak may not be defined by the time it takes for caterpillars to develop, but how synchronously larvae hatch out. If caterpillars hatch out synchronously they will all develop at roughly the same rate, leading to a potentially shorter and sharper peak. However, if they hatch asynchronously the time taken for them to reach pupation will be more drawn out, and could lead to a longer and shallower peak. Limited research has been undertaken investigating hatching synchrony, but at warmer temperatures hatching is suggested to be more synchronous (Embree, 1970), which may explain the shorter duration of peak observed in 2017 in this study.

There was no difference in duration of frass fall between tree species (Figure 2.8, Table 2.8), or the leafing phenology of the host tree (Table 2.9). Given timing of peak frass fall did not vary with any of these variables it is not overly surprising there was no effect of these variables on the duration of frass fall either, suggesting that neither host tree phenology nor tree species influences caterpillar phenology at this site.

2.5.2 Total frass fall

Previous research into caterpillar phenology, using frass fall, has typically focused on oak trees (e.g. Burgess et al., 2018; Smith et al., 2011). This focus has been justified through because the largest number of Lepidopteran species have been show to reside on oak trees (Kennedy and Southwood, 1984), and Lepidoptera typically form a large part of insectivorous birds, such as blue tits, diets (Betts, 1955; Shutt, 2017). The findings here support this focus on oak, due to the highest amount of frass fall being observed under oak trees (Table 2.11, Figure 2.10). However, frass fall was detected under all species investigated here (Figure 2.3, Figure 2.10), which suggests that four of the five most common broadleaved tree species in UK woodlands play a role in insectivorous birds feeding behaviour (with ash being unable to be tested in this study, due to there being a lack of suitable trees at this site). These findings suggest that none of the non-oak

tree species investigated here could provide Lepidopteran resources on the scale of oak, for birds nesting in oak-poor habitats, for example. Oak currently occupies the largest area in UK woodlands of any broadleaved species, but heterogeneous oak woodlands are uncommon (Forestry Commission, 2013). Due to the importance of oak for Lepidopteran larvae, demonstrated here, any changes to woodland composition in the future which would disadvantage oaks could lead to reduced breeding success and/or populations of insectivorous birds, due to reduced Lepidoptera larvae availability.

2.5.3 Probability of detecting a frass peak

In this study, 100% of oaks sampled produced a detectable frass peak, whereas in non-oak species between 35 and 54% produced a detectable frass peak, lower than stated by Veen et al. (2009). Here, I have shown the probability of a frass peak being detected significantly increased as oak density increased (Table 2.12, Figure 2.11). Taken in combination with the fact that there was no difference in the timing of peak frass fall between species, peaks detected under non-oak species could be as a result of windblown frass from nearby oaks being collected in traps, and therefore creating 'false positives'. Theoretically, all tree species investigated here could produce frass peaks (true positives) as they have all been shown to host Lepidopteran species (Kennedy and Southwood, 1984; Shutt, 2017). Therefore, further validation is required to clarify whether these peaks are true positives.

2.5.4 Conclusions

In conclusion, the results presented here support the oak focus of previous work, when Lepidopteran larvae are being investigated, due to oaks consistently producing the highest levels of frass fall. Lepidopteran larvae form a large proportion of insectivorous woodland birds' diets and these findings suggest birds are likely to be heavily reliant upon oak trees when foraging. Especially as frass fall detected under non-oak species could be due to contamination from nearby oaks. These findings also highlight that insectivorous woodland birds may face problems finding sufficient Lepidopteran food resources, if oaks are disadvantaged in UK woodlands in the future.

Temperature, either between years or across the site, was also shown here to not predict the timing of peak frass fall or frass fall duration, which is particularly pertinent given predicted future climate trends and insectivorous birds' reliance on this resource. The lack of intra-species variation in peak frass fall phenology, prevents non-oak species from providing a lepidopteran resource to breeding birds that have mistimed breeding events with Lepidopteran peaks on oak trees, reaffirming oaks importance within UK woodlands.

Chapter 3: Refining and comparing DNA extraction and amplification methods from avian adult and nestling faecal samples

3.1 Abstract

Advances in DNA technology, such as next generation sequencing (NGS), provide new opportunities to understand trophic interactions, and dependencies, to answer ecological questions. An essential pre-requisite of NGS is the ability to extract and amplify high quality DNA. Extracting DNA from environmental samples is often challenging due to DNA degradation and the presence of complex inhibitors. Faecal samples have the potential to provide a simple and non-invasive method to investigate species' resource usage. However, faecal samples contain highly degraded DNA, and high concentrations of substances that inhibit polymerase chain reactions (PCR), making DNA extraction and amplification difficult. Here, I provide details of methods developed to extract and amplify DNA from faecal samples of three bird species (nestling blue tit (*Cyanistes caeruleus*), nestling meadow pipit (*Anthus pratensis*) and adult starling (*Sturnus vulgaris*)), with the final goal of evaluating the diet of blue tit nestlings through NGS. DNA of sufficient quality for NGS was unable to be extracted from blue tit nestling faecal sacs with an off-the-shelf extraction kit (MO-BIO PowerSoil extraction kit). Improvements in DNA extraction were achieved through modifications to the off-the-shelf extraction kit, followed by a secondary extraction using solid phase reversible immobilisation with magnetic beads.

DNA extracted from nestling faecal sacs were tested with four invertebrate primer pairings to amplify invertebrate prey DNA during PCR. mICOIintF and jgLCO1490 (from Leray et al., 2013) provided the highest amplification success, but also likely amplified bird DNA. Amplification of DNA was much more readily obtained from adult starling and meadow pipit faecal samples, than from nestling blue tit faecal samples. Further work to increase amplification success, with specific invertebrate primers, would be required to sequence DNA from blue tit faecal samples, but this was not possible within the timeframe available. In conclusion, faecal samples can be highly variable both within and between species and, as such, a DNA extraction and amplification approach, which may be appropriate for one species may not be for another. Future work could investigate ways of increasing starting DNA concentration, such as through whole genome amplification, and further ways of removing chemicals which may be degrading DNA or inhibiting PCR.

3.2 Introduction

Recent climate change has altered species distributions, both spatially and temporally, putting pressure on existing species interactions and providing opportunities for new interactions to occur (Parmesan, 2006; Walther, 2010; Walther et al., 2002). An understanding of the interactions between species is essential to fully understand the implications of climate change (Walther, 2010). However, trophic interactions between species, such as predator-prey interactions, are challenging to study, due to foraging locations often being spatially extensive and difficult to observe. As a result, our understanding of food webs is often simplistic, with many missing interactions.

In temperate woodlands, the deciduous tree-herbivorous caterpillar-insectivorous bird tri-trophic system is often used as a model for understanding the effects of climate change on both individual trophic levels and their interactions (Burger et al., 2012; Burgess et al., 2018; Buse et al., 1999). Lepidoptera make up a large proportion of insectivorous nestling birds' diets, such as blue tits (*Cyanistes caeruleus*), however they are not the sole prey group (Betts, 1955). Therefore, in reality, this food chain is a more complex food web with multiple interactions. Nestling diet is important in maximising offspring fitness, which depends on the quality and quantity of prey items delivered (Wilkin et al., 2009). However, the specifics of diet composition, and the importance of specific prey groups are not well understood. Despite its importance, to date, studies of nestling diet in avian insectivores have typically only been able to be resolve prey items to a family level, with many prey items unable to be identified (e.g. Betts, 1955; García-Navas and Sanz, 2011; Grzędzicka, 2018; Wilkin et al., 2009). Traditional methods for elucidating diet are often labour intensive and require expert identification skills (e.g. microscopic analysis of prey remains in faecal matter, identification of prey from video footage), are invasive (e.g. ligatures (Johnson et al., 1980)) or require dead specimens (e.g. gizzard analysis (Bourgault et al., 2006)). These reasons combined, offer reason as to why diet composition often goes unstudied.

Technological advances, such as DNA based techniques, provide new opportunities to elucidate species interactions at a higher taxonomic resolution, and also through non-invasive methods. The most recent advance has been in next-generation sequencing (NGS, or parallel sequencing). NGS can sequence many individual DNA fragments within a sample, allowing the identification of the contents of complex mixtures, such as dietary samples (Shokralla et al., 2012). Due to recent advancements in technology, reduced costs and the increased number of plant and animal reference sequences available, NGS has widened potential to answer ecological questions (Valentini et al., 2009). Using this method, it is possible to assemble

detailed food webs, for the first time in some systems. NGS has been widely used to elucidate mammal diets (e.g. De Barba et al., 2014; Clare et al., 2009; Razgour et al., 2011) and is beginning to be used for avian diets (e.g. Jedlicka et al., 2013, 2016; Shutt, 2017; Trevelline et al., 2018).

NGS requires high quality DNA to be extracted and successfully amplified using an appropriate primer pairing of a 'barcode' region, through polymerase chain reaction (PCR) (King et al., 2008). During PCR, individual samples can be tagged, allowing samples to be tracked and more complex interactions, such as food webs, to be inferred (nested metabarcoding, see Kitson *et al.*, 2018 for details). NGS of PCR products result in large numbers of sequences that must be matched to reference sequence libraries for species identification. Ecological samples often prove difficult to produce DNA of sufficient quality for NGS applications, as DNA is often degraded, or contain high concentrations of PCR inhibitors (Schrader et al., 2012).

Faecal samples can be used for molecular dietary studies, as the methods used to elucidate diet from faeces are non-invasive, non-destructive and samples are relatively easy to collect. Despite being easy to collect, faecal samples are challenging to extract and amplify DNA of sufficient quality for NGS. This is due to excrement being chemically complex, with many chemicals inhibiting PCR as well as creating a hostile environment that can lead to further DNA degradation (McInnes et al., 2017; Monteiro et al., 1997; Oehm et al., 2011; Schrader et al., 2012). DNA recovery can be optimised through collection protocols, such as by collecting fresh samples from hard surfaces e.g. rock as opposed to soil (McInnes et al., 2017), through DNA extraction method (Braid et al., 2003; Schrader et al., 2012) and/or modifications in PCR protocol (e.g. additives (Kreader, 1996), dilution of template or polymerase selection (Schrader et al., 2012)).

Barcoding loci vary by taxon, and influences primer selection for PCR amplification. Typically in avian diet analyses cytochrome C oxidase subunit I (COX1) is selected (e.g. Trevelline et al., 2018) due to the large reference database available for this loci, which frequently permits sequence identification to species level (Hebert et al., 2003). A combination of loci may be used to elucidate diets for generalist species (Pompanon et al., 2012). PCR primers are barcode region specific, and can be species/taxa specific or universal (amplify multiple phyla or taxa). Universal primers are typically used in dietary studies due to not knowing *a priori* what the diet is likely to include. However, primer selection will introduce biases in terms of prey species returned, as different primers favour certain phyla or taxa. For example, the universal primers described by Leray *et al.,* (2013) amplify approximately 88 % of taxa within Arthropoda, which will affect the species recovered post-sequencing.

The aim of this chapter is to develop and test DNA extraction methods to extract DNA from nestling blue tit faecal sacs and to compare amplification success with universal invertebrate primers. The second aim is to sequence the extracted DNA, to evaluate nestling dietary composition and to relate this to local habitat components and nestling survival.

3.3 Methods

3.3.1 Sample collection and storage

3.3.1.1 Field collected faecal samples

3.3.1.1.1 Blue tit

Nest boxes containing active blue tit nests in Durham University woodlands (latitude: 54.7629, longitude:-1.5692) were monitored during March to July in both 2016 and 2017. Post-hatching, active nest boxes were checked every 2-4 days, until chicks were 15 days old, and a final check made once when nestlings were 20+ days old to ascertain assumed number of fledged chicks (i.e. last chick count minus any subsequent fatalities left in the nest) and approximate fledging date (estimated as the midpoint between the last day activity was detected and the final nest check. Activity refers to the date chicks' presence in nest boxes were detected audibly, without visual inspection, between days 15-20). Nests were not visually checked between 15 and 20 days post hatching to minimise the risk of premature fledging.

During each visit post-hatching (day 1 to 15), all chicks were removed from the nest and placed in the same cotton bird bag, lined with a clean paper bag, prior to sampling. Contamination between broods was minimised by lining cotton bird bags with a clean paper bag for each brood. Nitrile gloves were not used when handling small chicks; therefore alcohol gel was used to decontaminate hands after each brood was handled. Each chick was individually weighed, and in 2016 a unique combination of toes marked, using a non-toxic marker pen, allowing individuals to be identified prior to being large enough to metal ring (British Trust for Ornithology metal ring; under license to CJB). Faecal samples were collected opportunistically during routine handling, either from the bag, directly off hands or the floor or, most commonly, (once chicks were approximately five to seven days old) holding the chick directly over the tube, which often resulted in defecation. All faecal sacs were stored individually in 5 mL Eppendorf tubes (clip top for 2016 and screw top for 2017 samples) pre-filled with 4 mL 100% ethanol. Once in the ethanol, faecal sacs were broken up using stainless steel tweezers, decontaminated with ethanol and fire. Samples were stored at -20°C, within six hours of collection, for up to eight months.

3.3.1.2 Samples from captive birds

3.3.1.2.1 Starling

Faecal samples were collected from captive adult starlings (*Sturnus vulgaris*) at Newcastle University, which are fed a mixture of cat food, fruit, cereals and mealworms. Faecal samples were collected during routing cleaning of the birds' cages, using a disposable spatula to scrape

the solid portion of excrement from paper lining the cages. Faecal samples were stored in 5 mL screw top Eppendorf tubes, and were between zero and twelve hours old at collection. All samples originated from multiple individuals and were homogenised after collection, with more than one faecal sample being stored in the same tube. Samples were stored at -20°C for up to 24 hours after collection.

3.3.1.3 Tissue samples

Breast muscle from frozen puffin (*Fratercula arctica*) and kingfisher (*Alcedo atthis*), which both died of natural causes, and a toe from frozen coal tit (*Periparus ater)* and great tit (*Parus major*), which had both been previously kept for experimentation by Newcastle University and euthanised at the end of the study, were used to obtain positive bird DNA from known species. Invertebrate DNA was also extracted from three known species of moth (common wave (*Cabera exanthemata*), purple bar (*Cosmorhoe ocellata*) and silver ground carpet (*Xanthorhoe montanata*)); Geometridae larvae, Noctuid larvae, a tipulid (Tipulidae spp.), blue mussel (*Mytilus edulis*), ramshorn snail (Planorbidae spp.) whitefly (Aleyrodidae spp.) and thrip (Thysanoptera spp.), to obtain positive invertebrate DNA from known species. This allowed primers to be tested for both bird and invertebrate DNA amplification.

3.3.2 DNA extraction

Multiple DNA extraction methods were tested on nestling and adult faecal samples. The names used for each extraction method are referred to throughout this chapter, and any modifications made within the method described. All extracted DNA was stored at -20°C. Prior to DNA extraction being undertaken, faecal samples were defrosted and storage ethanol removed. Samples were heated to approximately 60°C for 30 minutes to remove residual ethanol and, a partially dry weight obtained (Mettler AE 166, ± 0.01 g).

Faecal samples from starlings were homogenized (to prevent individual biases e.g. specific inhibitors, differences in diet preferences) before adding to tubes to create samples of differing weights in the following classes, <0.01 g, 0.05 g, 0.2 g and 0.4 g. This allowed comparisons of the effects of modifications to DNA extraction methods on DNA recovery of different sizes of samples. The weight classes were representative of the weights of blue tit nestling faecal sacs obtained during field collection.

3.3.2.1 PowerSoil extraction kit

DNA from faecal samples were extracted using the MO-BIO PowerSoil DNA isolation kit following manufacturer's instructions (instruction manual Version 07272016, Qiagen, Valencia, CA.).

3.3.2.2 Chemical lysis extraction method

This is an optimised DNA extraction method for faecal samples, based on the MO-BIO PowerSoil extraction kit. All extraction buffer components are described in Table 3.1, and referred to throughout by the names used in the table. The chemical compositions of each solution, and the original extraction protocol, were devised by G. Sellers (original protocol in supplementary material, 3.6).

3.3.2.2.1 Cell lysis

Each blue tit nestling faecal sac, collected in 2016, was removed from the storage tube post drying and added to a new 5 mL tube that was prefilled with 2.4 g of sterile garnet (1 – 1.4 mm diameter) and 1460 µL lysis solution one. 530 µL of lysis solution two was subsequently added and the sample placed in SPEX SamplePrep Geno/Grinder 2010 at 1750 RPM for two minutes to fully lyse cells. This mixture was centrifuged (Thermo Scientific Heraeus megafuge 40R) at 4,000 xg for 1 minute at room temperature. Up to 1000 µL of supernatant was transferred to a clean 2 mL tube.

To simplify sample preparation in 2017, blue tit nestling faecal sample processing was slightly modified. 2200 µL lysis solution one, and 2.4 g garnet were added directly to the dried sample tube. This mixture was then ground at 1750 RPM for four minutes, centrifuged briefly for 30 s, and 800 µL lysis solution two added, and centrifuged at 4,000 xg for 1 minute with up to 1500 µL of supernatant removed and centrifuges at 10,000 xg for 1 minute (at room temperature, with centrifuging conditions remaining constant in all subsequent steps, unless stated otherwise). 500 µL of supernatant was retained for purification and any excess stored at -20°C.

Lysing volumes were modified in some experiments, with the following volumes also being tested: 2555 µL lysis solution one and 945 µL of lysis solution two, or 974 µL lysis solution one and 353 µL lysis solution two, but the rest of the lysis procedure remained the same.

3.3.2.2.2 DNA purification

200 µL of protein flocculant was added, briefly vortexed, and incubated on ice for 10 minutes before centrifuging. 500 µL of supernatant was removed and placed in a fresh tube with either 84 µL (2016 samples) or 200 µL (2017 samples) of a 1:1 mix of inhibitor flocculant 1 and 2 and the mixture centrifuged. Supernatant was transferred to a new 2 mL tube and 1568 µL of binding solution added. In a further test, binding solution volume was reduced to 1000 µL. A silica spin column (Biobasic EZ-10 DNA Mini Spin Column) was filled and flow through discarded until all the binding mixture had passed through. 392 µL of wash solution (or 375 µL of wash solution with 1000 µL of binding solution was also tested) was added directly to the

silica membrane and after being centrifuged, flow through was discarded and the spin column centrifuged again to dry the silica membrane. A new collection tube was provided, and 100 µL (313 µL and 50 µL also tested) of elution buffer, heated to 70°C, was applied directly to the silica membrane and left at room temperature for two minutes prior to centrifuging. DNA was collected in the new tube and stored at -20°C until use.

Half volumes for the whole DNA purification stage (based on original 2016 volumes) were also tested with all steps carried out as written above, or with a reduction to 750 µL binding solution.

Due to the chemical lysis extraction method, and solutions, being a modified version of the MO-BIO PowerSoil extraction kit, the modified solutions were also tested following the manufacturers protocol both for full DNA extraction, and just for DNA purification (step 8 onwards of manufacturer's protocol).

DNA extracted from meadow pipit faecal sacs, was provided by Lisa Malm who extracted it using the 2016 chemical lysis extraction method or the MO-BIO PowerSoil extraction kit.

Table 3.1: Descriptions of the chemical components, and concentrations, of each solution used during DNA extraction using the chemical lysis extraction method, modified from MO-BIOs PowerSoil DNA extraction kit. The names of the solutions here are used throughout the protocol descriptions.

Solution	Contents	Final concentration in solution	pH
Lysis solution one	Guanidine thiocyanate	147 mM	9.0 Adjusted using appropriate amount of 5M HCl
	Trisodium phosphate	228 mM	
	Sodium chloride	26 mM	
	Tris hydrochloride	67 mM	
	EDTA	27 mM	
Lysis solution two	Aluminium ammonium sulphate dodecahydrate	90 mM	-
	Sodium dodecyl sulphate (SDS)	1.25 %	-
Protein flocculant	Ammonium acetate	5 M	-
Inhibitor flocculant one	Aluminium ammonium sulphate dodecahydrate	180 mM	-
Inhibitor flocculant two	Calcium chloride dihydrate	204 mM	-
Binding solution	Guanidine hydrochloride	5.5 M	-
Wash solution	Ethanol	80%	-
Elution buffer (2016 samples)	Tris hydrochloride	10 mM	8
	EDTA	1 mM	
Elution buffer (2017 samples)	Tris hydrochloride	10 mM	8

3.3.2.3 Proteinase K digestion and additional inhibitor removal:

Enzymatic cell lysis, along with an additional inhibitor removal step, as described by Sokolov (2000), was tested on six great tit faecal samples. After samples were dried, and transferred to a 2 mL tube, 1 mL of enzymatic lysis solution (Table 3.2) was added to each sample, briefly vortexed and incubated at 55°C until fully digested (vortexed every 20 minutes, complete digestion in 1-2 hours). 100 µL of saturated potassium chloride was added to the lysate, mixed by repeated inversion, and incubated on ice for 5 minutes. After centrifuging at 10,000 xg for 10 minutes 500 µL of supernatant was removed and DNA purification carried out as described in chemical lysis extraction method.

This method was also tested on stored lysates from blue tit and meadow pipit faecal samples, from the chemical lysis extraction method. Either proteinase K (final concentration of 0.4 mg/mL) was added to each stored lysate and the steps described above followed, or 100 µL of saturated potassium chloride solution was added to stored lysates from the chemical lysis extraction method, incubated on ice for 5 minutes, centrifuged and 500 µL of supernatant

removed. Subsequent DNA purification was as described in the chemical lysis extraction method.

Table 3.2: Descriptions of the chemical components, and concentrations, of the lysis solution used during enzymatic cell lysis.

Solution	Contents	Final concentration in solution
Enzymatic lysis solution	Tris hydrochloride (pH 7.5)	50 mM
	Sodium chloride	100 mM
	EDTA	10 mM
	Sodium dodecyl sulphate (SDS)	1 %
	Proteinase K	0.4 mg/ml

3.3.2.4 Carboxylated paramagnetic bead clean up (SPRI, with magnetic beads)

After DNA was extracted from blue tit nestling faecal sacs (irrespective of first extraction method) all underwent a second DNA extraction, to remove any PCR inhibitors that were co-eluted, using solid-phase reversible immobilization with carboxylated paramagnetic beads. Magnetic bead solutions were made following Jolivet and Foley (2015), and were identical to AMPure XP and RNAClean XP beads (Beckman Coulter).

All samples were processed on 96 well plates. 90 µL of SPRI bead solution (20% PEG 8000, 10 mM Tris base, 1mM EDTA, 2.5 M sodium chloride, 0.05 % Tween 20) was added to 50 µL of extracted DNA solution and vortexed for 30 seconds. The mixture was incubated at room temperature for 5 minutes, placed on a magnetic plate and once supernatant had cleared, supernatant was removed and discarded. 200 µL of 70 % ethanol wash solution was added, left at room temperature for one minute, then removed and discarded. This step was repeated, and the beads left to air dry for 10-15 minutes on the magnetic plate, before 30 µL of 10 mM tris hydrochloride was used to elute DNA and magnetic beads removed.

3.3.2.5 E.Z.N.A tissue DNA kit

All DNA extracted from bird and invertebrate tissue was extracted using Omega bio-tek E.Z.N.A. tissue kit, following the manufacturer's protocol (August 2016 version, Product Manual D3396 Tissue DNA Kit Combo).

3.3.3 DNA amplification (PCR)

Universal invertebrate primers targeting the COX1 mitochondrial region were selected from the literature, and used in the pairings defined in Table 3.3, to amplify DNA. These

combinations were selected due to their ability to amplify likely invertebrate prey items in blue tit nestling diets.

mlCOIintF/jgHCO2198 and ZBJ-ArtF1-deg/mlCOIintF_revComp primers were modified to contain standard Illumina molecular identification tags (MIDs) and bridge sequences, as described in Kitson *et al.*, 2018. All other pairings, and ZBJ-ArtF1-deg/mlCOIintF_revComp in some instances, were tested in the untagged form with the sequences as written in Table 3.3.

Table 3.3: Descriptions of primers used, and the combinations used, to amplify invertebrate and bacterial DNA extracted from avian faecal samples during PCR.

Primer Name	Paired with	Sequence	Reference
mlCOIintF	jgHCO2198	GGWACWGGWTGAACWGTWTAYCCYCC	Leray et al. 2013
jgHCO2198	mlCOIintF	TAIACYTCIGGRTGICCRAARAAYCA	
mlCOIintF_revComp	ZBJ-ArtF1c or ZBJ-ArtF1c-deg	GGRGGRTAWACWGTTCAWCCWGTWCC	Modified from Leray et al. 2013
ZBJ-ArtF1c	ZBJ-ArtR2c or ZBJ-ArtR2c-deg	AGATATTGGAACWTTATATTTTATTTTTGG	Zeale et al. 2011
ZBJ-ArtR2c	ZBJ-ArtF1c or ZBJ-ArtF1c-deg	WACTAATCAATTWCCAAATCCTCC	
ZBJ-ArtF1c-deg	ZBJ-ArtR2c or ZBJ-ArtR2c-deg	AGATATTGGAACWTTATATTTTATHTTYGG	Modified from Zeale et al. 2011
ZBJ-ArtR2c-deg	ZBJ-ArtF1c or ZBJ-ArtF1c-deg	WACTAATCAATTWCCAAAHCCHCC	
LepF1	ZBJ-ArtR2c or ZBJ-ArtR2c-deg	ATTCAACCAATCATAAAGATATTGG	Brandon-Mong et al. 2015

PCR amplification was undertaken in 20 µL reactions, with a high fidelity Taq polymerase mix (either MyTaq Red HS Mix or MyFi Mix (BioLine, both at 2X dilution factor)), varying primer and magnesium concentration and template DNA (1 - 7 µL). The optimal conditions for each primer pair are described in Table 3.4.

PCR was undertaken on either an Applied Biosystems Veriti thermal cycler, or BIO-Rad T100 thermal cycler, but was consistent for each primer pairing after optimization.

Table 3.4: Optimal PCR chemistry and cycling conditions for each primer pairing.

Primer pairing	Magnesium concentration (mM)	Primer concentration (µM)	Cycles	Annealing temperature (°C)
mlCOIintF and jgLCO1490	2.0	0.6	45	51
ZBJ-ArtF1-deg (or ZBJ-ArtF1) and mlCOIintF_revComp ZBJ-ArtF1 and ZBJ-ArtR2 ZBJ-ArtF1-deg and ZBJ-ArtR2-deg LepF1 and ZBJ-ArtR2 (ZBJ-ArtR2-deg)	2.5	0.6	50	45

3.3.4 Gel electrophoresis

PCR products were visualized on 1.5% agarose gel, stained with ethidium bromide (3 µL/100 mL), under ultra-violet light, which had been run in 0.5x TBE buffer at 70 or 90V (dependent on the size of gel and tank being used) for 40 minutes. 5 µL of template DNA was used for visualization, along with bromophenol blue loading dye when MyFi Mix was used (1 µL/5 µL template). HS MyTaq Mix already contained loading dye, so no loading dye was added to template from reactions amplified with MyTaq. In each gel a DNA positive (labelled 'pos') and negative ('neg') are included to aid interpretation and to act as controls. The DNA positive shows successful DNA amplification with a band present in this lane and at the expected fragment length for the primers used. This acts as both a reference and a control to ensure that the PCR has been successful. The lane with the DNA negative should not have a band present, and is used as a control to ensure no contamination during PCR and PCR set-up. A DNA ladder (molecular weight marker) was also always included in the first lane of each gel, for reference, to establish the length of DNA fragment that is being expressed. EasyLadder I (Bioline) was used with each band representing 2000, 1000, 500, 250 and 100 base pairs (bp) from top to bottom of gel.

3.3.5 Sequencing

Although sequencing was originally planned to be undertaken, it was not possible within the timeframe of my PhD, due to issues with obtaining DNA and amplification success of suitable quality for NGS.

3.4 Results

696 blue tit nestling faecal samples were collected during 2016 and 2017 breeding seasons (334, and 362, respectively). Opportunistic collection of great tit (*Parus major)* nestling faecal sacs resulted in six faecal samples being collected over the two years. Collection dates varied by year, due to inter-annual phenological variation in nesting, with the mean sample collection date in 2017 being 10 days earlier than in 2016 (24th May and 3rd June, respectively, Figure 3.1). The earliest collection date in 2016 was 18th May, as opposed to 9th May in 2017, and the latest 15th June, 6th June in 2017 (Figure 3.1). Faecal samples were collected from blue tit nestlings from the day of hatching (referred to as day 1), until they were 18 days old (Figure 3.2), with 53% of faecal samples being collected from 13 to 15 day old chicks. Faecal samples ranged in weight from less than 0.01 g (marked as 0 g in Figure 3.3) to a maximum of 0.6g (mean 0.12 g), with samples in 2017, on average, being heavier than 2016 samples (0.15 and 0.08 g, respectively; Figure 3.3). Faecal sacs increased in weight with chick age (linear model: sample weight = 0.007 (± 0.0009) x chick age + 0.02 (± 0.01), $F = 75.32$, $df = 694$, adj. $R^2 = 0.10$, $p < 0.001$; Figure 3.4).

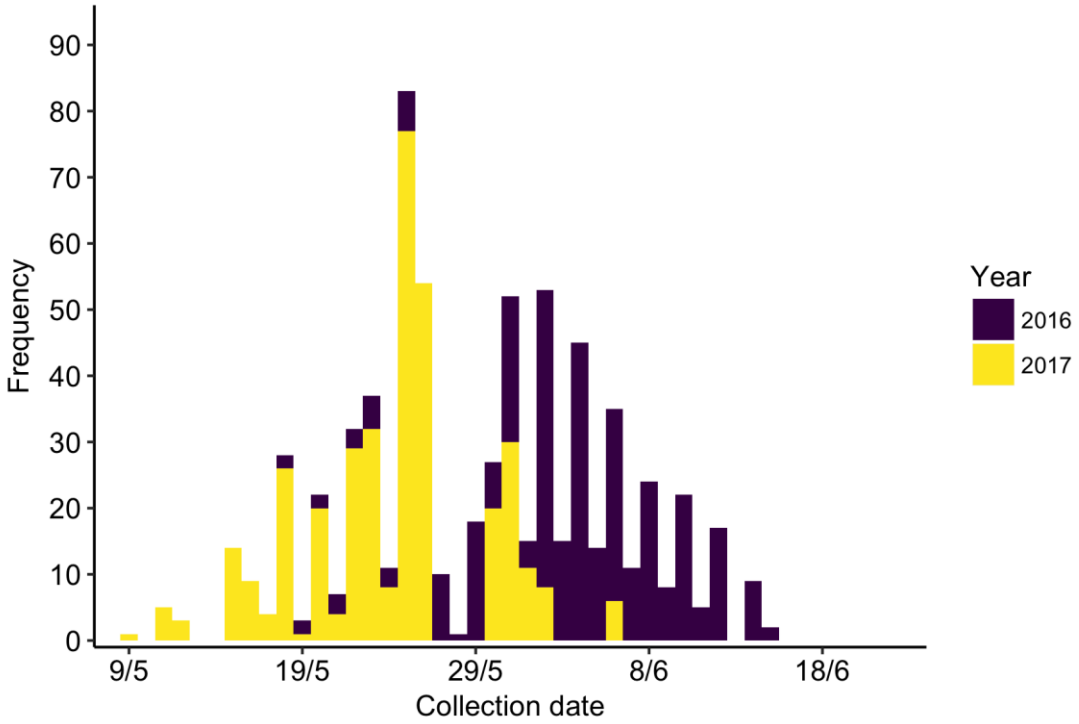

Figure 3.1: Number of samples collected on each calendar day (9th May until 15th June), per year.

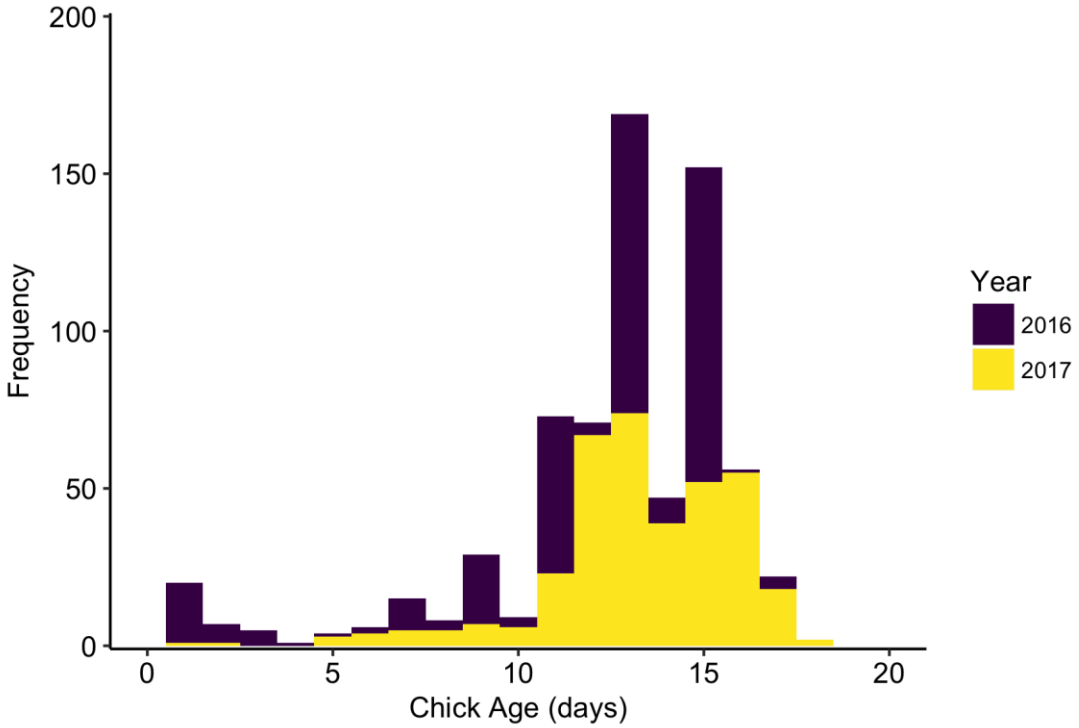

Figure 3.2: Total number of faecal samples collected at each different chick age (day 1 equals day of hatching) over the two collection years.

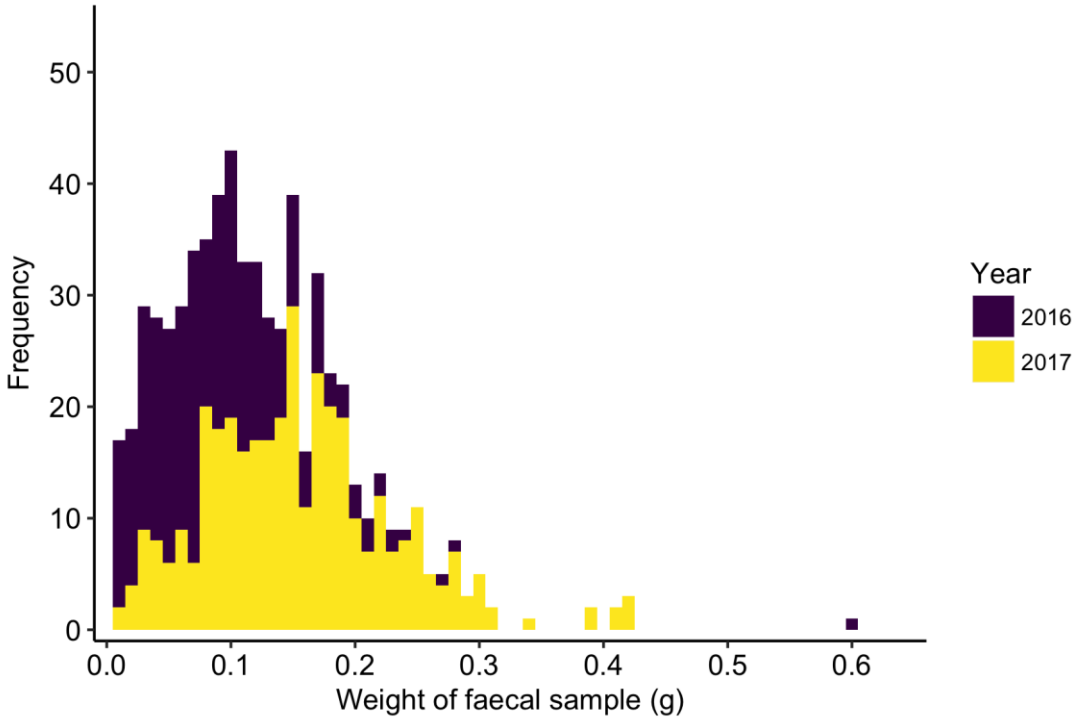

Figure 3.3: The number of blue tit faecal samples in each 0.01g weight class, over both years. The weight is a partially dry weight, after the sample had been incubated at 60°C for 30 minutes.

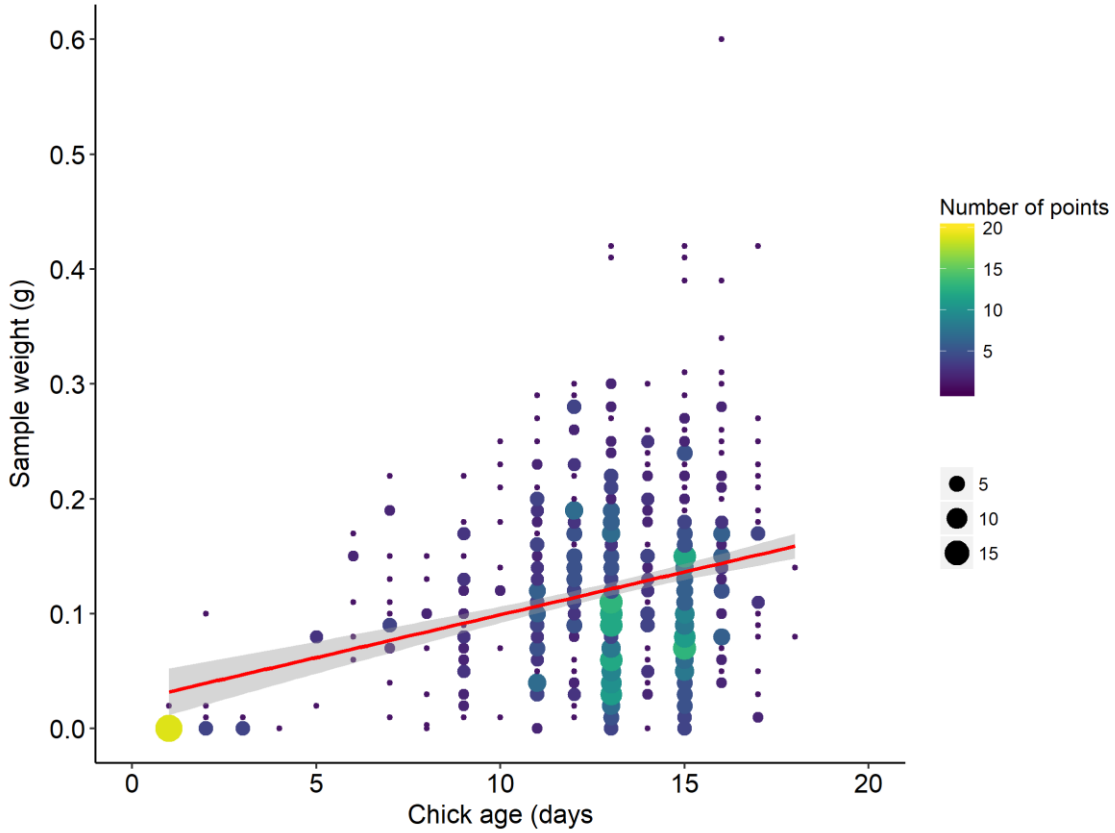

Figure 3.4: The relationship between weight of collected faecal sample and chick age (day 1 equals first day of hatching) at time of collection. The red line represents the fitted values from a linear model (weight = 0.007 (± 0.0009) x chick age + 0.02 (±0.01)), grey shaded area 95% confidence intervals, and the raw data plotted as points with size and colour denoting how many samples are at that position.

3.4.1 DNA extraction method

The chemical lysis extraction method had better success than the MO-BIO PowerSoil extraction kit, or any attempt to recreate/modify the protocol (Table 3.5). It is worth noting that during these tests each extraction was a separate faecal sac, as it was not possible to test more than two methods on the same faecal sac, so there could be unavoidable variation in extraction success due to this. Drying the faecal sac in the oven prior to DNA extraction did not improve DNA recovery, with approximately 60 % of samples amplifying successfully in each treatment (Table 3.5).

Table 3.5: A comparison of DNA extraction methods and modifications on DNA amplification success of blue tit faecal samples, using different universal invertebrate primer pairings.

Extraction method	Modification	Primer pairing amplification of blue tit faecal samples success rates (%)			
		ZBJ-ArtF1/ZBJ-ArtR2	mlCOlintF/ jgLCO1490	LepF1/ZBJ-ArtR2_deg	ZBJ-ArtF1_deg/mlCOlintF_revComp
Chemical lysis	Half volumes from DNA purification stage	< 1	-	-	-
	Eluted in 100 µL 10 mM tris hydrochloride	50	67	25	63
	1.5 mL lysis volume and eluted in 100 µL TE	< 1	-	-	-
	Increased inhibitor flocculant volume to 200 µL	-	67	-	-
	Addition of saturated potassium chloride as additional inhibitor removal step, eluted in 100 µL 10 mM tris hydrochloride	-	53	-	-
	Drying samples in oven before processing/not drying	-	Both 67	-	-
	SPRI bead clean up on extracted template		79		
MO-BIO PowerSoil extraction kit	Chemical lysis solutions following PowerSoil protocol throughout and standard solutions for DNA purification	33	-	-	-
	Chemical lysis solutions for DNA purification steps, following PowerSoil protocol throughout and standard solutions for lysis	< 1	-	-	-

Increasing the amount of inhibitor flocculant solution added in the chemical lysis extraction method did not affect amplification success of blue tit faecal samples, but amplification was weaker (Table 3.5), likely due to doubling elution buffer volume (and therefore halving DNA concentration).

Further inhibitor removal, by undertaking a second DNA extraction, post chemical lysis extraction, using SPRI beads, increased amplification success of DNA extracted from blue tit nestling faecal sacs from approximately 60% to 79% (Table 3.5).

The success of DNA extraction methods varieed between species (Table 3.6). Enzymatic lysis of great tit faecal samples did not produce amplifiable DNA (Table 3.6). The addition of saturated

potassium chloride solution, as an additional inhibitor removal step in the chemical extraction method, and decreasing elution volume (from 313 µL to 100 µL) produced amplifiable DNA in meadow pipit nestling faecal samples (Table 3.6). However, when this modification was tested on blue tit faecal samples amplification success was much lower than obtained with meadow pipit and the original unmodified chemical lysis extraction method on the same blue tit samples (Table 3.6).

Table 3.6: DNA amplification success of faecal samples from three nestling bird species extracted using two extraction methods, with or without modifications.

Extraction method	Species	Modification to extraction protocol	Amplification success (% samples) with mICOlintF/ jgLCO1490
Chemical lysis	Meadow pipit	None – eluted in 313 µL of TE	60
	Blue tit	Potassium chloride added to lysate and eluted in 100 µL tris hydrochloride	100
		None – eluted in 100 µL of TE	71
		Potassium chloride added to lysate and eluted in 100 µL tris hydrochloride	38
Proteinase K digestion and chemical lysis DNA purification	Great tit	None	<1

Faecal samples from captive adult starlings, with known diets, amplified more successfully than blue tit samples with 86% and 66% amplification success rate, respectively, when extracted using the same extraction protocol . There was no difference in amplification success rates of starling faecal samples extracted using G. Sellars modified MO-BIO PowerSoil extraction protocol (supplementary material 3.6.1) or the chemical lysis method (Table 3.7). Starling samples lysed in a total volume of 3 mL had the best amplification success across all weight categories, in comparison to lysis in 2 and 3.5 mL (Table 3.8). Heavier faecal samples (0.4 g), when lysed in 3 mL, failed to amplify more often than lighter samples (<0.01 and 0.05 g, 0.2 g) with 67% and 100% amplification success rate, respectively (Table 3.8).

Table 3.7: Amplification success of DNA extracted from adult starling faecal samples using the original modification of the MO-BIO PowerSoil extraction kit (by G. Sellers) and the modified chemical lysis method.

Sample weight (g)	Amplification success (% samples amplified) with mICOIintF/ jgLCO1490 with each extraction method	
	G. Sellers modified MO-BIO PowerSoil extraction protocol	Chemical lysis
< 0.01	100	100
0.05	100	100
0.2	100	100
0.4	67	67

Table 3.8: Testing the interaction of lysis volume and sample weight on DNA amplification success of faecal samples from captive adult starlings.

Sample weight (g)	Amplification success (% samples amplified) with mICOIintF/ jgLCO1490 with different lysis volumes (mL)		
	2	3	3.5
< 0.01	67	100	67
0.05	100	100	100
0.2	100	100	67
0.4	100	67	67

3.4.2 DNA amplification (PCR)

3.4.2.1 mICOIintF and jgLCO1490

DNA amplification was stronger, at all primer and magnesium concentrations, when DNA was eluted in water rather than TE. Primer concentration was more important for DNA amplification than magnesium concentration, with high primer concentrations giving stronger amplification, but also non-specific binding (especially in samples eluted in TE) with the production of additional fragments around 1000 base pairs. The optimal chemistry for PCR was 2.5 mM magnesium and 0.6 µM primer concentration. The optimal annealing temperature was 51°C, consistent with Kitson et al., 2018, when all other factors remained constant

mICOIintF and jgLCO1490 strongly amplified all bird DNA tested (Table 3.9). Amplification efficiency was stronger and cleaner in PCRs conducted with MyFi, as opposed to MyTaq, under optimal conditions, and as such was preferentially used.

3.4.2.2 ZBJ-ArtF1 and ZBJ-ArtR2

The ZBJ-ArtF1/ZBJ-ArtR2 primer pairing was more sensitive to magnesium concentration than primer concentration with optimal DNA amplification at 2.5mM magnesium and 0.6 µM primer concentration. This primer pairing did not amplify any bird DNA and only amplified DNA from Planorbidae spp. and Lepidoptera species (Table 3.9).

Table 3.9: Amplification success or failure of each bird or invertebrate species with each primer pairing tested.

	Species	Primer pairing			
		mICOIintF/ jgLCO1490	**ZBJ-ArtF1/ZBJ-ArtR2**	**LepF1/ZBJ-ArtR2(_deg)**	**ZBJ-ArtF1/mICOIintF_revComp**
Birds	Kingfisher (*Alcedo atthis*)	✓	×	✓	✓
	Great tit (*Parus major*)	✓	×	×	×
	Coal tit (*Periparus ater*)	✓	×	×	×
	Puffin (*Fratercula arctica*)	✓	×	×	✓
Terrestrial invertebrates	Geometridae larvae	✓	✓	✓	✓
	Common wave (*Cabera exanthemata*)	✓	✓	✓	✓
	Silver ground carpet (*Xanthorhoe montanata*)	✓	✓	✓	✓
	Tipulid spp.	✓	✓	✓	✓
Aquatic invertebrates	Blue mussel (*Mytilus edulis*)	✓	×	×	×
	Planorbidae spp	✓	✓	✓	✓

3.4.2.3 LepF1/ZBJ-ArtR2 (and ZBJ-ArtR2-deg)

LepF1 when paired with ZBJ-ArtR2 amplified DNA most successfully at intermediate magnesium concentration (2.5 mM), and mid to high primer concentrations (0.4-0.6 µM), with high magnesium concentration (3 mM) inhibiting DNA amplification. This primer pairing amplified a wide range of invertebrate DNA but did not amplify bird DNA well (Table 3.9).

3.4.2.4 ZBJ-ArtF1-deg (or ZBJ-ArtF1)/mICOIintF_revComp

Pairing ZBJ-ArtF1-deg and mICOIintF_revComp at 0.6 µM concentration and 2.5 mM magnesium concentration with MyFI Mix and 4 µL of template DNA produced a clean reaction with invertebrate DNA. When tested further, no improvements to amplification strength or specificity could be made by modifying either annealing temperature and/or annealing time.

This pairing amplified all moth species tested (unknown Geometridae larvae, common wave adult and silver ground carpet adult) but did not amplify bird DNA well, in comparison to the other primer pairings previously tested (e.g. mICOIintF and jgLCO1490,Table 3.9).

The addition of unique identification tags to primers, to allow nested metabarcoding, caused disruptions in amplification with variation in amplification success dependent on tag combination (Figure 3.5).

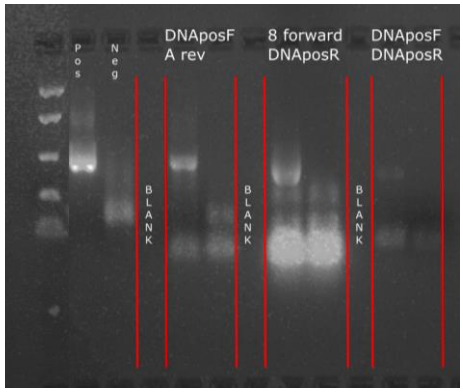

Figure 3.5: Amplification success of DNA extracted from carpet wave moth, with untagged primers (pos) and different combinations of tagged primers.

3.4.3 Comparing amplification success rates of DNA from blue tit faecal sacs with different primer pairings

As all primer pairings successfully amplified a variety of Lepidopteran species, the amplification success of DNA extracted from blue tit nestling faecal sacs could be compared. mICOIintF and jgLCO1490 amplified DNA from blue tit nestling faecal sacs the best out of all combinations tried, with 79% of faecal samples tested amplifying successfully (Table 3.5). However, mICOIintF and jgLCO1490 also strongly amplified bird DNA as well (Table 3.9). No other primer pairing tested amplified bird DNA as readily as mICOIintF and jgLCO1490 (Table 3.9).

ZBJ-ArtF1/ZBJ-ArtR2 and mICOIintF/jgLCO1490 exhibited similar amplification success rates during small scale tests (Table 3.5) but amplification success rates dropped during wider testing. No improvement in amplification with ZBJ-ArtF1/ZBJ-ArtR2 was observed when template DNA volume was reduced (Figure 3.6 and Figure 3.7). Amplification success of DNA from blue tit faecal sacs was only 25% with LepF1/ZBJ-ArtR2-deg (Table 3.5). During small scale tests, ZBJ-ArtF1-deg (or ZBJ-ArtF1)/mICOIintF_revComp showed higher amplification success than LepF1/ZBJ-ArtR2-deg (Table 3.5), but the addition of unique identification tags to ZBJ-ArtF1-deg (or ZBJ-ArtF1)/mICOIintF_revComp caused amplification success to reduce to 12.5%.

Due to spending extended time testing and refining DNA extraction methods, testing primer pairings and none of the primer pairings that did not amplify bird DNA giving satisfactory levels of DNA amplification, sequencing was not undertaken.

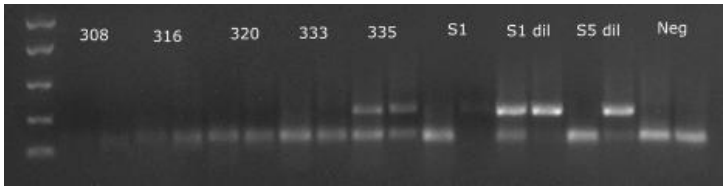

Figure 3.6: Visualising the effect of reducing template DNA volume (2 and 1 µL on left and right of each sample pair, respectively) on amplification success of DNA from blue tit nestling faecal sacs and amplified with ZBJ-ArtF1/ZBJ-ArtR2 primers at 0.4 µM concentration and 3.0 mM magnesium concentration. EasyLadder I is in the first well for reference.

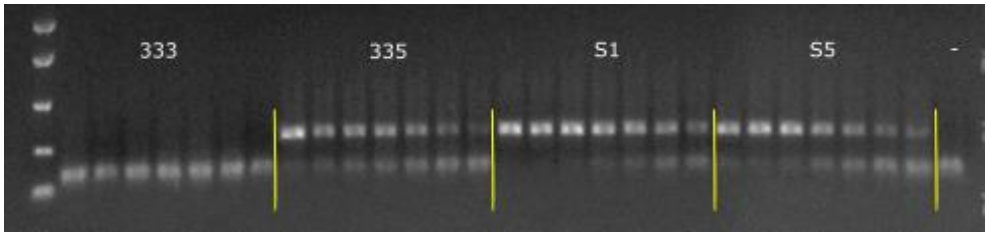

Figure 3.7: Serial dilutions (1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64, respectively) of DNA from two blue tit faecal sacs (333 and 335, diluted with TE) along with DNA from Planorbidae spp. eluted in TE (S1) and H_2O (S5). PCR chemistry was as follows: 2 µL of template DNA, and ZBJ-ArtF1 and ZBJ-ArtR2 at 0.4 µM, with 3mM magnesium concentration. EasyLadder I is in the first well, for reference.

3.5 Discussion

The results I have presented here highlight the difficulties of trying to obtain high quality DNA and high DNA amplification success from faecal samples, both of which are essential pre-requisites for NGS. I extensively tested DNA extraction methods and PCR modifications, such as primer choice and cycling conditions, to maximise amplification success.

3.5.1 Sample collection

Faecal sample collection demonstrated a peak in collection date each year (Figure 3.1), with samples being more readily obtained from older chicks (Figure 3.2). This will need to be considered, in the future, when interpreting sequencing results as the dietary requirements of chicks may differ with age (Betts, 1955), but younger chicks will not be as well represented as older chicks in this dataset. Therefore, the species richness of younger chick's diets may not highlight all important taxa.

3.5.2 DNA extraction method

The MO-BIO PowerSoil extraction kit did not reliably provide amplifiable DNA for PCR from the blue tit faecal samples, and as such was modified to improve results (Table 3.5). There was no difference in DNA extraction success between the original modifications to the MO-BIO PowerSoil protocol (supplementary material 3.6.1) and the chemical lysis method (Table 3.7). Due to the likelihood that DNA in faecal samples was present in low concentrations, further modifications were made to try to concentrate extracted DNA. Modifications such as eluting in smaller volumes and lysing in smaller volumes did not provide an improvement in DNA amplification success rates (Table 3.5). When concentrating DNA during extraction any inhibitors which are present in the sample, and may co-elute with DNA (such as polysaccharides, which behave like DNA (Schrader et al., 2012)), will simultaneously be concentrated.

An oversight in the chemical lysis extraction protocol for 2016 samples was to elute DNA in TE buffer (tris hydrochloride and EDTA). EDTA prevents DNA degradation during storage, but reduces DNA amplification success during PCR by chelating metal ions (magnesium, in particular), which stabilise DNA and are cofactors for polymerase enzymes (Rossen et al. 1992). The protocol was modified for samples processed in 2017, and all samples were eluted in 10 mM tris hydrochloride instead.

Prior to extraction, removal of storage ethanol is required as ethanol carried through to PCR can reduce, or inactivate, polymerase enzymes (Rossen et al., 1992). There was no difference in amplification success between samples which had been dried at 60°C, to further remove any

residual ethanol, or processed without drying (Table 3.5). This suggests that ethanol was not a likely cause of inhibition in these samples, but as a precaution all samples were dried at 60°C prior to DNA extraction.

Inhibitors can be removed in a number of ways during DNA extraction, such as through silica spin columns, chemical flocculation and/or enzymatic break down (Schrader et al., 2012). Here, I tested whether silica spin columns, chemical flocculation and/or enzymatic lysis could remove inhibitors sufficiently so that amplifiable DNA could be obtained from nestling faecal sacs. Silica spin columns have been shown to remove inhibitors co-extracted with DNA during chloroform extractions (Kemp et al., 2006), and are often used in commercial extraction kits designed for inhibitor rich samples (such as MO-BIO PowerSoil extraction kit), and as such spin columns were used in every DNA extraction method tested here. Chemical flocculation successfully removes inhibitors present in soil samples and is routinely used in drinking water purification (Braid et al., 2003). As some inhibitors found in water samples are also likely present in faecal samples chemical flocculation should improve amplifiable DNA recovery in faecal samples. Increasing the volume of inhibitor flocculant, to facilitate the removal of more inhibitor molecules, and simultaneously increasing elution volume, to dilute inhibitors carried through with eluted DNA, had no effect on PCR amplification success of the blue tit samples (Table 3.5). These two modifications reduced the strength of the bands when visualized on an ethidium bromide gel, which represent amplified DNA, due to concurrently diluting inhibitors and DNA. Therefore, an increased volume of inhibitor flocculant was used, but with a reduced elution volume for all faecal samples collected in 2017, and is recommended when using this method. Enzymatic cell lysis, using proteinase K, instead of chemical lysis can reduce inhibitor activity, when coupled with effective DNA purification (An and Flemming, 1991). However, using enzymatic cell lysis and DNA purification steps from the chemical lysis method, gave no detectable DNA from great tit nestling faecal sacs (Table 3.6). This could possibly have been due to enzyme inactivation by either storage ethanol or an inhibitor present in the samples, preventing lysis from occurring (Rossen et al., 1992).

Polysaccharides are a common inhibitor present in faecal samples (Monteiro et al., 1997; Schrader et al., 2012), and behave in a similar way to nucleic acids (Monteiro et al., 1997; Peist et al., 2001; Schrader et al., 2012), with their concentration varying with time, even within the same individual due to variation in diet (Monteiro et al., 1997). A membranous sac encases nestling excrement, containing both faecal matter and uric acid, to facilitate the removal of excrement from the nest (Herrick, 1900; Weatherhead, 1984). The membranous sac itself may be comprised of polysaccharides (visual observation during DNA extraction and J. Nicholls, pers. comm. 2018). Satisfactory levels of amplification have been reported in blue tit nestling

faecal samples when the sac was removed prior to extraction (J. Nicholls pers. comm., 2018), suggesting the sac itself could be a potential source of inhibition. The storage method of the samples used in this study prevented this modification being tested, as faecal sacs were homogenised upon collection in an effort to prevent DNA degradation. Due to polysaccharides behaving in a similar way to nucleic acids (Schrader et al., 2012) they can compete with nucleic acids during extraction and be co-eluted with, or even instead of, DNA. Molluscan and plant tissue are rich in polysaccharides, and as such suggestions of methods to extract amplifiable DNA, whilst removing polysaccharides, are common in these fields (e.g. Sharma et al., 2002; Sokolov, 2000; Souza et al., 2012; Wang and Stegemann, 2010). Phenol-chloroform and cetyl trimethylammonium bromide (CTAB) extractions are often used for inhibitor rich samples (Schrader et al., 2012). However, phenol-chloroform extractions require the use of hazardous chemicals, and are not particularly suited to large scale studies, and as such this method was not used. DNA extracted using the CTAB method can be degraded during the extraction process (Fang et al., 1992), which could negate any benefits of inhibitor removal in faecal samples where DNA is already likely highly degraded. Another method for removing polysaccharides is through the addition of a saturated potassium chloride solution to lysates, to precipitate polysaccharides and proteins from the sample after cell lysis and before DNA elution (Sokolov, 2000). Removing polysaccharides prior to eluting DNA is preferable, due to polysaccharides behaving in a similar way to nucleic acids (Schrader et al., 2012). When tested with faecal samples, the addition of potassium chloride restored amplification of DNA extracted from meadow pipit nestling faecal sacs (Table 3.6). However, this approach reduced DNA amplification success in blue tit nestling faecal samples, with reduced amplification success observed in comparison to the chemical lysis extraction method (Table 3.5). This suggests that it was concentrating DNA, not additional inhibitor removal, which restored amplification in the meadow pipit samples. Being unable to restore amplification of DNA in both species tested highlights the differences encountered when extracting DNA from different species faeces, and that a one-size-fits all extraction approach is not possible. It also further suggests that inhibitors could be originating from specific prey items present in blue tit nestling diets, but not in meadow pipit, or variation in digestion physiology between the two species could be responsible for differing success between species.

Blue tit faecal samples varied in weight from <0.01 up to 0.6 g (Figure 3.3), and faecal sac weight was positively related with age (Figure 3.4). Therefore, it was essential to ensure that the extraction protocol was optimised for all possible weights. Modifying lysis volume, and therefore diluting inhibitors, was tested using faecal samples from adult starlings. DNA from adult starling faeces amplified more successfully, at all weights and lysis volumes, than DNA from blue tit nestling samples. Only heavy samples (0.4 g or 0.2 g) lysed in large or small

volumes (3 ml or 2 ml), exhibited reduced amplification success (Table 3.8). This suggested lysing in 2.5 ml total volume sufficiently diluted any inhibitors present whilst maintaining DNA in a high enough concentration for amplification. The high DNA amplification success of adult starling faeces further suggests that the reduced success in blue tits may be due to prey items consumed or nestling physiology.

A secondary DNA extraction can also further remove inhibitors which have been carried through the first extraction (e.g. Kemp et al., 2006). This presents increased costs (in both time and money), but can be the only solution for extremely complex samples, such as the blue tit nestling faecal sacs. SPRI beads are ideal in this situation as they reversibly bind DNA leaving inhibitors to be discarded and DNA eluted in a smaller volume to further concentrate extracted DNA. DNA from blue tit faecal samples that were extracted using the chemical lysis extraction method and underwent a secondary extraction using SPRI magnetic beads exhibited increased DNA amplification (Table 3.5), suggesting SPRI beads successfully removed inhibitors which had been co-eluted with DNA in the first extraction and further concentrated DNA. This secondary extraction method could be developed further (as in Vo and Jedlicka, (2014)) to be used as a single extraction method, maintaining the benefits for recovery of amplifiable DNA, but reducing the incurred time and monetary costs.

3.5.3 DNA amplification (PCR)

mICOIintF and jgLCO1490 primer pairing amplified 79% of nestling blue tit faecal samples, after undergoing a second extraction with SPRI beads, the highest success rate of all the primer pairings tested (Table 3.5). However, this pairing is a universal primer which amplifies all phyla, apart from Entoprocta (Leray et al., 2013), and successfully amplified all bird DNA tested (Table 3.9). The high amplification success with mICOIintF and jgLCO1490 could be due to blue tit DNA, which is likely to be present in most samples, being amplified over prey DNA.

Amplification success with other primer pairings, which did not readily amplify bird DNA, such as ZBJ-ArtF1/ZBJ-ArtR2, LepF1/ZBJ-ArtR2 and ZBJ-ArtF1-deg/mICOIintF_revComp (Table 3.5) may be more representative of faecal sacs that contain prey DNA, or these primers may be more sensitive to inhibitors than mICOIintF/jgLCO1490. Prey DNA concentration has been shown to naturally vary within excrement and can be absent altogether when chicks are undergoing periods of fasting, as has been demonstrated in shy albatross (*Thalassarche cauta*) (McInnes et al., 2017). This may offer an alternative explanation for why amplification success exhibits no discernible patterns with primers that do not amplify bird DNA. However, chicks did not appear to be routinely starving, although brood reduction was common in most broods and faecal samples may have been obtained from these individuals, which may not have been

fed as frequently. A further set back with using ZBJ-ArtF1-deg/mICOIintF_revComp primer pairing was due to an apparent effect of the addition of unique MIDs and bridge sequences on amplification success (Figure 3.5), suggesting the unique MIDs were interacting and inhibiting amplification during PCR when used in certain combinations.

3.5.4 Future work

There is still additional work that could be undertaken to improve DNA extraction and amplification success, prior to sequencing, outlined here.

DNA amplified with mICOIintF and jgLCO1490 could be sequenced as is, despite the likelihood a large proportion of reads would be blue tit. These effects could be minimised by using a sequencing platform with increased read depth (i.e. Illumina HiSeq as opposed to MiSeq) which may provide sufficient prey reads, in addition to blue tit reads, to elucidate trophic interactions. Even a sub-optimal approach would provide an advancement in knowledge, as to date NGS has not been used in this system, apart from to describe adult pre-breeding diet (Shutt, 2017). To try and maximise prey reads during sequencing of DNA amplified by mICOIintF/jgLCO1490, SPRI beads, used here in secondary DNA extraction, could be diluted to a lower concentration, therefore only allowing a predetermined amount of DNA to bind regardless of DNA concentration in the starting mixture This would normalise the amount of DNA from each sample that makes up each library (Hosomichi et al., 2014). Reducing the variation in DNA concentration between each sample within each library, may prevent prey reads being swamped during sequencing when bird reads are the dominant signal. Alternatively, blue tit blocking primers could be used, in addition to universal invertebrate primers, to prevent amplification of blue tit DNA whilst still allowing amplification of prey DNA during PCR (e.g. Vestheim and Jarman, 2008).

New invertebrate primers could be designed, or sourced from the literature (e.g. Folmer et al., 1994), that are likely to amplify prey items found in blue tit diets, but do not amplify bird DNA, and these primers tested to see if amplification of DNA from faecal samples improves. Testing primers that amplify a different locus e.g. 16S may also improve amplification success. However, using a different locus may not provide as much species level detail as COX1 due to a shorter barcoding region making species discrimination harder than with COX1 primers

If low DNA concentration in extractions is what is preventing amplification, whole genome amplification could be undertaken to improve DNA quality, and concentration, prior to amplification with invertebrate primers (Cheung and Nelson, 1996).

Modifications to the faecal sac collection protocol could be made to try to reduce inhibitors at this stage. As previously discussed, the membranous sac itself may be a source of polysaccharides, and other PCR inhibitors, as well as likely containing a large amount of bird DNA. The faecal sac could be dissected and removed at time of collection to try and circumvent this. However, this does increase the risk of contamination.

Finally, to further establish the effects of fasting and diet composition on amplifiable DNA recovery, feeding experiments on captive birds could be undertaken. In addition, the time it takes for prey items to be detected in faecal samples after consumption could also be established using this approach and could then be translated into field studies. This would allow prey phenology in the nesting environment to be deduced from faecal samples.

3.5.5 Conclusion

Using NGS to elucidate the diet of nestling blue tits still remains a promising approach, and would shed light on the trophic interactions which these birds depend on during the breeding season. Here, I have shown that despite faecal samples being hostile environments, with degraded DNA and high concentrations of inhibitors, DNA of sufficient quality for amplification by PCR can be extracted. However, the methods presented may not be directly applicable (without modification) from one species to another.

3.6 Supplementary material

3.6.1 Modified MO-BIO PowerSoil extraction protocol (G. Sellers)

Described here is the original chemical lysis extraction protocol, based on MO-BIO PowerSoil DNA extraction kit, devised by Graham Sellers. All extraction buffer components are described in Table 3.1, and referred to by names stated in the table throughout.

3.6.1.1.1 Cell lysis:

Samples are processed dry and volumes are based upon 1 g of starting material. Dried samples are added to a 5 mL Eppendorf tube, prefilled with 2 g of sterile garnet (1-1.4 mm diameter) and shaken briefly. 2200 µL of lysis solution one added and mixture briefly vortexed, followed by 800 µL of lysis solution two and the sample ground using appropriate apparatus (e.g. SPEX SamplePrep Geno/Grinder 2010 at 1750 RPM for two minutes). This mixture is then centrifuged (Thermo Scientific Heraeus megafuge 40R) at 4,000 xg for 1 minute at room temperature. Supernatant is then transferred to a clean tube, centrifuged (10,000 xg for 1 minute – these conditions from now on) and supernatant removed.

3.6.1.1.2 DNA purification:

500 µL of supernatant is carried through for purification at all stages, however this volume can be modified, and all subsequent solution volumes should be modified accordingly if the starting volume is different. 200 µL of protein flocculant is added to the removed supernatant, briefly vortexed, and incubated on ice for a minimum of 10 minutes. The mixture centrifuged, supernatant removed and placed in a fresh tube with 125 µL total volume of a 1:1 mix of inhibitor flocculant 1 and 2, the mixture vortexed and incubated on ice for a minimum of 10 minutes. The mixture centrifuged and supernatant transferred to a new 2 mL tube with 1000 µL of binding solution. This solution is used to fill a silica spin column to capacity, which is centrifuged and flow through discarded, with this step repeated until all the binding mixture had passed through. 375 µL of wash solution is added directly to the silica membrane and after being centrifuged, flow through discarded and centrifuged again to dry the silica membrane. A new collection tube is used and 300 µL of elution buffer, applied directly to the silica membrane, centrifuged and DNA is now in the collection tube.

Chapter 4: The effect of climate and habitat on blue tit phenology in the United Kingdom

4.1 Abstract

Phenological synchrony between trophic levels in food webs is crucial to maximise fitness of higher trophic levels. The tri-trophic deciduous tree-herbivorous caterpillar-insectivorous bird food chain has become a model ecological system for investigating the impacts of climate change on phenology. Understanding the cues blue tits (*Cyanistes caeruleus*) use to correctly time their breeding is of great value in informing predictions on how bird species may fare in a changing climate. To date, the effect of climate on insectivorous birds' phenology has largely been investigated without considering the effects of habitat. For insectivorous birds, nesting habitat defines the food resources available for chicks later in spring. Consequently, if warming springs affect the temporal patterns of food availability, differently across habitats, the impacts of climatic change may operate differently among habitats. Blue tits are thought to optimise their breeding to coincide with peaks in their main prey the winter moth caterpillar (*Operophtera brumata*). I hypothesise that, after controlling for effects of spring temperature and latitude, birds nesting in areas with a high proportion of early leafing tree species would have early breeding phenology and vice versa. I use data from 34 long-term nest-recording sites across the UK to test this hypothesis.

I found there has been a significant temporal advancement in both first egg and hatching date, since 1980, with blue tits at these sites laying their first eggs and hatching approximately 10 and 7 days earlier, respectively. I found that the main driver in the advancement of breeding phenology, was mean spring temperature. There was no evidence for differences in blue tit breeding phenology in relation to local tree composition, i.e. there was no fine tuning of breeding to local tree phenology. This suggests that birds are using temperature cues as the proximate cue to time breeding, and not local habitat cues that may indicate when the local peaks in food are likely to occur. The lack of association between bird breeding phenology and habitat, suggest mismatch could become a problem. This will be especially problematic if resource peaks differ between habitats, as birds do not appear to be adapted to local habitat phenology, especially as birds and caterpillars already differ in their responses to climate change.

4.2 Introduction

Phenology is defined as the timing of cyclic or periodic events. In living organisms this is usually in relation to the timing of cyclic life events, such as migration or reproduction, of which many coincide with meteorological seasons. Phenology is often finely tuned such that organisms can exploit seasonal pulses in resources, which may only occur for a short period of time , and/or in a specific location, and are characteristic of seasonal environments. For example the pied flycatcher (*Ficedula hypoleuca*) breeds in temperate Europe during spring, exploiting food resources and a moderate climate in which to reproduce, before migrating to Africa for the non-breeding season (Lundberg and Alatalo, 2010).

In recent decades the phenology of many organisms has been changing (Walther et al., 2002), and in general spring activities such as breeding (e.g. Crick et al., 1997; Moyes et al., 2011), return of migrant species to their breeding grounds (e.g. Jonsson and Jonsson, 2009) and emergence of butterflies (Dell et al., 2005) have all advanced. Many of these phenological changes have been shown to be strongly temperature dependent and attributable to changes in climate (Thackeray et al., 2016; Walther et al., 2002). Similarly, the breeding phenology of many avian species has advanced in recent decades (Crick et al., 1997; McCleery and Perrins, 1998) and correlates negatively with temperature (McLean et al., 2016).

Blue tits (*Cyanistes caeruleus*) are a small resident passerine species, with a distribution limited to the Western Palearctic (Birdlife International, 2014; Stenning, 2018). They are an obligate secondary cavity nesting species and are therefore reliant on the presence of natural cavities, such as disused woodpecker cavities or natural tree cavities, in which to breed (Perrins, 1979). Blue tits have received a lot of research attention due to their abundance and willingness to inhabit man-made nest boxes (Perrins, 1979), making it easy to monitor many aspects of their life cycle.

Blue tits are a relatively short lived species, typically only living for three years, and breeding in their first year (Robinson, 2018). They are usually single brooded (Perrins, 1979), meaning the correct timing of breeding is vital. During the breeding season blue tits are insectivorous (Perrins, 1991), and predominantly feed young on Lepidopteran larvae, typically winter moth (*Operophtera brumata)* larvae, and also other invertebrates including spiders and flies (Cowie and Hinsley, 1988; García-Navas and Sanz, 2011; Serrano-Davies and Sanz, 2017). Winter moth are believed to be the preferred prey item and nestlings fed a higher proportion of winter moth larvae are generally in better condition at fledging (Wilkin et al., 2009). Winter moth larvae are only available for a short period during spring, with larvae feeding on newly emerged leaves of deciduous trees, predominantly oak (*Quercus spp.)* but occurring in lower numbers on other

species (Wint, 1983). Pupation takes place in the ground, at which point caterpillars become unavailable to foraging blue tits. Peak nestling demand for food must therefore coincide with the peak availability of winter moth caterpillars, and for this to occur timing the onset of breeding is crucial.

Blue tits typically lay one egg per day and begin incubation after the penultimate or final egg, to ensure synchronous hatching. Incubation typically lasts 13 to 15 days (Perrins, 1979; Robinson, 2018; Stenning, 2018) and nestlings usually hatch over one or two days. The mean clutch size in the UK is nine eggs (min-max, 2-16; Robinson, 2005), requiring adult birds to correctly predict, approximately 30 days in advance, when the peak in prey availability will occur. Once birds have begun laying eggs an opportunity remains to advance or delay hatching through a number of mechanisms. This may be required if their initial timing was sub-optimal or the environment changes after the onset of egg laying. Egg pauses, where an egg is not laid for one or more days after the first or subsequent eggs have been laid, can be used to delay breeding. Delaying or pausing during incubation, after clutch completion, is another mechanism to delay hatching. In contrast, birds can advance hatching by reducing their clutch size or by varying attentiveness during incubation.

Adult blue tits have been shown to use a number of cues to initiate breeding, and to attempt to predict when the peak invertebrate availability will occur (Visser and Lambrechts, 1999). The proximal cue for the onset of breeding is thought to be the change in day length which triggers slow and then rapid gonadal growth (Visser and Lambrechts, 1999). The rate of rapid gonadal growth increases with increased ambient air temperature during the onset of spring, the latter also acts as a predictor for the emergence of Lepidopteran larvae (Salis et al., 2016). There are a number of environmental cues that adult birds could use to fine tune breeding phenology, following the initial physiological response. Such cues might include local tree phenology and food availability at the time of laying. The strength of these cues, and the species they use as a cue, varies by population and location. For example, the phenology of great tits (*Parus major*) in Norway is closely correlated with bud-burst in birch trees (*Betuala spp.*; Slagsvold, 1976) where as in England and Holland breeding phenology is closely correlated with oak bud-burst or leafing (Burgess et al., 2018; Cole et al., 2015; Visser and Lambrechts, 1999). In the UK, oak forms 18% of 1.3 million hectares of broadleaved woodland (Forestry Commission, 2013) and is therefore likely to be vital to woodland birds. However, birch also covers a similar area to oak, with much of the woodland in the UK being a mix of broadleaved species (Forestry Commission, 2013). Assessing the importance of these species in providing food, and therefore cues, to breeding birds can help to inform future woodland management.

The tri-trophic deciduous tree-herbivorous caterpillar-insectivorous bird system, where blue tits are secondary consumers, has become a model ecological system for investigating the impacts of changing climate and phenology. In general, most long-term phenological studies of blue tits (and great tits, a sympatric species, with similar ecology), have demonstrated advancements in first egg date (FED) in relation to warming spring temperatures (Dolenec, 2007; Matthysen et al., 2011; Perrins, 1991; Potti, 2009; Sanz, 2002; Thorley and Lord, 2015). With this advancement comes the risk of trophic mismatch, where one trophic level advances and lower level/s either do not change, or change at a different rate, resulting in an uncoupling of a once synchronous relationship (Durant et al., 2007). If the lower two trophic levels, in this tri-trophic system, do not advance, or advance at a different rate, blue tits may no longer be able to exploit the best food resource for their chicks (Buse et al., 1999; Wilkin et al., 2009). This could result in prey switching to a sub-optimal prey item and could result in lower productivity (Buse et al., 1999; Wilkin et al., 2009). With elevated spring temperatures, caterpillar development will also occur more rapidly (Buse et al., 1999), as well as advancing the phenology of oak trees (Tansey et al., 2017). To date, different populations have exhibited differing evidence of mismatch, even within the same country, between trophic levels. Some populations in the UK (Charmantier et al., 2008; Cresswell and Mccleery, 2003) and Belgium (Matthysen et al., 2011) are maintaining synchrony with the peak in caterpillar abundance. However, in the Netherlands, and in a nationwide study in the UK, some populations are not maintaining synchrony across the trophic levels (Both et al., 2009; Burgess et al., 2018; Visser et al., 1998, 2006).

To minimize the potential for mismatch with local resources, it would be expected that birds would fine tune their breeding phenology to match local tree and caterpillar phenology. As the winter moth is polyphagous (Wint, 1983), blue tits could feasibly use a number of deciduous tree species as cues to time their breeding. Those few studies that have considered habitat cues mainly focused upon differences between deciduous versus coniferous woodlands, finding mixed evidence that birds are using local habitat cues (Amininasab et al., 2016; Blondel et al., 1993). Experimentally, the presentation of leafing branches earlier, to represent advancement in spring phenology, had no impact on FED in great tits (Schaper et al., 2011).

The advancements in FED that populations have been exhibiting in response to warmer springs have occurred too rapidly to have been brought about by evolution, or natural selection alone (Charmantier et al., 2008). The relationship between phenology and temperature is the same at population and individual levels, further suggesting advancements in phenology are likely to be as a result of phenotypic plasticity (Charmantier et al., 2008). Phenotypic plasticity is where an organism changes its phenotype, in this case timing of egg laying, in response to changes in their environment without it being genetically fixed, allowing populations to rapidly respond to

environmental change (West-Eberhard, 1989). Although phenotypic changes themselves are not genetically fixed, plasticity itself is a selectable trait and can become fixed within populations (Nussey et al., 2005).

The aim of this chapter is to investigate the impact of tree species, temperature and latitude on blue tit breeding phenology across the UK. To my knowledge, this is the first time fine-scale tuning of phenology, in regard to habitat, has been investigated across such an extensive range and across multiple populations. I hypothesise that as different tree species exhibit different leafing phenology (Roberts et al., 2015), blue tits breeding in areas with high proportions of late-leafing species such as ash (*Fraxinus excelsior*) and/or oak will lay later, and therefore hatch later, than blue tits in areas with higher proportions of early leafing species, such as sycamore (*Acer pseudoplatanus*) and/or birch.

4.3 Methods

4.3.1 Nest recording

Nest records generated annually by volunteer nest recorders, across the UK, working as part of the British Trust for Ornithology's (BTO) Nest Record Scheme (NRS) were used. Each record included the following data: species, year, grid reference, altitude, and for each visit: the date, number of eggs/young and stage of development of nest/eggs. Finally the outcome of the nesting attempt (if known), was recorded. If precise records of first egg date (FED) are not recorded (due to visiting after the first egg has been laid), these dates are back calculated, assuming one egg is laid per day. Similarly, if hatching date was missed, it was back calculated based on the developmental stage of chicks when first observed. For the period 1962 – 2015 73,612 digitised blue tit nest records were available in the NRS database.

4.3.2 Site and nest record selection

Figure 4.1: Black circles represent the locations of sites with long running blue tit nest box schemes, selected from BTO NRS database, and that were included in this study.

The NRS blue tit database was cleaned to remove records containing submission and/or inputting errors (70,743 records remained). Nest recorders submit the location of nests using a minimum accuracy of a four-figure grid reference (1 km x 1 km). Initially, all grid references were converted to four-figure grid references to highlight grid references where multiple records had been submitted over multiple years, aiding the identification of individual sites with long term datasets. Any four-figure grid references where the same observer had submitted records for a minimum of 10 years, and was active in 2015, were selected. These records were then used to identify focal sites, and the recorder contacted to confirm each such site was likely to be a single population. Thirty four sites, with records from 1979 – 2015, were identified and used in subsequent analyses (Figure 4.1).

As citizen scientists collected these data, there was variation in the frequency of visits, leading to uncertainty in some of the FED and/or hatching date estimates, resulting in minimum and maximum estimates of date variables to be created. Consequently, a further clean-up step was undertaken. For records to be used in the FED analysis, records were removed if the difference between the upper and lower estimates of FED/hatching were greater than 10 days, leaving 10,406 FED records for

the selected sites and time period aforementioned, and 5,944 hatching date records spanning 1980-2015.

4.3.3 Habitat

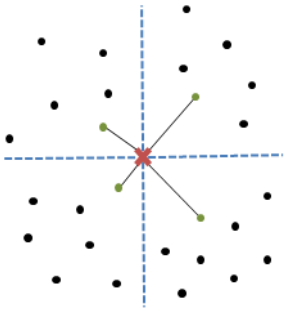

Figure 4.2: X represents the sampling point and the dashed lines the quarters to be used for sampling. In each quarter the distance (black solid line) to the nearest tree (green filled circle), was measured.

Total tree density and species specific densities were estimated at each site using modified Point Centered Quarter Method (PCQM) (Khan et al., 2016). PCQM has been shown to give similar species density estimates to other distance sampling approaches and quadrat sampling (Cottam and Curtis, 1956). This sampling approach was chosen as a way of sampling the current available habitat, which is unlikely to have changed dramatically over the period of the bird observations as all sites were mature woodlands. Twenty five stratified random sampling points were selected at each site, stratified using the location of nest boxes to ensure the sampled habitat was likely to be used by nesting pairs. Each point was split into four equal quadrants and the distance to, and species of, the nearest tree recorded (using a digital laser rangefinder (Bosch PLR 25), Figure 4.2). Trees were only ever recorded in one point, to ensure each individual sampling point was independent. Saplings (any single stemmed tree with a diameter at breast height (DBH) of less than 5 cm) were not sampled and the next nearest tree with a DBH greater than 5 cm included. Sapling density is likely to change more rapidly than more mature tree density over the sampling period.

Total tree density was estimated using the following equation (Khan et al., 2016):

$$\rho = 4\,(4N - 1)/\pi \sum_{i=1}^{N} \sum_{j=1}^{4} R^2_{(1)ij}$$

Where:

ρ is overall tree density;

R_{ij} is the distance from i^{th} random point to the closest tree in the j^{th} quadrant;

N is the number of random points (always 25 in this study).

Individual species were grouped into species categories following categorisation used in Forestry Commission (2012), and groupings used are outlined in Table 4.1.The density of a single species (ρ_k) at each site was calculated in the following way (Mitchell, 2010):

$$\rho_k = \frac{Number\ of\ quadrants\ with\ species\ k}{4N} \times \rho$$

Where:

k is a single species category recorded at the site;

ρ is the calculated total tree density of the site;

N is the number of random points (always 25).

Species densities were then converted to proportions of the total tree density at each site (Figure 4.3).

Table 4.1: Description of the species which comprise each general tree category, as referred to throughout this chapter

Category	Species
Ash	European ash *Fraxinus excelsior*
Beech	Common beech *Fagus sylvatica*
Birch	Downy birch *Betula pubescens;* Silver birch *Betula pendula;* Common hornbeam *Carpinus betulus*
Oak	English oak *Quercus robur;* Sessile oak *Quercus petraea;* Red oak *Quercus rubra*
Sycamore	Sycamore *Acer pseudoplatanus*
Willow	Bay willow *Salix pentandra;* Crack willow *Salix fragilis;* Goat willow *Salix caprea* Grey willow *Salix cinerea oleifolia*
Other	Alder spp. *Alnus spp.* Apple spp. *Malus spp.* Aspen *Populus tremula* Bird cherry *Prunus padus;* Blackthorn *Prunus spinose;* Wild cherry *Prunus avium* Purging buckthorn *Rhamnus cathartica* Caucasian fir *Abies nordmanniana* Cedar *Cedrus libani;* Japanese red cedar *Cryptomeria japonica* Corsican pine *Pinus nigra laricio;* Scots pine *Pinus sylvestris* Douglas fir *Pseudotsuga menziesii* Elder *Sambucus nigra* English elm *Ulmus minor'Atinia';* Wych elm *Ulmus glabra* European larch *Larix decidua* Common hawthorn *Crataegus monogyna* Common hazel *Corylus avellana* Common holly *Ilex aquifolium* Horse chestnut *Aeculus hippocastanum* Leylandii *Cupressocyparis leylandii* Lime spp. *Tilia spp.* Field maple *Acer campestre;* Norway maple *Acer platanoides* Norway spruce *Picea abies;* Sitka spruce *Picea sitchensis;* Spruce spp. *Picea spp.* Poplar spp. *Populus spp.* Coast redwood *Sequoia sempervirens* Common rowan *Sorbus aucuparia* Spindle *Euonymus europaeus* Sweet chestnut *Castanea sativa* Tulip tree *Liriodendron tulipifera* Walnut *Juglans regia* Western hemlock *Tsuga heterophylla* Yew *Taxus baccata*

4.3.3.1 Testing tree density estimates

To ensure 25 sampling points per site was sufficient to estimate tree density accurately, the methodology was tested at three sites that were also used in the final analyses. The three sites used to test the methodology varied in area covered, 124 Ha (Highnam Woods, SO7819), 172 Ha (Minsmere, TM4667) and 510 Ha (Nagshead, SO6008). Each nest box within the site was used as a potential sampling point that resulted in 67, 89 and 407 points for Highnam, Minsmere and

Nagshead, respectively. The methodology was tested using 25, 30 or 35 random points at each site. Sampling points were randomly selected from the total points available at each site, and total tree density and individual species densities calculated, with 500 bootstraps of randomly selected points for each site-point combination. There was no significant difference in the mean total tree density calculated at each site with an increase in the number of sampling points (one way ANOVA: Highnam: DF = 2, SS = 26785, MS = 13392, F = 2.06, p = 0.13; Minsmere: DF = 2, SS = 679, MS = 340, F = 0.37, p = 0.70; Nagshead: DF = 2, SS = 400, MS = 200, F = 0.07, p = 0.93). However, variance declined as the number of points sampled increased (supplementary material 4.6.1, Table S.1).

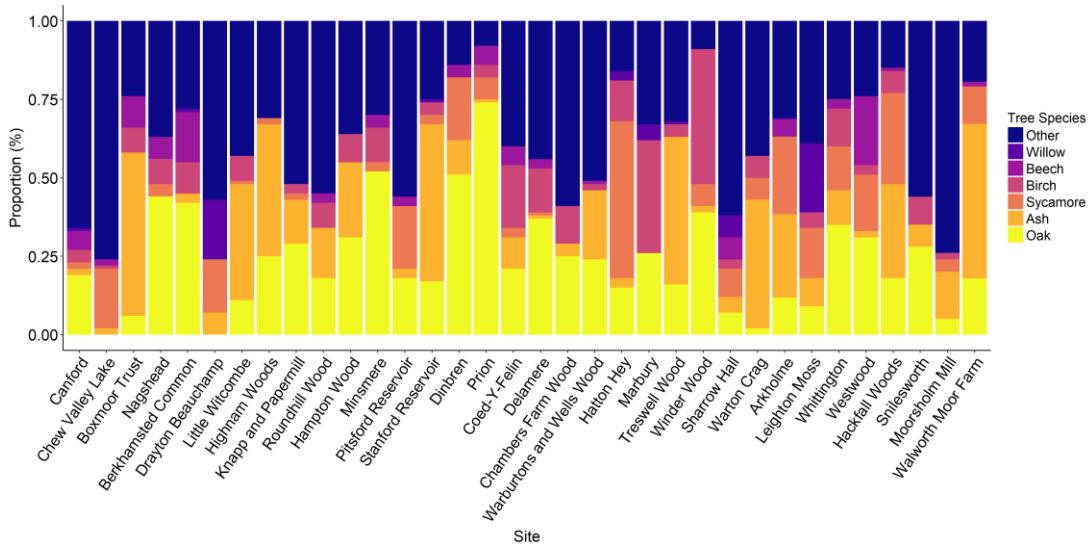

Figure 4.3: Proportion of each tree genus present at each of the 34 sites included in the study, ordered from south to north.

4.3.4 Climate

Interpolated daily mean temperature, from across the UK, for the period spanning the bird data, at a resolution of 5 x 5 km (Perry et al., 2009; Perry and Hollis, 2005; https://www.metoffice.gov.uk/climatechange/science/monitoring/ukcp09/) was used to relate bird nesting traits to climate. Each nest record was associated with the climate data of the encompassing 5 x 5 km grid square. The mean daily temperature between day 75 and 128 (16th March to 8th May) was calculated for each nest record, as this period has previously been shown to best predict spatial and temporal variation in FED (Phillimore et al., 2016). In addition, the mean first egg date was 27th April (min: 29th March, max: 14th June), falling within the chosen time period for temperature, and mean hatching date 19th May (min: 22nd April, max: 21st June). It is likely this time period (day 75-128) influenced hatching phenology, as females would have been incubating, or deciding when to commence incubation, during this period. A within subject mean centring approach was taken to allow the spatial and temporal slopes to be explored, with a mean

yearly temperature for the aforementioned time period for each site derived, and also the deviation from this for each nest record at that site (van de Pol and Wright, 2009).

4.3.5 Statistical Analysis

In all models, phenology (either FED or hatching date) was used as the response variable with temperature and latitude as fixed effects along with the following cross-classified random effects: year, site and habitat (as random intercepts), and the within site temperature slope was also allowed to vary. All models were implemented as Bayesian mixed effects models, using 'MCMCglmm' (Hadfield, 2010), in 'R' v.3.5.1 (R Core Team, 2017). All response variables were interval censored Gaussian responses, specifying that the response variable fell between a minimum and maximum value (either minimum or maximum FED or hatching date, for the respective models), and were assumed to follow a normal distribution. All continuous fixed effects were scaled and mean centred. Priors were defined as the default inverse-Wishart distribution for the residual term and flat, parameter expanded priors for the variance terms in all models. The burn in period was set to 20,000 in all models, with total number of iterations varying dependent on the model. Full model structures are described in Table 4.2, and models did not undergo any simplification.

In all analyses habitat variables were included as a multiple membership random effect. Hence, a presence/absence matrix was created for each tree species categories with 1 denoting it was present at a site and 0 absent. The presence/absence matrix was then weighted by the proportion of each species present at each site and used cumulatively in the model. Weighting by proportion, rather than using raw species density data, was required in this modelling approach due to the assumption of summing to unity. Including habitat as a multiple membership random effect allowed the effect of each individual tree species, as well as the total habitat effect to be explored, as one species may contribute to the habitat effect more than another. To ensure the conversion from densities to proportions did not mask any habitat effects the same models were constructed, but the multiple-membership habitat variable removed, and replaced with oak density (as a fixed effect). Oak was chosen *a priori* for this test as I expected oak to exert the greatest effect on phenology, based on previous research (Wilkin et al., 2007) and results from Chapter 2. The oak model suggested no masking of habitat effects; hence I only present results using the multi-membership habitat variable in the main text. The results of the oak model, and further information on rationale, are presented in the supplementary materials (supplementary material 4.6.2: Table S.2 and Table S.3).

Table 4.2: Summary of response, fixed and random effect variables and model structures used in the Bayesian general linear mixed models implemented in this study. All models were implemented using the 'R' package 'MCMCglmm' and after full models were created, no model simplification was undertaken.

Response variable (min and max of each used)	Fixed effects				Random effects					Iterations	Thinning interval
	Year (continuous)	Mean site March/April temperature	Deviation from mean site temperature	Latitude	Site	Year	Year:Site	Deviation from mean site temperature:Site	Habitat		
First egg date	✓	×	×	×	✓	✓	✓	×	×	50,000	30
	✓	✓	✓	✓	✓	✓	✓	✓	✓	650,000	500
Hatching date	✓	×	×	×	✓	✓	✓	×	×	50,000	30
	✓	✓	✓	✓	✓	✓	✓	✓	✓	650,000	500

Model convergence was assessed visually from trace and posterior density plots. The number of iterations and the thinning interval used (stated for each model in Table 4.2) were defined to maintain an effective sample size above 1000, whilst ensuring autocorrelation between successively stored iterations did not exceed 0.1 (Hadfield, 2017).

There was evidence for residual spatial autocorrelation in the first egg date model, but not in the hatching date model, with full methods and results presented in the supplementary material (supplementary material 4.6.3).

4.4 Results

4.4.1 First egg date

Across the 34 study sites, blue tits showed a significant temporal advancement in first egg date (FED) of 0.27 days per year, i.e. 2.7 days advance in FED per decade, which equates to an advancement of 9.7 days during the study period (1979-2015; Table 4.3).

Blue tit FED was negatively related to mean spring temperature at a site level (Figure 4.4, Table 4.4), and positively related to latitude (Figure 4.5, Table 4.4). For every degree Celsius increase in mean spring temperature, FED was on average 3.4 days earlier. After controlling for temperature, for each degree increase in latitude (heading north in the UK) blue tit FED was, on average, 1.6 days later. Within site temperature did not predict FED (Table 4.4).

No single tree species alone induced a significant advance or delay in FED in this study (Figure 4.6, Table 4.5). If a site was composed of a single species the advance or delay to FED is unlikely to be greater than 15.2 days, interpreted from the upper variance limit from the habitat effect (Table 4.5).

Table 4.3: Model estimates for the fixed and random effects, from Bayesian general linear mixed models, where either first egg date (FED) or hatching date was the response variable being investigated. Rows in bold denote significant effects.

Fixed effects	**Posterior mean**		**95% credible interval**		**Effective sample size**		**pMCMC**	
	FED	**Hatch date**	**FED**	**Hatch date**	**FED**	**Hatch date**	**FED**	**Hatch date**
Intercept	***655.9***	**567.3**	***345.0-972.0***	**236.7-931.5**	***1000***	**1000**	***<0.001***	**0.002**
Year	***-0.27***	**-0.21**	***-0.42 – -0.11***	**-0.39--0.04**	***1000***	**1000**	***0.006***	**0.012**
Random effects	**Posterior mean variance**		**95% credible interval**		**Effective sample size**			
	FED	**Hatch date**	**FED**	**Hatch date**	**FED**	**Hatch date**		
Year	**20.2**	**18.6**	**8.6-31.4**	**9.2-29.4**	**643**	**299**		
Year:Site	**20.8**	**22.8**	**17.7-24.3**	**19.1-26.5**	**1000**	**1072**		
Residual	**36.5**	**25.7**	**35.4-37.5**	**24.8-26.7**	**1000**	**1000**		

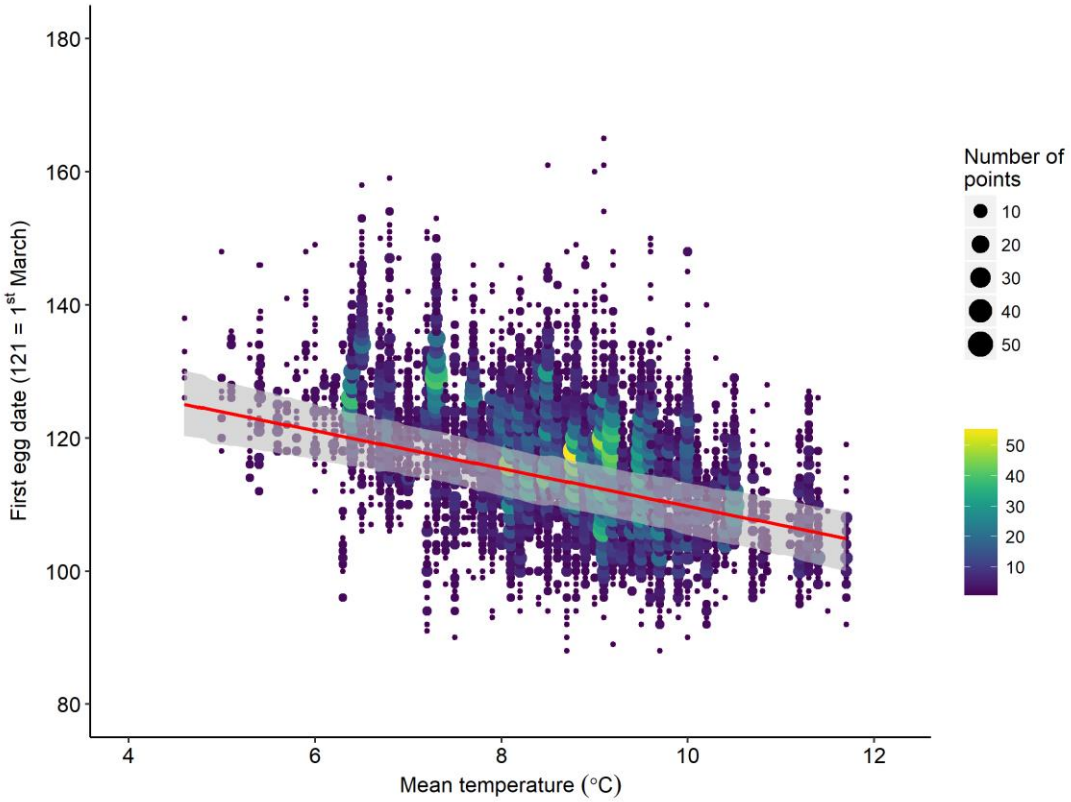

Figure 4.4: Model prediction for change in blue tit first egg date (FED) with mean spring temperature (day 75 – 128). Points represent single nest records from the BTO NRS data from the same sites in different years. The size and colour of the underlying point represents the number of data points at that position. The prediction line (red) is generated from the general linear mixed model, at the mean latitude and year, with the 95% credible interval shown surrounding the line.

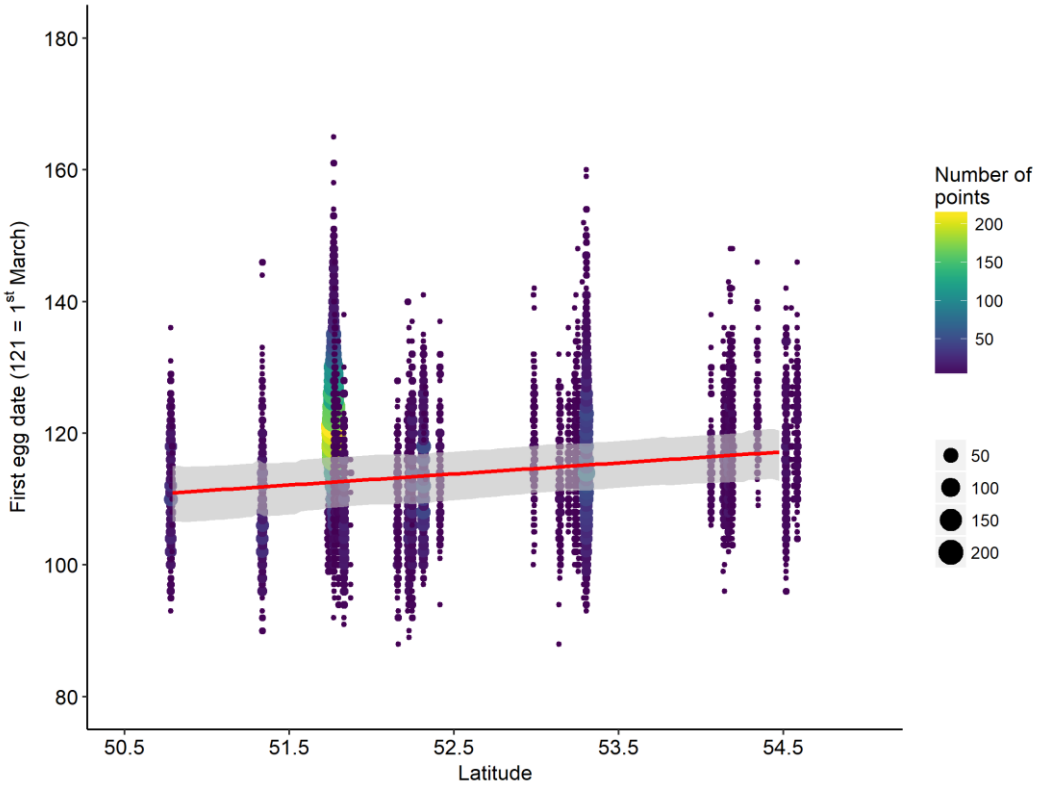

Figure 4.5: Model predictions for blue tit first egg date (FED) in relation to latitude. Points represent single nest records, from the same sites in different years, with the size and colour of the point being indicative of the number of data points at that position. The prediction line (red) is generated from the general linear mixed model holding temperature and year at their mean value. The shaded area surrounding the line represents the 95% credible intervals.

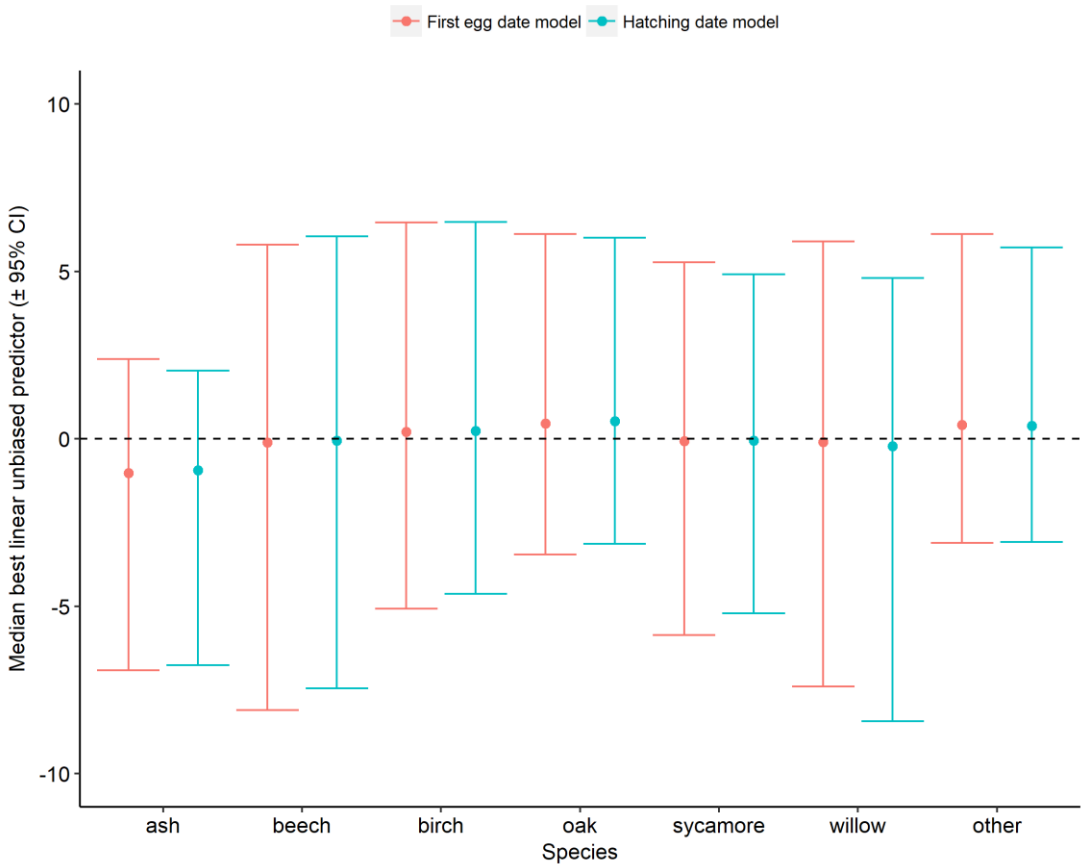

Figure 4.6: Best Linear Unbiased Predictors (BLUPs) for each tree species, calculated using their posterior distributions, with the median BLUP from all stored iterations and 95% credible intervals (CI). BLUPs represent the effect of habitat on the response variable (either first egg date, or hatching date), on top of temperature and latitudinal effects. Credible intervals that cross zero do not depart from the main effect. For example, on average, first egg date when ash is present at a site is 1.8 days earlier than predicted given the temperature and latitude effects. However, as the 95% CIs cross zero, there is no significant effect of ash on first egg date.

4.4.2 Hatching Date

Hatching date and FED were highly correlated (Pearson's correlation coefficient = 0.9). Hatching date exhibited a significant temporal trend during the period of study, with an advancement of 0.2 days per year, which equates to a 7 day advancement over the period of this study (1980 – 2015; Table 4.3). The advancement in hatching date is less than the advancement observed in FED.

Blue tit hatching date was negatively related to temperature (Figure 4.7, Table 4.4) with blue tits hatching 4.1 days earlier for each degree Celsius increase in mean spring temperature at a site level i.e. comparing between sites. Temperature within a site did not predict hatching date (Table 4.4). Hatching date was positively related to latitude, consistent with the relationship between latitude and FED (Figure 4.8, Table 4.4), and was delayed by 1.8 days for each degree increase in latitude.

There was no effect of any individual tree species on blue tit hatching date (Figure 4.6). However, if a site was to comprise of a single species the advance or delay to hatching date is unlikely to be greater than 13.7 days, interpreted from the upper variance limit of habitat (Table 4.5).

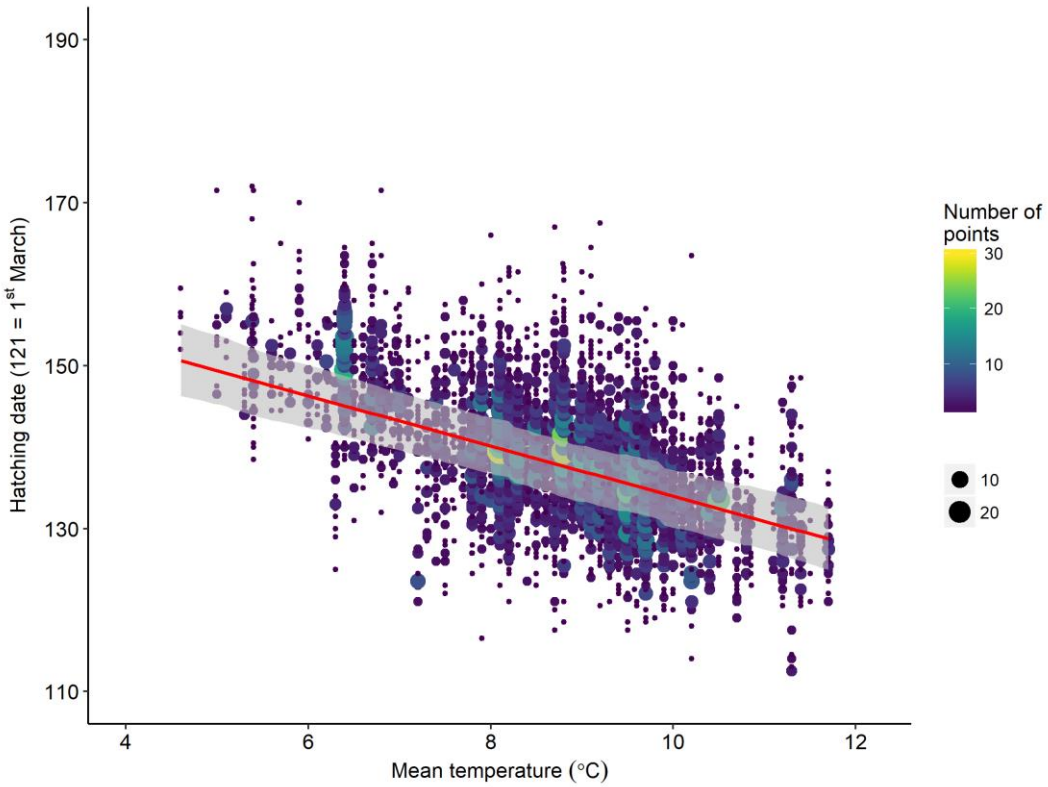

Figure 4.7: Model prediction for the change in blue tit hatching date with mean spring temperature (day 75 – 128, red line) between sites in across 35 years. Underlying point size and colour represent the number of bird records at that temperature and hatch date. The prediction line (red) is from the general linear mixed model, with all other variables held at their mean value, with the 95% credible intervals shown by the shaded grey area.

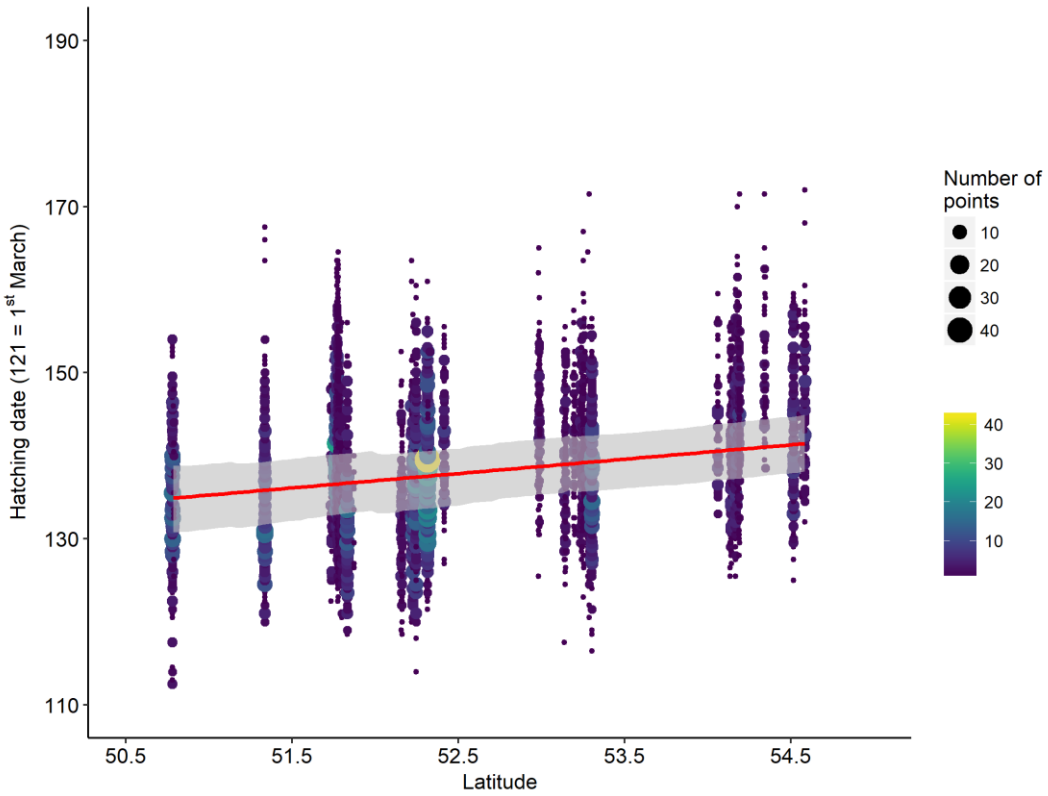

Figure 4.8: Model predictions for change in blue tit hatching in relation to latitude (red line). Points represent single nest records, with colour and size indicating how many records are at this position. The prediction line (red) is generated from the general linear mixed model holding all other variables at their mean values. Ninety five percent credible intervals are denoted by the shaded area.

Table 4.4: Model estimates for the fixed effects, from the Bayesian general linear mixed models, where either first egg date (FED) or hatching date was the response variable being investigated. Rows in bold denote significant effects.

	Posterior mean		**95% credible interval**		**Effective sample size**		**pMCMC**	
	FED	**Hatch date**	**FED**	**Hatch date**	**FED**	**Hatch date**	**FED**	**Hatch date**
Intercept	**113.63**	**137.91**	**109.66-117.11**	**134.05-140.90**	**1260**	**1260**	**<0.001**	**<0.001**
Between site mean temperature (°C)	**-3.44**	**-4.09**	**-4.35--2.32**	**-5.06--3.21**	**1260**	**1260**	**<0.001**	**<0.001**
Within site temperature (°C)	-0.31	-0.25	-4.19-2.28	-1.73-1.50	1260	1260	0.62	0.57
Latitude	**1.58**	**1.78**	**0.63-2.50**	**0.83-2.71**	**1376**	**1260**	**<0.001**	**<0.001**
Year	**-1.19**	-0.33	**-2.32--0.13**	-0.93-0.22	**1260**	1260	**0.03**	0.25

Table 4.5: Model estimates for the random effects from the Bayesian general linear mixed models, where either first egg date (FED) or hatching date was the response variable being investigated. Rows in bold denote significant effects.

Group	Type	Posterior mean variance		95% credible interval		Effective sample size	
		FED	Hatch date	FED	Hatch date	FED	Hatch date
Year:Site	**Intercept**	**5.51**	**4.79**	**4.27-6.75**	**3.49-6.01**	**1260**	**1533**
Year	**Intercept**	**10.58**	**6.85**	**5.36-18**	**3.29-11.59**	**1091**	**1260**
Site	**Intercept**	**7.32**	**6.49**	**3.12-11.74**	**3.14 – 10.63**	**1160**	**1260**
	Slope	17.77	4.64	1.4×10^{-5}-48.43	3.7×10^{-6} – 11.71	1260	1260
Habitat	Intercept	13.46	12.73	1.4×10^{-5} – 60.5	2.2×10^{-5} – 49.1	999	1066
Residual		**36.5**	**25.89**	**35.55-37.61**	**24.81-26.85**	**1365**	**1260**

4.5 Discussion

The aim of this study was to investigate the effect of habitat, in addition to temperature and latitude, on breeding phenology of blue tits in the UK. To my knowledge, this is the first study to investigate the effect of tree species composition on phenology at such a wide scale. Previous research has investigated the impact on blue tit phenology of single tree species, within single populations, or has contrasted phenology between two habitat types (e.g. Blondel et al., 1993).

Overall, there was no evidence for differences in blue tit phenology, either in terms of FED or hatching date, in relation to the proportion of any single tree species present (Figure 4.6, Table 4.5), after controlling for temperature and latitude. However, it should be noted that information about laying gaps and any variation in length of incubation, which ultimately delays or advances hatching dates, were not included in these analyses due to not being available in the dataset. FED and hatching date were also highly, but not perfectly, correlated (Pearson's correlation coefficient = 0.9). This suggests that both the phenology analyses are similar in this dataset, and it is not surprising that variables that influenced FED also influenced hatching date. However, it is still important to consider both FED and hatching date when investigating changes in phenology, as an opportunity to adjust phenology still remains after egg laying (Tomás, 2015), so these two measures of phenology may not always be highly correlated in every dataset.

I hypothesised phenology would be earlier when large proportions of early leafing species, such as sycamore or birch were present, and later when large proportions of later leafing species were present, such as ash and/or oak. This was expected as it has been shown that caterpillars emerge around the time of bud-burst, as younger leaves are more nutritious and contain less tannin than more mature leaves (Feeny, 1970). Therefore, peak food availability for blue tits is likely to be just after bud burst, and birds will need to time peak nestling demand to coincide with peak food availability. Winter moth, blue tits preferred prey item, are polyphagous and, therefore, any of the tree species considered in this study could act as a cue for birds to time reproduction to, as well as these species hosting a number of other invertebrate species (Kennedy and Southwood, 1984).

There are a number of possible reasons as to why no relationship between habitat and blue tit phenology was found. The first is that blue tits do not use local habitat as an additional cue to fine tune their breeding. An experimental study, in which leafing branches were presented to great tits earlier than usual, found that the presentation of early leafing branches did not advance breeding phenology (Schaper et al., 2011). Nor do birds consume buds or leaf scales regularly, or in high enough quantities, to obtain chemical signals from the trees (Bourgault et al., 2006). It may be that the birds are constrained by their physiology, and are unable to process or interpret

habitat cues. My findings, which are the first extensive examination of the potential role of habitat in modifying phenology, substantiate the experimental work that suggested habitat plays an insignificant role in breeding phenology of great tits (Schaper et al., 2011). Day length has been shown to be the proximal cue used to initiate breeding, starting gonadal growth, and temperature alters the rate of gonadal growth (Visser and Lambrechts, 1999). My results highlight that with warming spring temperatures blue tits are breeding earlier (Figure 4.4, Figure 4.7, Table 4.4). However, even after accounting for temperature, FED and hatching date are later in the north (at a higher latitude; Figure 4.8, Table 4.4) than in the south of the UK. If physiology is constraining FED phenology preventing birds from processing habitat cues, a difference in the response of hatching date to habitat would be expected. Hatching date is less constrained by physiology as birds can be flexible in incubation strategies to advance, or delay, hatching to ensure peak nestling demand coincided with peak prey availability (Vedder, 2012). However, hatching date was not found to vary with habitat (Table 4.5).

A second potential explanation, for the lack of effect of individual tree species on phenology, could be due to habitat composition being relatively homogeneous across the 34 sites (Figure 4.3), resulting in small individual species effects that are difficult to detect. Although I tried to maximise the variation across the study sites, it is possible nest recorders preferentially chose to place nest boxes in particular habitats. As many of these sites were established to monitor pied flycatcher, which preferentially nest in oak dominated woodland (Lundberg and Alatalo, 2010), this could have inadvertently reduced habitat variation between sites. For example, a transect in Scotland, established to monitor blue tit phenology, randomly selected 40 woodland sites from the available woodland habitat, and these sites exhibited greater between site habitat variation than those in this study (Shutt et al., 2018). This suggests nest recorder bias in nest box locations could be an issue when investigating the effects of habitat with this dataset. Many of the study sites have a diverse tree community, which could result in cues from single tree species being diluted and not strong enough to tease apart from the overall effect of habitat. The spatial scale that habitat was recorded (at a site level) may also have been too broad to detect fine scale effects, with fine scale studies reporting that environmental variables sampled at between 25-75 m of the nest showed the strongest associations with FED (Wilkin et al., 2007).

Finally, in mixed deciduous, non-oak dominated, habitats; there may not be substantial enough caterpillar biomass or no difference in peak timing, as inferred from frass fall (Chapter 2), to warrant close cueing of individual tree species. However, resource peaks have been observed on non-oak tree species (Shutt, 2017), suggesting it is still possible that individual tree species could be likely cues. Although, caterpillars are more likely to be present, and in higher numbers, on oak trees (Kennedy and Southwood, 1984; Shutt, 2017). Blue tits have a varied diet (Cowie and

Hinsley, 1988; García-Navas and Sanz, 2011; Serrano-Davies and Sanz, 2017), and although nestlings fed a higher proportion of caterpillars are in better condition at fledging (Wilkin et al., 2009), this fitness benefit may come at a cost to the parent in increased energy expenditure during foraging when oak is not abundant. In non-oak dominated woodlands it may be more beneficial to feed nestlings on a larger quantity of easy to find sub-optimal prey, such as spiders and aphids, which may be in higher abundance but not subject to such strong peaks in availability as caterpillars. Therefore, there may be no reliable cue which the birds can use to predict future availability of sub-optimal invertebrate prey.

There being no detectable effect of individual tree species on blue tit breeding phenology suggests blue tits are not fine tuning their breeding phenology in relation to cues from their surrounding habitat. The implications of this could be great, if resource peaks also differ in phenology between habitats. Mismatch between trophic levels can occur when trophic levels respond to climate change at different rates (Cushing, 1990; Durant et al., 2007). Generally, blue tits are advancing their FED more slowly than caterpillars (Burgess et al., 2018; Buse et al., 1999). The lack of association between breeding phenology and habitat composition, demonstrated here, suggests mismatch could become more of a problem in the future, as blue tits are responding to temperature but not cueing into habitat to maintain synchrony with their food resources. There is evidence blue tit populations in the UK are showing signs of mismatch, more so in warm springs (early years) than in cold springs (Burgess et al., 2018). This again reinforces my findings here that temperature is the proximate cue blue tits use to time their breeding.

Here I have demonstrated the effects of temperature and habitat on both FED and hatching date. Both FED and hatching date are subject to similar cues, such that temperature and latitude, but not individual tree species, influenced breeding phenology. Both measures of phenology have been presented here as blue tits may be able to make more decisions about fine tuning hatching date rather than FED, with FED potentially being more constrained by physiology and nutrient availability (e.g. Smith and Smith, 2013). Therefore, when looking at the effects of habitat on phenology, hatching date may be more informative (Tomás, 2015), as this could be more finely tuned to synchronise with local prey availability through egg pauses and changes to incubation strategies.

Previous research has investigated whether responses of blue tit FED to temperature, and in a sympatric species the great tit, is a plastic response. In general, the conclusion has been that such responses can be attributed to plasticity (Charmantier et al., 2008; Cresswell and Mccleery, 2003; Nussey et al., 2005; Porlier et al., 2012) as the response is unlikely to have been able to become genetically fixed in such a short period of time (Charmantier et al., 2008). Therefore, the earlier breeding due to warmer spring temperatures observed in this study is also likely result from

phenotypic plasticity, despite within site temperature not predicting phenology (Table 4.4). This lack of association is likely to be due to the scale that temperature data were available (5 x 5 km resolution), and the spatial accuracy of nest records. Nest recorders often used the same four-figure grid references for the majority of their site, for ease during data entry, despite in reality the site covering multiple grid references. Both of the factors combined, resulted in little across site variation in temperature, which is not likely to be representative of reality. Although, there is still variation in both FED and hatching date between sites, even after controlling for temperature and latitude that is not explained by habitat (Table 4.5). This could be due to other characteristics of these sites, which haven't been accounted for in these models. One such characteristic could be the underlying geology of the site, which may influence, for example, calcium and other nutrient availability (Briggs and Mainwaring, 2017), constraining the female's ability to commence egg laying or to sustain her through incubation.

In conclusion, the results show that both temperature and latitude are predictors of blue tit breeding phenology across the UK, and temperature is likely to be the proximal cue for blue tit breeding phenology. However, I found no evidence to support the hypothesis that blue tit breeding phenology differs in relation to tree species composition, suggesting blue tits are not using habitat cues to fine tune their breeding phenology, leaving them at increased risk of trophic mismatch in light of warming springs.

4.6 Supplementary Material

4.6.1 Validating habitat sampling method

Table S.1 Variance in total tree density estimates from each methodology at each site

Site	25 random points; 500 iterations (trees/ha)	30 random points; 500 iterations (trees/ha)	35 random points; 500 iterations (trees/ha)
Highnam	8935.4	6426.1	4189.5
Minsmere	1137.9	887.4	726.9
Nagshead	3143.8	2659.6	2306.1

4.6.2 Alternative model structures

Here, I present results from alternative model structures (Table S.2 and Table S.3) than those presented in the main results section (Table 4.4). These alternative model structures were used to ensure converting habitat variables to proportions did not mask any habitat effects. Converting to proportions did not take into account that overall tree density varied between sites (Figure S.1; min: 6.7, mean 462.03, max: 1075.3 trees per hectare), which may impact how birds use the available resources. For example, if two sites have different oak densities (e.g. 1 vs. 10 trees per hectare) this could become masked when converting to proportions as both sites may have 25% oak, depending on overall tree density. A site with a higher tree density of a particular species may provide a stronger cue to the bird, hence why it is necessary to ensure the results are consistent when investigated with oak density instead. It was decided *a priori* to only include oak density as this species is believed to provide blue tits with most of their food resources during the breeding season (Gibb and Betts, 1963; Peck, 1989) and their optimal habitat (Perrins, 1979). In addition to this, oak is one of the most abundant species in UK woodlands (Forestry Commission, 2013), therefore being a species blue tits are likely to use as a cue.

The results do not differ from the models presented in the main text, with between sites mean spring temperature, latitude and year being significant predictors of blue tit breeding phenology (Table S.2). Blue tit breeding phenology (either FED or hatching date) is not predicted by within site temperature or oak density. As the results do not differ from those presented in the main text (Table 4.4 and Table 4.5) it suggests that, even though some information is lost when species density is converted into a proportion of all trees present, this does not impact the results and interpretation.

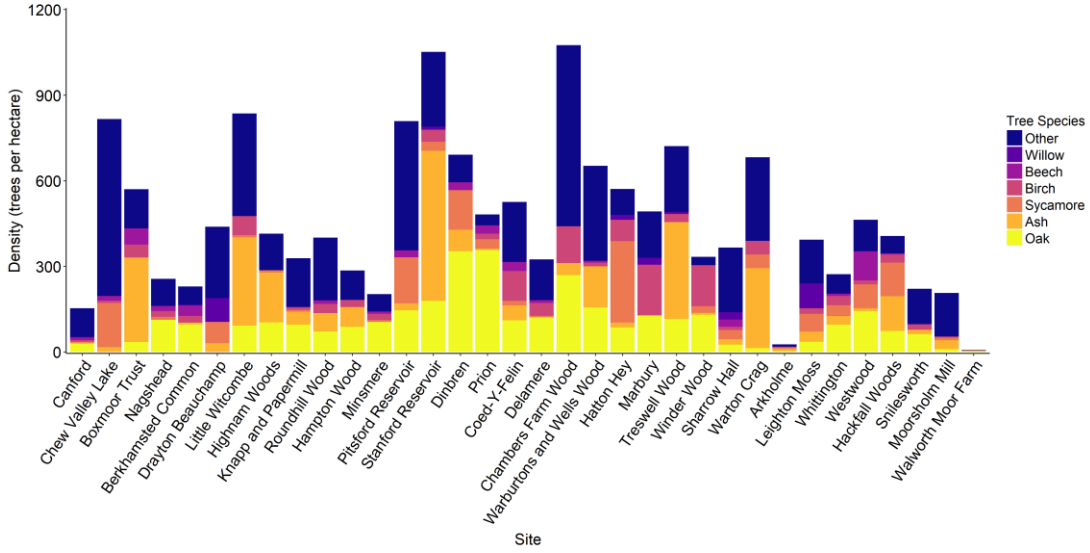

Figure S.1: Densities of each tree species category used in the main analyses at each of the 34 sites included in this study. The total height of each bar represents the overall tree density at each site.

Table S.2: Model estimates, from the Bayesian general linear mixed models, for the fixed effects from models including Quercus density instead of proportions of the seven most important genera for both first egg date (FED) and hatching date. Rows in bold denote significant effects.

	Posterior mean		95% credible interval		Effective sample size		pMCMC	
	FED	Hatch date	FED	Hatch date	FED	Hatch date	FED	Hatch date
Intercept	**114.06**	**138.26**	**112.36-115.69**	**136.68-139.84**	**970**	**1261.7**	**<0.001**	**<0.001**
Between site mean temperature (°C)	**-3.48**	**-4.07**	**-4.62--2.40**	**-5.12--3.14**	**970**	**1000**	**<0.001**	**<0.001**
Within site temperature (°C)	-0.31	-0.17	-3.38-1.92	-12.26-1.00	970	1097	0.62	0.61
Latitude	**1.50**	**1.74**	**0.51-2.44**	**0.84-2.72**	**859**	**899**	**0.008**	**0.002**
Oak density	0.17	0.34	-0.61-0.93	-0.59-1.17	970	1000	0.65	0.43
Year	**-1.09**	-0.34	**-2.09--0.11**	-0.94--0.25	**1110**	1000	**0.03**	0.25

Table S.3: Model estimates for the random effects from both first egg date (FED) and hatching models, using the alternative structure where Quercus density is included as a fixed effect. Rows in bold denote significant effects.

Group		Posterior mean		95% credible interval		Effective sample size	
		FED	Hatch date	FED	Hatch date	FED	Hatch date
Year:Site	**Intercept**	**5.53**	**4.76**	**4.40-6.79**	**3.60-6.10**	**970**	**1000**
Year	**Intercept**	**10.39**	**6.93**	**5.42-16.69**	**3.30-11.28**	**849**	**778**
Site	**Intercept**	**7.84**	**6.70**	**4.09-12.93**	**3.20-10.93**	**825**	**833**
	Slope	14.17	4.85	4.3×10^{-7}-40.62	1.62×10^{-7}-13.72	970	739
Residual		**36.49**	**25.91**	**35.46-37.57**	**24.93-26.98**	**1177**	**1000**

4.6.3 Checking for spatial autocorrelation

Models were checked for spatial autocorrelation by computing Moran's I on the residuals (using 'Moran.I' from 'ape' R package (Paradis et al., 2004)) from a model with the same structure (minus the multiple membership random effects term) implemented using the R package 'lme4' (Bates et al., 2015), due to being unable to extract residuals from models fitted using MCMCglmm. There was evidence of spatial autocorrelation in the residuals of the first egg date model (First egg date: Observed = 0.005, Expected = -9.61×10^{-5}, p = 0.02), suggesting, that despite the random effect structure implemented, there is still remaining spatial patterning that I have not accounted for. There was no evidence residual spatial autocorrelation in the hatching date model (Moran's I: Hatching date; Observed = 0.008, Expected = -0.00017, p = 0.06).

Chapter 5: The impact of habitat and climate on blue tit productivity in the United Kingdom

5.1 Abstract

Understanding the drivers of reproductive success is vital to further our knowledge of population processes, especially in light of climate and habitat change and observed population declines in many avian species. In the UK, increasing spring temperatures have led to changes in blue tit (*Cyanistes caeruleus)* breeding phenology. Such changes in breeding phenology can lead to phenological mismatch between resources and users, and therefore impact upon productivity. However, to date, the influences of habitat in non-oak dominated woodlands on productivity is poorly understood. Understanding the drivers of reproductive success is important when predicting the possible impacts of environmental change.

Here, I quantify the effect of habitat (canopy tree species), spring temperature and latitude on blue tit clutch size and the risk of nest failure, using breeding data spanning 1979-2015 from across the UK. Increasing spring temperature and later clutch initiation both reduced blue tit clutch size, supporting studies on other species. Clutch size was positively related to oak density, but no other tree species investigated. Increases in temperature reduced the risk of failure, at both egg and young stage, but clutches initiated later in the breeding season experienced higher failure risk than those initiated earlier. There was a latitudinal effect on nest success, with more northerly nests being less likely to fail than their southern counterparts. Despite increased clutch sizes at high oak densities, surprisingly, no effect of habitat composition on the risk of nest failure could be detected, suggesting failure may not be limited by food availability, but instead driven by climatic conditions and nesting phenology. When envisaging the effect of climate change on this system, the effects of mismatch also need to be considered, which may not be uniform across habitat. More information on resource usage is required to establish the full impact of mismatch in this system. The impacts of these results upon population size will depend on whether recruitment remains stable under climate change, and should be a priority for future research.

5.2 Introduction

Understanding the drivers of reproductive success is crucial to track population level responses to environmental change, as well as to gain a better understanding of what influences population processes (McLean et al., 2016). Reproductive success is reliant upon resource availability throughout the reproductive cycle, as well as being proximally limited by environmental factors such as climatic conditions, habitat and predation. The importance of each of these factors is likely to differ during each reproductive stage, and as such each nesting stage needs to be considered independently before looking for overarching drivers of reproductive success.

The model system of deciduous tree-herbivorous caterpillar-insectivorous bird, is commonly used when investigating phenological change in temperate woodlands (Both et al., 2009; Burgess et al., 2018), with most studies investigating the effects of climate on blue and great tits (*Cyanistes caeruleus* and *Parus major*, respectively) phenology. Most research into the effects of climate change are conducted at single sites, which are typically oak dominated (Both et al., 2009; Charmantier et al., 2008; Hinks et al., 2015), making results difficult to extrapolate to populations in non-oak dominated woodlands. This is despite tits being habitat generalists and found across a variety of habitat and woodland types (Robinson, 2018; Stenning, 2018).

The effect of habitat on tit productivity has previously focussed on comparisons between broad habitat types, such as deciduous vs. evergreen woodland (Atiénzar et al., 2010; Van Balen, 2002; Blondel et al., 1993; Gibb and Betts, 1963; Lambrechts et al., 1997), or urban vs woodland (Glądalski et al., 2017). Typically, birds nesting in deciduous areas have higher productivity (clutch size and number of fledglings) than birds nesting in evergreen or urban areas (Van Balen, 2002; Blondel et al., 1993; Glądalski et al., 2017). Smaller scale variation in habitat, such as at a territory scale, has been shown to influence productivity both within single sites (Amininasab et al., 2016; Perrins, 1979; Wilkin et al., 2009) and between sites (Marciniak and Nadolski, 2007; Tremblay et al., 2003). It is widely accepted that oak (*Quercus spp.*) is the optimal breeding habitat for nesting blue and great tits (Perrins, 1979), and rarely are the effects of other tree species considered (except Shutt et al., 2018). Shutt et al. (2018) demonstrated non-oak tree species, such as birch (*Betula spp.),* oak and sycamore (*Acer pseudoplatanus)* positively correlated with fledging success, but did not influence clutch size. Blue and great tits tend to forage mostly in oak trees (Gibb, 1954; Peck, 1989). However, blue tits also forage in a range of other species, only showing avoidance of beech (*Fagus sylvatica),* spruce (Norway and sitka spruce, *Picea abies* and *Picea sitchensis*, respectively) and western hemlock (*Tsuga heterophylla*), but these results are confounded by oak being the most abundant tree species at these study sites (Gibb, 1954; Peck, 1989). In the UK, oaks support a greater richness of Lepidoptera, and other invertebrate species, than any other native tree species (Kennedy and Southwood, 1984). This may explain birds'

foraging preference for oak, when available, and the focus on oak in insectivorous bird studies. Woodland maturity also influences productivity with higher fledging success (Arriero et al., 2010), but lower hatching success (Atiénzar et al., 2010), in mature woodland territories.

Climatic conditions also influence productivity, with warmer temperatures during May reducing the number of blue tit fledglings, in a single site study (Potti, 2009), but not affecting clutch size (Dolenec, 2007; Potti, 2009). If nestlings are exposed to extreme warm temperature, heat stress can lead to a reduction in appetite and a consequent reduction in growth rate and muscle mass, as shown in other species (Belda et al., 1995; Geraert et al., 1996). These findings are corroborated in blue tits, tested at a single UK site, with warmer temperatures leading to lower mass gain in nestlings than cooler temperatures (Mainwaring and Hartley, 2016). In recent decades, warming springs have led to an advancement in breeding phenology of many UK bird species (Crick et al., 1997) and concerns of nestling demand mismatching with peak resource availability (Burgess et al., 2018). With increased mismatch between demand and resources, both nestling condition and adult survival decreases due to additional foraging costs (Thomas et al., 2001). Decreased adult survival can result in a reduction in the number of chicks successfully fledging, and can even ultimately cause complete brood failure, with recent findings at one site attributing nearly all complete brood failures to the predation of one parent (Santema and Kempenaers, 2018).

Precipitation reduces the adults' ability to find sufficient food to sustain chicks. Long periods of intense rainfall have been shown to reduce the quality of nestlings through reduced growth rates (Kelleri and Van Noordwijk, 1994). However, one study from the UK found that increased precipitation actually increased the mass gain of nestlings, (Mainwaring and Hartley, 2016) and hypothesised this could be due to caterpillars increasing in weight due to being porous and having a higher water content during periods of rainfall (Speight, 1979).

In addition to climate and environmental effects, female age and breeding phenology influences clutch size with clutch size peaking around a female's third year, and older females producing smaller clutches (Amininasab et al., 2017). The timing of nest initiation during the breeding season also impacts clutch size, with birds initiating nests later in the breeding season typically laying smaller clutches than earlier laying conspecifics (Crick et al., 1993). This is believed to be in an effort to correctly time breeding with seasonal resource peaks in prey availability, with later laying birds trying to speed up their reproductive cycle to ensure resource peaks are not missed, maximising their chances of successfully fledging young (Perrins and McCleery, 1989).

In this study I aim to use a long-term dataset of nesting attempts made by blue tits, across a network of 34 sites in the UK, to investigate whether environmental factors, such as temperature and precipitation, and/or habitat (a proxy for food availability) impact upon blue tit productivity.

Here, I define productivity as the following:

1. Clutch size: The number of eggs produced in a breeding attempt and therefore the potential maximum number of offspring that could be produced in a given nesting attempt.
2. Risk of failure: the risk of the nest failing during two distinct nesting stages: (1) egg stage: from the date of the first egg being laid to that when the first chick hatches; and (2) young stage: from first hatching until fledging (when chicks leave the nest). I also consider the risk of failure over the entire nesting period. Here, failure is defined as either no eggs hatching or chicks fledging, dependent on the nesting stage being investigated.

Firstly, I hypothesise that blue tits nesting in areas with more oak trees will have larger clutch sizes, but clutch size will have a negative relationship with temperature. Secondly, I hypothesise that the risk of failure during egg and young stage is likely to be higher in non-oak dominated woodlands than oak dominated woodlands, and in cooler and/or wetter springs.

5.3 Methods

5.3.1 Nest Recording

All blue tit breeding data were collected by volunteer nest recorders and are collated annually by the British Trust for Ornithology (BTO) Nest Record Scheme (NRS). Each nest record included the following data: species, year, grid reference, altitude, and for each visit: the date, number of eggs/young and stage of development of nest/eggs, and finally the outcome of the nesting attempt (if known). Clutch size is taken as the maximum number of eggs recorded during a nesting attempt and first egg date (FED)/hatching date is the midpoint between minimum and maximum date estimates. Minimum and maximum FED is estimated where precise records of FED are not available (due to visiting after first egg has been laid) and FED is back calculated on the assumption of one egg being laid per day. Hatching date estimates (minimum and maximum hatching date) are back calculated based on the developmental stage of chicks when first observed. In total, 73,612 digitised blue tit nest records, for the period 1979 – 2015, were available in the NRS database.

5.3.2 Site selection

Identical site selection criteria were used as in Chapter 4. Sites were selected on the basis of having long term datasets available and for a full description of the criteria for inclusion in the study see Chapter 4. This resulted in the same 34 sites, from across the UK, being included in the study (Figure 5.1).

Figure 5.1: Locations of all sites included in this study, denoted by filled black circles, with names of sites, as labels. These names are used in reference to specific sites, when discussed in the results and discussion.

5.3.3 Habitat

Habitat was recorded at all sites in the same way as described in Chapter 4. In short, a distance sampling technique was used to estimate individual tree species densities at each site. Species were categorised based upon groupings used in the National Forestry Inventory (Forestry Commission, 2013), where closely related species are combined as an aggregate taxa (Table 4.1). These aggregations were used as one of the species within the grouping often replaced each other at sites, and the species in each grouping can host a similar invertebrate community.

Species densities were converted to the percentage of total species density at a given site for analyses that required habitat data to sum to unity.

5.3.4 Climatic data

Interpolated daily mean temperature and daily total precipitation data from across the UK, at a resolution of 5 x 5 km (Perry et al., 2009; Perry and Hollis, 2005; https://www.metoffice.gov.uk/climatechange/science/monitoring/ukc), for the period spanning the bird data were used to relate clutch size and risk of nest failure to climate. When investigating clutch size mean daily temperature was calculated for each record over March and April (mean March/April temperature). This period was used to relate clutch size and climate as the mean first egg date for this dataset was 28th April (min: 29th March, max: 14th June), therefore this time period is likely to influence the female's condition and therefore inform clutch size. A within subject centring approach was undertaken (following van de Pol and Wright, 2009), with a mean site temperature for each year and a deviation from the site mean for each record being used as temperature variables, allowing within and between site effects to be investigated. For egg stage analyses a bespoke time period was used for each record, with total temperature and precipitation sums calculated for each record from the mid first egg date estimate, until the end of the egg stage (calculated as mid first egg date + clutch size + mean incubation time (14 days, (Robinson, 2018))). In analyses investigating young stage the time period used was from hatching until fledging (hatching date (mid-point of minimum and maximum hatching date until 20 days after this (mean time to fledging (Robinson, 2018)). In whole nesting stage analyses, both of the time periods previously described were summed and used (first egg date until 20 days after hatching date).

5.3.5 Quality control of datasets

Due to nesting data being collected by citizen scientists, with no strict recording protocol regarding visit frequency, this can lead to uncertainty in some FED and hatching estimates,

resulting in minimum and maximum estimates being created based on information provided. Therefore, further quality control was undertaken. Described below for each dataset used.

5.3.5.1 Clutch size

Records were removed when maximum clutch size was less than 2, or greater than 16, and if the difference between minimum and maximum FED was greater than 10 days. Records where the maximum nest contents recorded by the observer was greater than the recorde d maximum clutch size were also removed, as this suggested the clutch was incomplete at the time of the maximum egg count. Due to being unable to ascertain whether small clutches were full clutches or potentially incomplete clutches, all clutches of two or above were used in analyses. There were 8,290 records for the selected sites spanning 1979-2015.

5.3.5.2 Risk of failure

The BTO NRS uses an approach described by Mayfield (1961) to calculate the number of exposure days during each nesting stage (egg stage, nestling stage and both combined to give overall number of exposure days for the entire nesting period). An exposure day is defin ed as a day where the nest was observed to be active (i.e. egg laying has commenced for egg stage, chicks have hatched/are hatching for nestling stage) and therefore at risk of failure. Exposure day one refers to the first day a nest was observed during the corresponding nesting period, and does not necessarily indicate the first day an egg was laid, for example, as the nest may have been found part way through egg laying. This needs to be taken into account when interpreting the results, especially of the egg stage analyses, as many nests will have been found during egg laying and are likely to have an artificially short number of exposure days for this period.

Records were removed if the maximum exposure days recorded during the nesting period under investigation (egg, young or whole nest stage) was 0 (indicating the nest had not been monitored during this stage), and/or the range of exposure days (calculated as the difference between the minimum and maximum exposure days) was greater than 10. The midpoint of the minimum and maximum exposure days was used in all analyses as the number of exposure days.

A binary coding system was used to denote nest success (0) and nest failure (1), with a nest being classified as a success if it reached the end of the stage being monitored, even if it then subsequently failed during a later stage. These data sets spanned the time period 1980 – 2015. Records where the outcome of the nest was unknown were removed. These quality control steps left 5,887 records in the egg failure data set (262 that are egg failures), 4,723 records in the young failure data set (644 that are nestling failures) and 3,538 in the overall nest failure data set (781 that are nest failures).

5.3.6 Statistical Analyses

5.3.6.1 Clutch size

Clutch size in relation to climatic, phenological and environmental variables was analysed through constructing a Gaussian Bayesian mixed effects model, in 'MCMCglmm' (Hadfield, 2010) using 'R' v.3.5.1 (R Core Team, 2017), with clutch size as the response variable. The full model structure included temperature, FED and latitude as fixed effects with year, site and habitat as cross-classified random (intercept) effects, and the temperature slope was allowed to vary between sites. The full model structure, described in Table 5.1, was created *a priori* based upon biological reasoning, and no further model selection was undertaken. Flat, parameter expanded priors were used for all variance terms in the model, and the default inverse-Wishart distribution was used for the residual term (Hadfield, 2010). Habitat variables were included as a multiple membership random effect. For a full description of how the habitat multiple membership random effect was set up, see Chapter 4. In brief, a presence/absence matrix was created and weighted by the proportion of the total tree density each species represented. The species categories included were ash, beech, birch, oak, sycamore and willow, and were included due to being present across many sites, and also likely to be key foraging resources for blue tits during the breeding season as they are host to many invertebrate species (Kennedy and Southwood, 1984). An 'other' species category was also included, which represented every species present at the site not included in any of the aforementioned categories, due to the multiple membership weightings being required to sum to unity. To ensure converting habitat variables to percentage of each species did not mask any habitat effects, this model was re-run with oak density as a fixed effect instead of including the multiple membership habitat variable, as *a priori* oak was thought to be blue tits optimal habitat (Perrins, 1979). The model structures are described in Table 5.1. In all analyses, fixed effects were mean centred (mean is subtracted from each value) and scaled (after centring each value is divided by the standard deviation), prior to inclusion in the model. Model convergence was assessed visually through trace and posterior density plots and autocorrelation between successive iterations was not allowed to exceed 0.1, whilst keeping effective sample sizes above ~1000 (Hadfield, 2017). These criteria determined the number of iterations and thinning interval needed (Table 5.1).

Model residuals were checked for spatial autocorrelation, using the model with oak density, fitted using 'lme4' due to being unable to extract residuals from 'MCMCglmm' (Bates et al., 2015; Hadfield, 2017). There was no evidence for residual spatial autocorrelation (Moran's I: observed = 0.0005, expected = -0.0001, sd = 0.003, p = 0.83), suggesting spatial dependencies have been adequately accounted for in the model structure.

Table 5.1: Model structure with the response, fixed and random effect variables used in the Bayesian mixed effect models investigating differences in blue tit clutch size.

Response variable	Fixed effects					Random effects					Iterations	Thinning Interval	Burnin
	Site mean March/April Temperature	Deviation from site temperature	Mid first egg date	Latitude	Oak density	Year	Site	Year:Site	Deviation from site temperature:Site	Habitat			
Clutch	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	50,000	40	10,000
size	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	450,000	400	30,000

5.3.6.2 Risk of failure

The risk of failure (at each stage: egg, nestling and overall) was investigated using mixed effects cox regression (survival) models, implemented through the 'coxme' package in R 3.5.1 (R Core Team, 2017; Therneau, 2018). The response variable is a survival matrix, with the number of exposure days (i.e. time monitored until failure) along with a binomial code for whether an event (failure) was observed. If the nest survived to the next nesting stage, or survived all of the stages, these records were denoted by a 0 and represent right-censored failure. The model structures used in each stage specific risk of failure model are described in Table 5.2. Cox's regression uses cox proportional hazard function, and was selected on the basis that there is no assumption as to the distribution of survival times. One drawback of this approach is the assumption that the effects of the covariates are constant with time. However, as exposure days (number of days the nest has been monitored during the defined stage) is the only time metric available, it is reasonable to assume that the effect of these covariates are constant for each day monitored, as this does not necessarily equate to nest/chick age.

Table 5.2: Descriptions of model structures used to investigate the effects of environmental covariates on the risk of nest failure at three stages during blue tit breeding attempts. In all models the response variable was a survival matrix, with survival as a binomial outcome (1, failure, 0, success) and the number of exposure days (days the nest was monitored) for each record.

Response variable	Fixed effects						Random effects	
	Stage specific temperature sum	Stage specific precipitation sum	Oak density	First egg date	Hatching date	Latitude	Site	Year
Risk of egg stage failure	✓	✓	✓	✓	×	✓	✓	✓
Risk of nestling stage failure	✓	✓	✓	×	✓	✓	✓	✓
Risk of overall nest failure	✓	✓	✓	✓	×	✓	✓	✓

5.4 Results

5.4.1 Clutch size

The median clutch size, over the 36 year period investigated (1979 – 2015), was 9 eggs (SD: ± 2.2 eggs).

There was a negative relationship between clutch size and site level temperature, and for every degree Celsius increase in site level mean March/April temperature clutch size decreased by 0.65 eggs (95% credible interval (CI): -0.83 to -0.50; Table 5.3, Figure 5.2). Temperature within a site did not, however, affect clutch size (Table 5.3). After controlling for temperature, clutch size decreased when breeding commenced later in the year, with a decrease of 0.14 eggs (95% CI: -0.14 to -0.13) for each increase in calendar day (Table 5.3, Figure 5.3), between 29th March and 14th June. However, there was no latitudinal effect on clutch size (Table 5.3).

Clutch size did not vary significantly with habitat (effect of ash, beech, birch, oak, sycamore, willow and other combined), after accounting for the fixed effects (temperature, latitude and first egg date; Table 5.4), as the credible interval effectively crossed zero, despite having the largest posterior mean. No individual species alone exerted significant effects, although oak was the only species to have a median best linear unbiased predictor which departed from zero, however the effect was not significant as the credible intervals crossed zero (Figure 5.4). Clutch size varied significantly with year and site, with mean clutch sizes between 8 and 10 in 95% of years and in 95% of sites (Table 5.4).

Table 5.3: Model estimates, for the fixed effects, from the Gaussian Bayesian general linear mixed effects model, investigating the effects of climate, habitat and phenology on clutch size. Rows in bold denote significant predictors of blue tit clutch size. All continuous predictors were scaled, with their scaling factors denoted below the table, which needs to be considered when interpreting parameter estimates.

	Posterior mean	95% Credible Interval	Effective Sample Size	pMCMC
Intercept	8.90	8.01 - 9.65	1050	<0.001
Mean site Mar/Apr temperature (°C) •	**-0.71**	**-0.91 – -0.55**	**1050**	**<0.001**
Temperature deviations from site mean (°C) ∇	**0.005**	**-0.46 - 0.33**	**1050**	**0.83**
Latitude ◊	0.04	-0.46 - 0.20	1050	0.66
First egg date *	**-1.35**	**-1.40 – -1.29**	**1050**	**<0.001**

Scaling factors: • = 1.09, ∇ = 0.04, ◊ = 0.88, * = 9.88

Table 5.4: Parameter estimates for the random effects, from the Gaussian Bayesian linear mixed model, with clutch size as the response variable and 'habitat' referring to the percentage occurrence of ash, beech, birch, oak, willow and other within each site. Significant effects are marked in bold, and were considered significant if the credible interval did not cross zero.

Group		Posterior Mean	95% Credible Interval	Effective Sample Size
Year:Site	**Intercept**	**0.17**	**0.11 – 0.24**	**1050**
Year	**Intercept**	**0.29**	**0.11 – 0.52**	**1050**
Site	**Intercept**	**0.23**	**0.1– 0.40**	**1050**
	Slope	0.21	1.88×10^{-6} – 0.53	1050
Habitat	Intercept	0.81	6.82×10^{-8} – 2.75	1050
Residual		**2.98**	**2.89 – 3.07**	**1244**

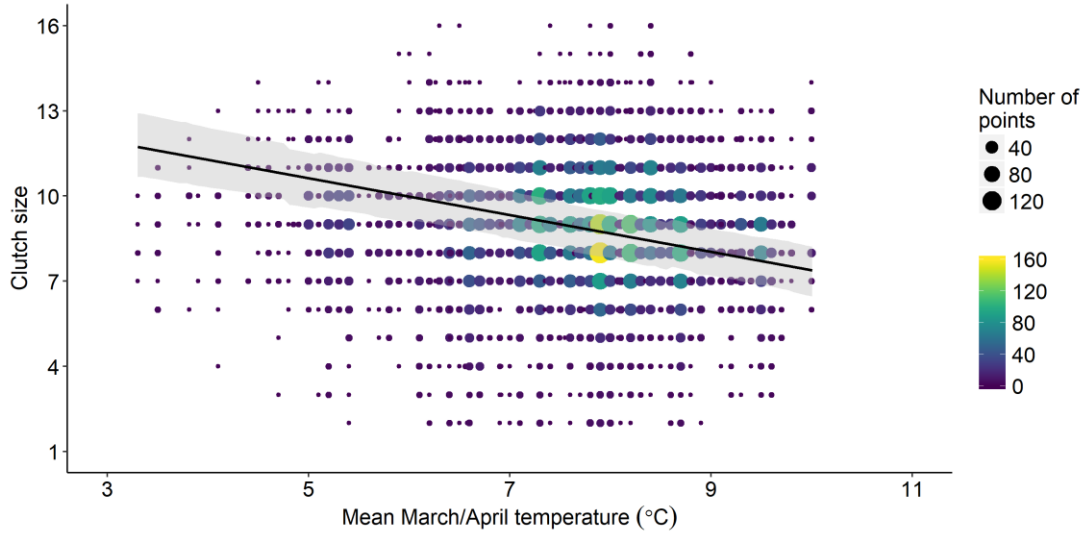

Figure 5.2: The relationship between site level mean March/April temperature and blue tit clutch size across the UK. The black line represents the fitted line from a Bayesian mixed effects model, controlling for the effects of latitude and first egg date, with the lighter shaded areas showing the 95% credible intervals. The points show the raw underlying data from which the model was fitted, with size and colour representative of the number of points at that position.

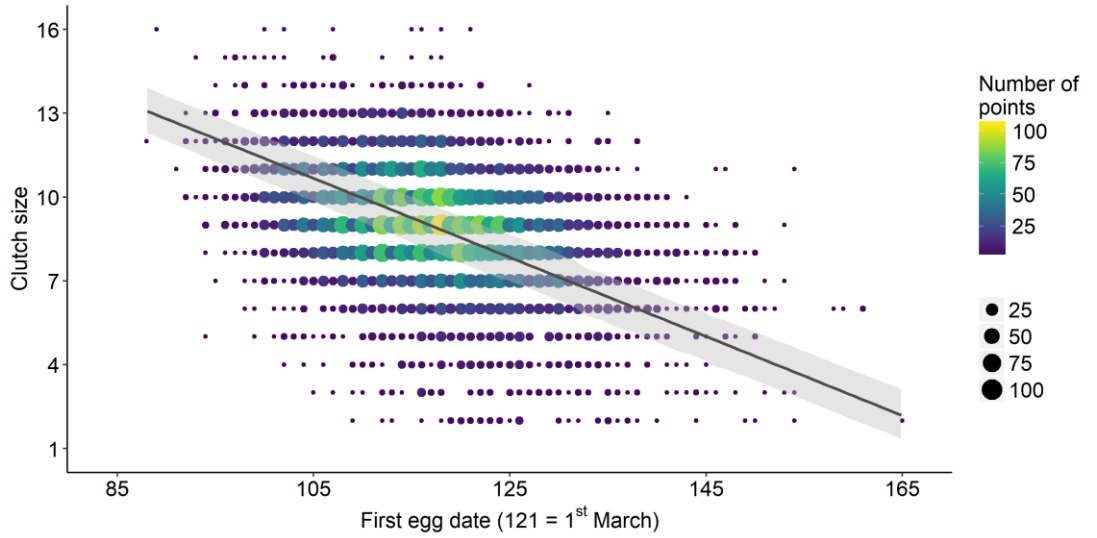

Figure 5.3: The relationship between clutch size and first egg date, after controlling for temperature and latitude. The solid black line depicts the fitted values from a Bayesian mixed effects model, with the shaded area representing the 95% credible interval. The underlying data are shown by the points, with the size and colour depicting how many records are at that position.

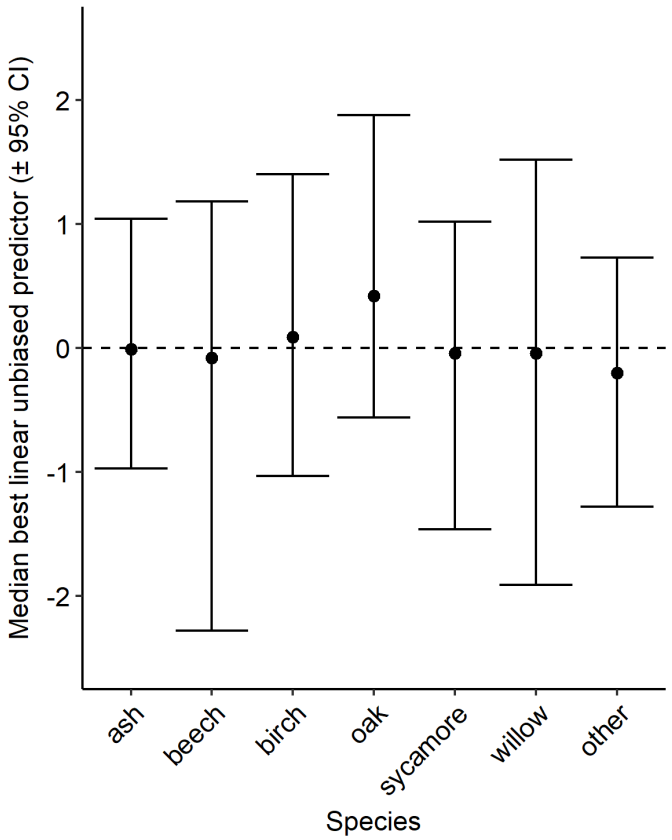

Figure 5.4: The median best linear unbiased predictors from the multiple membership random effect from the Bayesian mixed effects model for each species category, depicted by a solid black circle. The error bars show the 95% credible intervals. This represents the change in clutch size, once the fixed effects have been accounted for, based on the presence of each species. Due to all the credible intervals crossing zero, none of the species categories included have any significant effect on clutch size.

Oak density, as opposed to percentage of total tree density that comprised oak, predicted clutch size with larger clutch sizes at higher oak densities (Table 5.5). All other effects remained, with similar effect sizes to when oak percentage was used (Table 5.3, Table *5.4* and Table 5.5)

Table 5.5: Model estimates, for the fixed effects, from a Gaussian Bayesian general linear mixed effects model, with clutch size as the response variable and including oak density. Rows in bold denote significant predictors of blue tit clutch size. All continuous predictors were scaled, with their scaling factors denoted below the table, which needs to be considered when interpreting parameter estimates.

	Posterior mean	95% Credible Interval	Effective Sample Size	pMCMC
Intercept	**8.96**	**8.67 - 9.26**	**1000**	**<0.001**
Mean site Mar/Apr temperature (°C) •	**-0.75**	**-0.92 – -0.59**	**1000**	**<0.001**
Temperature deviations from site mean (°C) ∇	**0.01**	**-0.41 - 0.42**	**1000**	**0.96**
Latitude ◊	0.04	-0.13 - 0.21	**1000**	0.64
First egg date *	**-1.43**	**-1.50 – -1.38**	**1140**	**<0.001**
Oak density ♣	**0.15**	**0.02 - 0.29**	**1000**	**0.03**

Scaling factors: • = 1.09, ∇ = 0.04, ◊ = 0.88, * = 9.88, ♣ = 61.76

Table 5.6: Estimates for the random effects, from the Gaussian Bayesian linear mixed model, with clutch size as the response variable and including oak density as a fixed effect. Significant effects are marked in bold, and were considered significant if the credible interval did not cross zero.

Group		Posterior Mean	95% Credible Interval	Effective Sample Size
Year:Site	**Intercept**	**0.17**	**0.11 – 0.24**	**1130**
Year	**Intercept**	**0.26**	**0.11 – 0.44**	**1192**
Site	**Intercept**	**0.53**	**0.12– 0.45**	**901**
	Slope	0.12	1.35×10^{-6}– 0.50	885
Residual		**3.32**	**3.23 - 3.41**	**1000**

5.4.2 Risk of failure

5.4.2.1 Egg stage

The risk of the nest failure during the egg stage (from first egg date until the end of incubation) decreased when temperature sums increased, and all other parameters were held constant (Table 5.7). For each 1°C increase in temperature sum the risk of a nest failing was 0.82 times lower, between 116 and 464°C. For example a nest laid where the temperature sum was 126°C, was 8.2 times less likely to fail during the egg stage than a nest where the temperature sum was 116°C. The risk of failure increased when breeding commenced later in the breeding season (Table 5.7). For each calendar day increase in first egg date the risk of failure during the egg stage was

1.89 times higher, between 29th March and 14th June, equating to an 18.9 times higher risk of egg failure for a nest laid on the 7th April compared to a nest laid on 29th March. Neither the total amount of precipitation, nor the density of oak within the nesting area influenced the risk of the nest failing during the egg stage (Table 5.7). There was also a large amount of variation in the risk of failure between years and sites, with between sites showing the greatest variation in risk (Table 5.7; year, Figure 5.5; site, Figure 5.6). Treswell Wood was predicted to have the greatest risk of failure (3.53 times higher than the average site) and Warburtons and Wells Woods the lowest risk (0.6 times lower), during the egg stage, of all the sites included in this study (Figure 5.6).

Table 5.7: Egg stage survival coefficients describing the risk of failure with covariates investigated in the mixed effects cox proportional hazard regression. The hazard ratio represents the risk of egg failure due to the covariate under investigation and all others held constant. A hazard ratio of 1 represents no effect of the covariate, > 1 an increased risk of failure and < 1 a decreased risk of failure. Significant covariates are denoted in bold.

Fixed effects				
Variable	**Coefficient ± SE**	**Hazard ratio**	**Z**	**P value**
Temperature sum	**-0.19 ± 0.08**	**0.82**	**-2.63**	**0.009**
Precipitation sum	-0.02 ± 0.08	0.98	-0.25	0.81
Oak density	0.19 ± 0.12	1.21	1.68	0.09
First egg date	**0.64 ± 0.08**	**1.89**	**8.39**	**<0.001**
Latitude	-0.01 ± 0.13	0.99	-0.11	0.91

Random effects		
Group	**Standard deviation**	**Variance**
Year	0.46	0.21
Site	0.56	0.31

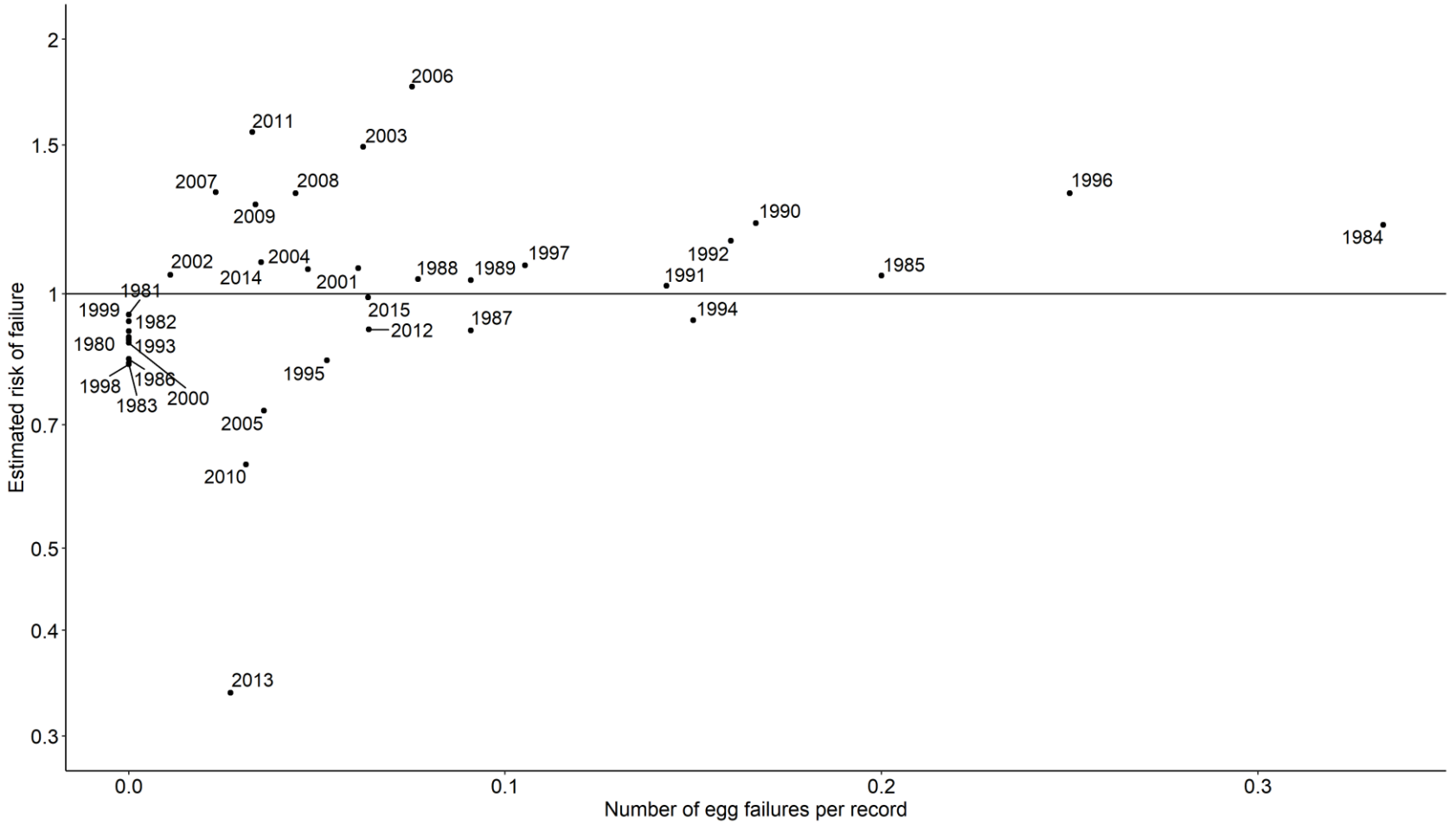

Figure 5.5: The predicted risk of failure during the egg stage for each year against the number of egg failures per record submitted (to correct for years where there are a large number of records). The horizontal solid black line represents the average risk of failure, therefore any point above the line has a higher than average risk of failure, and any below a lower than average risk of failure. To interpret the risk, a risk of 2 is a 2 times higher risk of failure than the average site and a risk of 0.8 is a 0.8 times lower risk of failure than the average site. Note, the y axis is plotted on a logarithmic scale to avoid over plotting.

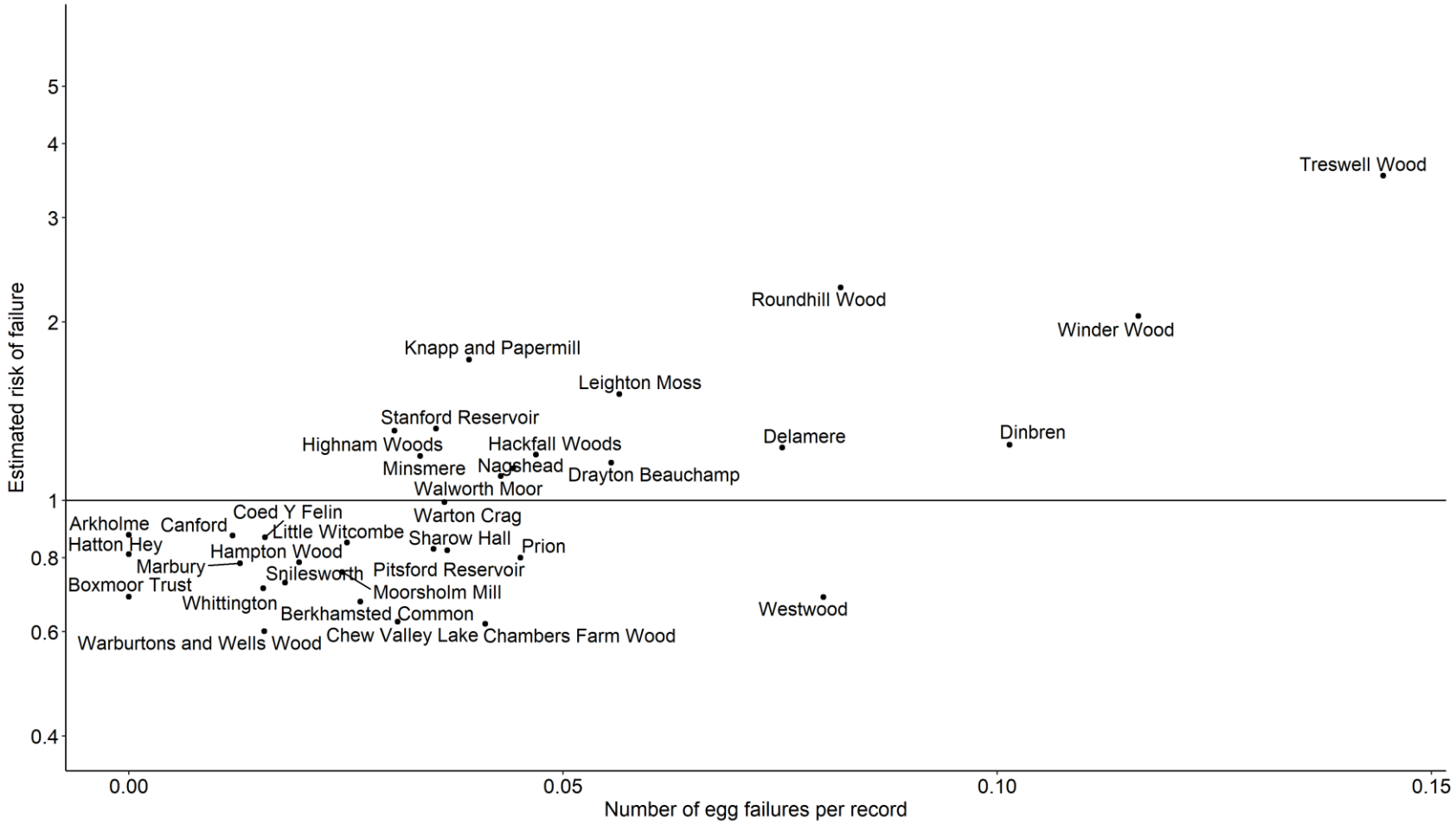

Figure 5.6: The predicted risk of failure during the egg stage for each site against the number of egg failures per record submitted (to correct for sites with higher number of nest boxes, or an increased submission rate and therefore a large number of records). The horizontal solid black line represents the average risk of failure, therefore any point above the line has a higher than average risk of failure, and any below a lower than average risk of failure. To interpret the risk, a risk of 2 is a 2 times higher risk of failure than the average site and a risk of 0.8 is a 0.8 times lower risk of failure than the average site. Note the logarithmic scale on the y-axis, to aid interpretation and avoid over plotting.

5.4.2.2 Young stage

The risk of a nest failing during the young stage decreased when the temperature sum increased (Table 5.8), with the risk of nest failure 0.80 times lower for each 1 °C increase in temperature sum during the chick rearing period, between 185 and 370°C. The risk of failure also decreased when precipitation sums increased, as well as with latitude (i.e. higher latitude equals more northerly, in the UK). Risk of failure during nestling stage decreased by 0.88 times and 0.62 times for each millimetre increases in precipitation sums (between 0.6 and 147 mm) and degree of latitude (between 50.7 and 54.5 degrees), respectively (Table 5.8). The risk of failure during the nestling stage increased when chicks hatched later, with nests being 1.6 times more likely to fail for each calendar day increase between 24th April and 21st June (Table 5.8). The density of oak trees at the site did not affect the risk of young failure. Similarly to the risk of failure during the egg stage, there was a large variation in the estimated risk of failure with year and site. 2013 had the lowest risk of failure with nests being 0.26 times less likely to fail, and in 2007 nests were 3.92 times more likely, than average, to fail (Figure 5.7). Similarly to the site level risk of failure during egg stage Treswell Wood had the greatest risk of failure, with nests 4.03 times more likely to fail than average (Figure 5.8). Young were least likely to fail in Hackfall Wood, with nests being 0.41 times as likely to fail, than average (Figure 5.8).

Table 5.8: Young stage coefficients describing the risk of failure for covariates investigated in the mixed effects cox proportional hazard regression, when each other covariate is held constant. The hazard ratio represents the risk of young failure due to the covariate under investigation and all others held constant. A hazard ratio of 1 represents no effect of the covariate, > 1 an increased risk of failure and < 1 a decreased risk of failure. Significant covariates are denoted in bold.

	Coefficient ± SE	Hazard ratio	Z	P value
Temperature sum during nestling period	**-0.22 ± 0.07**	**0.80**	**-2.98**	**<0.001**
Precipitation sum during nestling period	**-0.13 ± 0.05**	**0.88**	**-2.33**	**0.02**
Oak density	-0.18 ± 0.11	0.84	-1.57	0.12
First hatching date	**0.47± 0.07**	**1.60**	**6.62**	**< 0.001**
Latitude	**-0.47 ± 0.11**	**0.62**	**-4.27**	**< 0.001**

Random effects

Group	Standard deviation	Variance
Year	0.73	0.54
Site	0.54	0.29

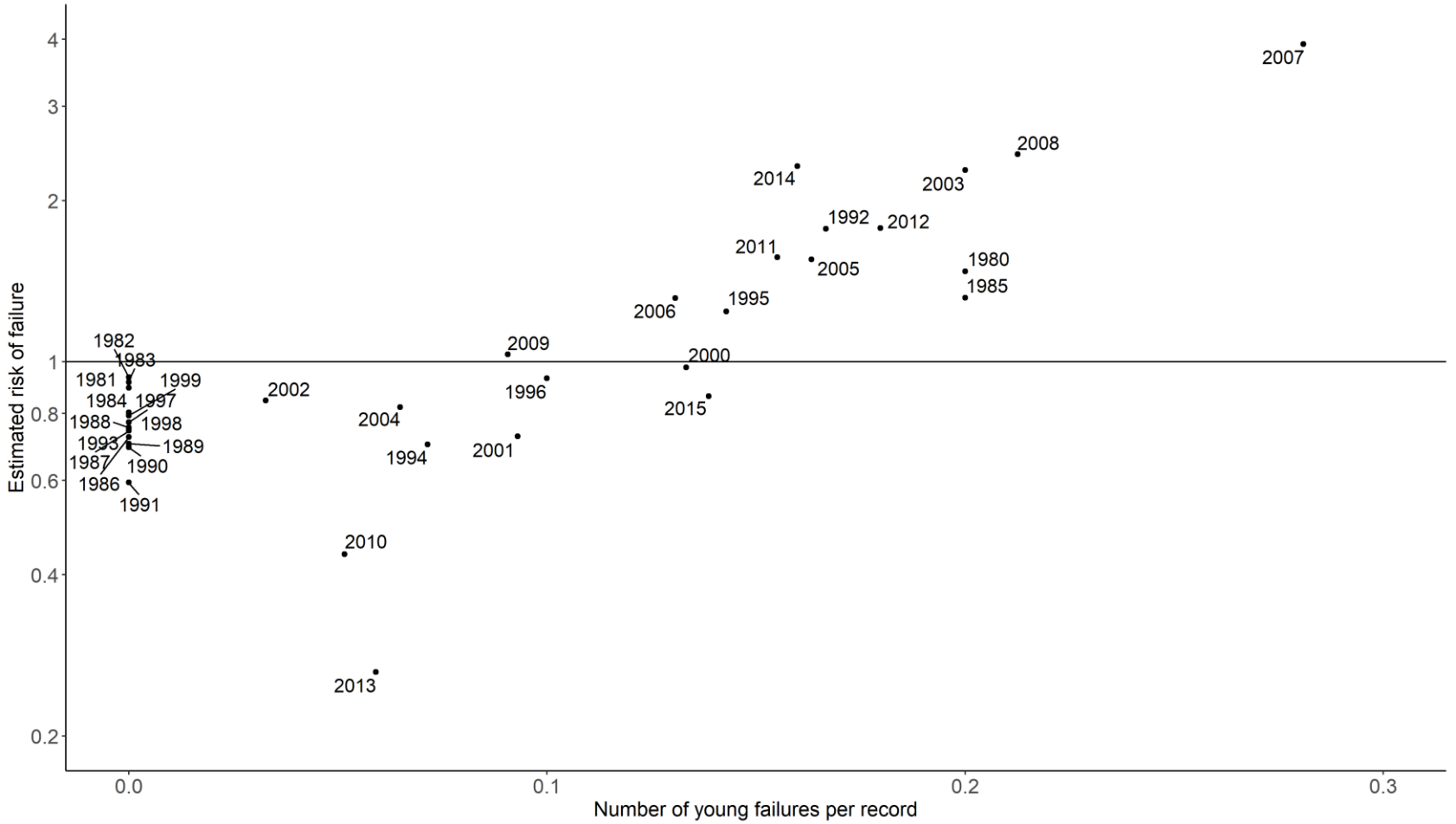

Figure 5.7: The risk of nest failure during young stage for each year versus the number of young failures per nest record submitted (to correct for years where there are a large number of records). The horizontal solid black line represents the average risk of failure, with any point above the line representing a higher than average risk of failure, and any below a lower than average risk of failure. To interpret the risk, 2 is a 2 times higher risk of failure than the average site and 0.8 is a 0.8 times lower risk of failure than the average site. Note the logarithmic scale on the y axis for plotting, to aid interpretation and avoid over plotting.

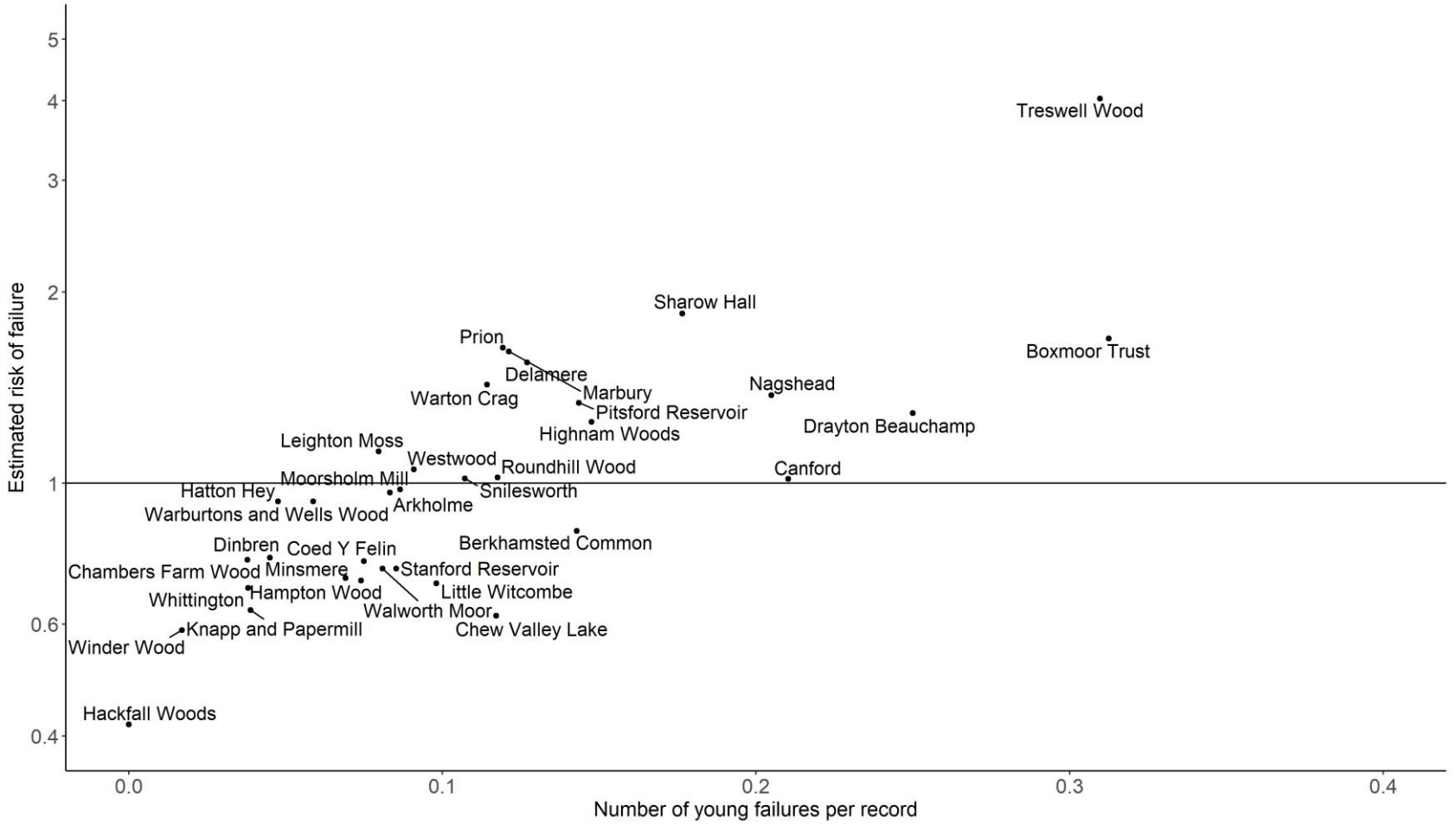

Figure 5.8: The predicted risk of nest failure during the young stage for each site, against the number of young failures per record submitted (to correct for sites with higher number of nest boxes, or an increased submission rate and therefore a large number of records). The horizontal solid black line represents the average risk of failure, therefore any point above the line has a higher than average risk of failure, and any below a lower than average risk of failure. To interpret the risk, 2 is a 2 times higher risk of failure than the average site and 0.8 is a 0.8 times lower risk of failure than the average site.

5.4.2.3 Overall nest failure

In contrast to when each nesting stage was considered individually, when failure at any point during the nesting stage was investigated, there was no effect of either temperature or precipitation on the overall risk of nest failure (Table 5.9). There was also no effect of oak tree density on the risk of nest failure (Table 5.9). There was an increased risk of the nest failing when eggs were laid later during the breeding season, between 31st March and 28th May. For each calendar day increase, risk of nest failure increased 1.49 times (Table 5.9). However, there was a decreased risk of nest failure with increased latitude, with nests 0.79 times less likely to fail with each increase in latitude (between 50.7 and 54.6 degrees). There was large variation in the estimated risk of failure between years and sites (Table 5.9). The estimated risk of overall nest failure varied inter annually, with the risk of failure being lowest in 2013 (0.36 times less likely to fail than the average year) and highest in 2007 (2.31 times more likely to fail than the average year; Table 5.9). Nests in Treswell Wood had the highest risk of failure with the risk being 2.65 times higher than the average site (Figure 5.8). Whittington had the lowest risk of failure, with the risk being 0.45 times than that of the average site (Figure 5.8).

Table 5.9: Coefficients for the risk of nest failure at any point during the nesting period with each covariate investigated in the mixed effects cox proportional hazard regression, when each other covariate is held constant. The hazard ratio represents the risk of nest failure due to the covariate under investigation and all others held constant. A hazard ratio of 1 represents no effect of the covariate, > 1 an increased risk of failure and < 1 a decreased risk of failure. Significant covariates are denoted in bold.

	Coefficient ± SE	Hazard ratio	Z	P value
Temperature sum during nesting period	-0.07 ± 0.05	0.93	-1.65	0.10
Precipitation sum during nesting period	0.12 ± 0.07	1.12	1.77	0.08
Oak density	-0.10 ± 0.09	0.91	-1.17	0.24
First egg date	**0.40 ± 0.05**	**1.49**	**8.36**	**< 0.001**
Latitude	**-0.24 ± 0.08**	**0.79**	**-2.97**	**0.003**

Random Effects		
Group	**Standard Deviation**	**Variance**
Year	0.50	0.25
Site	0.41	0.17

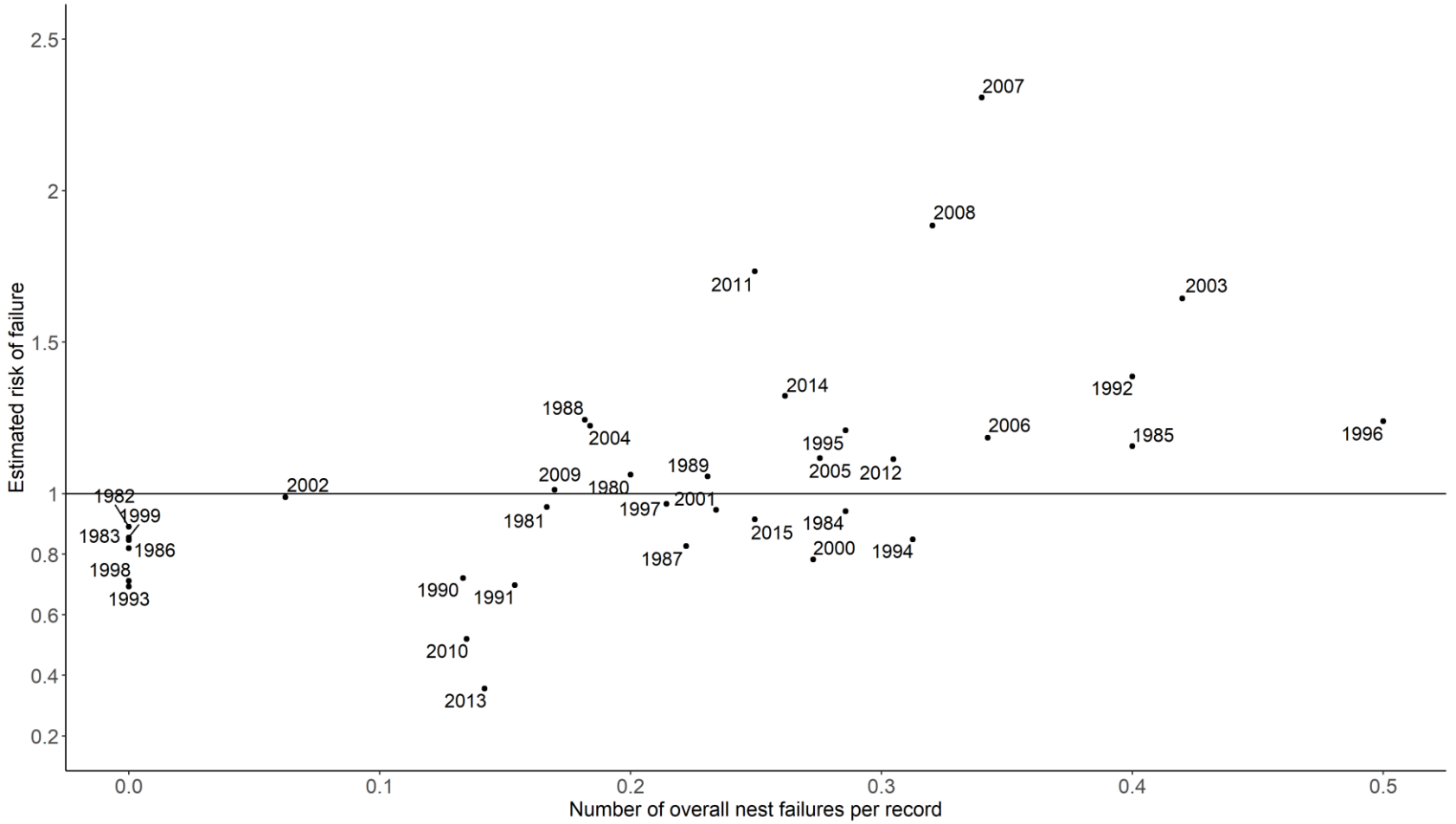

Figure 5.9: The predicted risk of overall nest failure at any point during a nesting attempt for each year, versus the number of failures per record submitted (to correct for years where there are a large number of records). The horizontal solid black line represents the average risk of failure, with points above the line representing a higher than average risk of failure, and points below a lower than average risk of failure. To interpret the risk, 2 is a 2 times higher risk of failure than the average site and 0.8 is a 0.8 times lower risk of failure than the average site. Note the logarithmic scale on the y-axis, to aid interpretation and avoid over plotting.

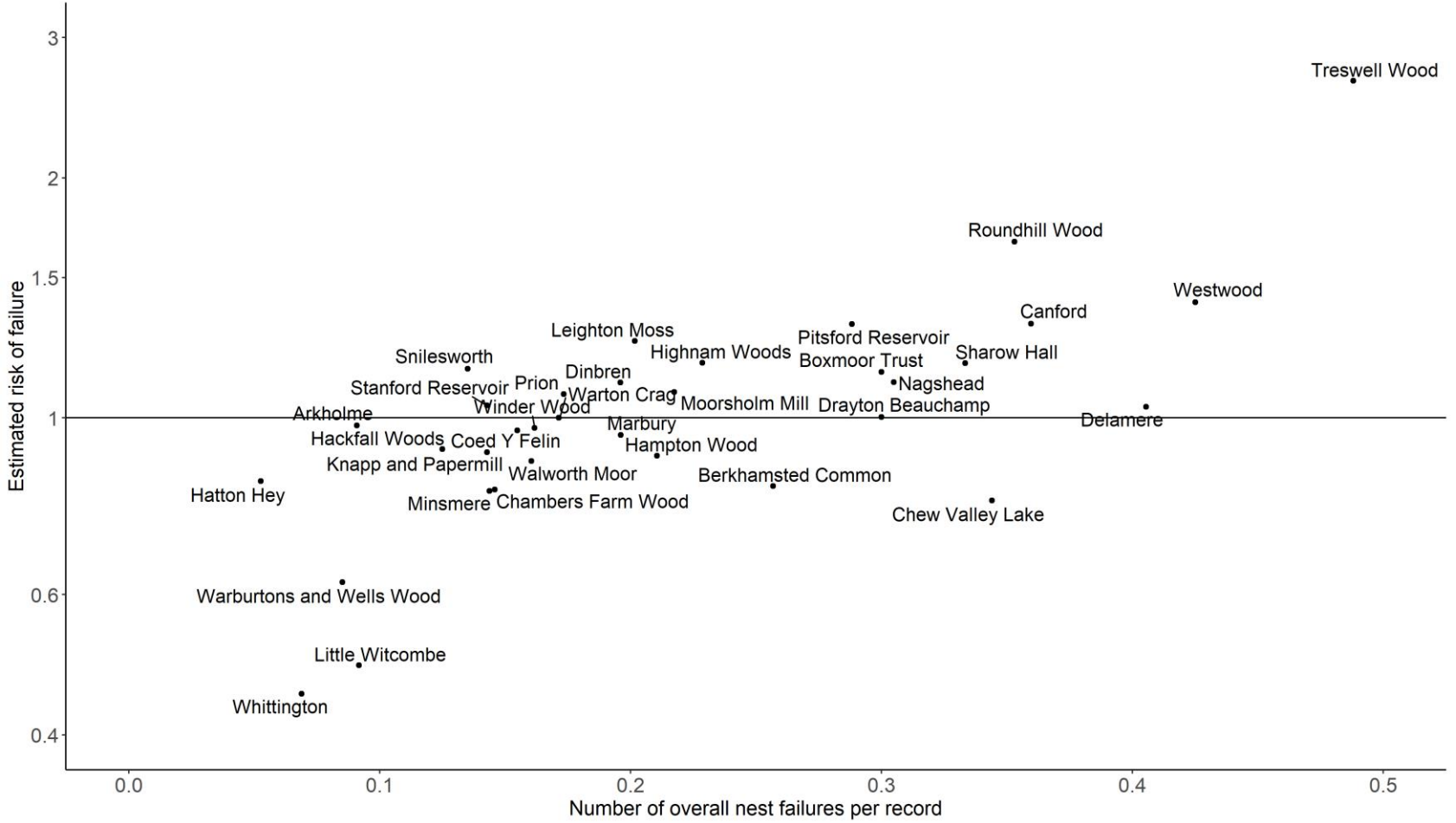

Figure 5.10: The predicted risk of overall nest failure at any point during a nesting attempt for each site included in this study, versus the number of failures per record submitted (to correct for years where there are a large number of records). The horizontal solid black line represents the average risk of failure, with points above the line representing a higher than average risk of failure, and points below a lower than average risk of failure. To interpret the risk, 2 is a 2 times higher risk of failure than the average site and 0.8 is a 0.8 times lower risk of failure than the average site. Note the logarithmic scale on the y-axis, to aid interpretation and avoid over plotting.

5.5 Discussion

High levels of productivity, and recruitment, are needed to maintain adequate population numbers and for populations to be resilient to environmental change. Here, I show that nesting habitat influences productivity but not survival, and that climatic variables such as temperature and precipitation exert complex effects. I discuss these findings in respect to the effect of climate, habitat and phenology on blue tit clutch size and risk of nest failure.

5.5.1 Clutch size

The average clutch size (calculated across all years and sites in this study) is in line with the national average (9 eggs, for both SD: ± 2.2, ± 2.14, this study and nationally (Robinson, 2018), respectively) suggesting the sites included here are representative of sites nationally.

Here, I have shown that clutch size decreases as spring temperatures increase (Figure 5.2 and Table 5.3), even after controlling for first egg date. This relationship has not previously been demonstrated in this species, or at a large scale. No relationship between temperature and clutch size has been found previously in the UK (Shutt, 2017) or Croatia (Dolenec, 2007). Ambient temperature is likely to exert effects upon the female during egg laying through influencing resource availability, as invertebrate prey phenology has been shown to be closely related to temperature (e.g. Buse and Good, 1996). Temperature could also act as a cue for when peak invertebrate availability may occur, and therefore influence bird breeding parameters to allow synchrony between nestling demand and resource availability. An example of such alterations could be to the onset of incubation, either through laying fewer eggs or commencing incubation prior to laying the final, or penultimate, egg which is most common in blue tits (Perrins, 1979). Alterations to the onset of incubation have been shown to help synchronise nestling demand and food resources (Simmonds et al., 2017). However, temperature within a site did not predict clutch size (Table 5.3). This is likely due to the spatial resolution of the temperature data, and nesting locations, being too course to accurately predict this relationship. Birds that commenced breeding later in the breeding season laid fewer eggs (Figure 5.3 and Table 5.3), supporting previous findings in single brooded species across the UK (Crick et al., 1993). Egg production, and therefore clutch size, is likely to be constrained by the female's ability to gain the required resources (fat, protein, calcium and water) to successfully produce eggs (Boyce and Perrins, 1987). In Chapter 4, I showed that birds are commencing breeding earlier in warmer springs. Females may be resource limited earlier in the year, if ample food is not available and consequently may be unable to produce larger clutches (Boyce and Perrins, 1987), offering a potential explanation for the reduction in clutch size observed with temperature. Female age has also been shown to influence clutch size, with older females typically laying smaller clutches later in the breeding season

(Amininasab et al., 2017). Female age was unable to be included with this dataset and could be contributing to the negative effect of temperature on clutch size, and this should be noted when interpreting these results. However, the negative relationship between clutch size and temperature remains even when controlling for first egg date, so this analysis should adequately account for differences in clutch size due to differences in breeding phenology due to female age.

Clutch size varied between both sites and years, even after controlling for temperature (Table 5.4). This again provides support for clutch size being dependent upon resource availability, as resource availability will likely vary between sites and years. No differences in clutch size with latitude were observed (Table 5.3). Studies investigating clutch size differences over large geographical ranges (27 degrees of latitude, as opposed to approximately 4 degrees in this study) have found a non-linear relationship of clutch size and increasing latitude (Fargallo, 2004), with largest clutch sizes predicted in the central region of the latitudinal gradient. Fargallo (2004) showed these differences could be explained by habitat, with evergreen habitats coinciding with lower latitudes, for example. However, when percentage occurrence of individual species at each site were considered there were no significant effects of any of the tree species on clutch size (Figure 5.4and Table 5.4). Oak's median best linear unbiased predictor (BLUP) was positive and was the only species that departed from zero, however the confidence interval overlapped zero, so the effect was non-significant. The inclusion of oak density, without any other species and not as a percentage occurrence, did predict clutch size showing a positive relationship between the two variables (Table 5.5), providing some support for Fargallo (2004). This suggests that converting to percentages decreases the power to detect relationships. However, as none of the other species median BLUPs depart from zero, it is unlikely they would exert an effect on clutch size, even if included as individual densities. The effect of oak density is still relatively weak, with the lower confidence interval approaching zero (Table 5.6). The lack of strong habitat effect may be due to birds being unable to predict nesting site quality during the initial nesting period (Shutt et al., 2018).

The implications of reduced clutch sizes with warming spring temperature could be great, given future climate projections for warming (IPCC, 2014). However, how this translates into the effect on population size will depend whether recruitment remains stable (Reed et al., 2001). The reductions in clutch size due to temperature, could be buffered slightly by birds nesting in oak dominated woodland, due to increased clutch size with increasing oak density. However, the relationship with temperature is steeper than that of habitat, so habitat alone is unlikely to be able to counteract the negative effects of temperature.

5.5.2 Risk of failure

Climatic factors decreased the risk of nest failure when each nesting stage, egg and young, were considered independently. Increasing temperature sums decreased the risk of failure during both egg and young stage, whereas increases in precipitation sums only decreased the risk of failure during young stage (Table 5.7, Table 5.8).

Complete nest failure, as opposed to brood reduction, has been shown to be linked to the disappearance of one, or both, parents due to predation (Santema and Kempenaers, 2018). In the dataset used here brood reduction could not be deduced, nor were the number of fledglings recorded, which prevented finer scale analyses of the effects of climate and habitat on productivity. Therefore, the results presented here mostly relate to the effects on egg and chick physiological requirements (e.g. thermal requirements for development) and the parents predation risk.

Decreased risk of nest failure during egg stage with increasing temperature sums (Table 5.7) corroborate experimental results, where blue tit eggs incubated at higher temperature had increased hatching success (Nord and Nilsson, 2011). Warmer temperatures require females to expend less energy on thermoregulation, coupled with less energy expenditure required during foraging (te Marvelde et al., 2011) and incubation, allowing greater energy investment in egg production and incubation. Eggs will also chill less rapidly whilst the female is absent from the nest at warmer temperatures, decreasing the likelihood of failure. Similarly, during the young stage decreased energetic demands are placed upon both adults and nestlings at warmer temperatures, with lower energy is requirements for thermoregulation, allowing nestlings to expend more energy on growth (Nord and Nilsson, 2011) and adults less energy on brooding and more on foraging. Experimentally, there is no evidence of differences in survival between nests when nest temperature was artificially raised (Andreasson et al., 2018), however hatching success did increase (Nord and Nilsson, 2011). Increased precipitation decreased the risk of failure during the young stage, but had no effect during egg stage (Table 5.7, Table 5.8). Increased rainfall has been shown to increase nestling mass (Mainwaring and Hartley, 2016) and therefore assumed survival, due to heavier mass at fledging being indicative of higher quality chicks (Wilkin et al., 2006). This is the opposite to what has been found in closely related great tits, where nestling mass was reduced with increased rainfall (Kelleri and Van Noordwijk, 1994), showing the complex effects climatic variables can exert. These complex effects of climatic variables are highlighted in this study, where neither temperature nor precipitation had any detectable effects on the risk of survival when risk was considered over the entire nesting period (Table 5.9), despite their being detectable effects when nesting stages were considered independently. The effects of

precipitation are particularly hard to quantify, as a combination of intensity, duration and volume of rainfall are likely to be important.

Contrary to the potential negative implications of warming temperatures on clutch size, the implications for the risk of failure are the opposite. With warmer and wetter springs, blue tits could see a reduced risk of nest failure, which should translate into more successful nests. However, the effects of temperature may become detrimental, by increasing energetic demand due to heat stress, after a certain point and therefore increase the risk of failure.

Delays in breeding phenology, either FED or hatching date, resulted in increased risk of nest failure, at all nesting stages (Table 5.7, Table 5.8 and Table 5.9). Taking this result, and as clutch size exhibits a seasonal decline, I have consistently showed that productivity and success decreases as the breeding season progresses, which is consistent with findings in a number of bird species (Verhulst and Nilsson, 2008). During the egg stage this could be due to poorer quality females only being able to allocate enough resources to egg production later in the breeding season, reduced resource availability later in the season, or a combination of both. During the young stage, increased nest failure for nests initiated later could be indicative of mismatch, due to nestling demand missing the peak in resource availability. If the peak in resource availability is missed, nestlings will likely be fed on alternative, potentially sub-optimal, prey items and may be less able to assimilate the resources needed for growth and survival (Arnold et al., 2010; Pollock et al., 2017).

Latitudinal effects on the risk of nest failure were complex, with no detectable effect during the egg stage (Table 5.7). There was, however, a reduction in the risk of nest failure during the young stage with increased latitude (Table 5.8). Suggesting northern populations are at a lower risk of failing than southern counterparts. Regional population trends have not been explored for blue tits, however have been documented in other species, such as the willow warbler (*Phylloscopus trochilus)*, suggesting that spatially variable trends are possibly due to local-scale drivers (Morrison et al., 2010). The latitudinal differences in the risk of blue tit nest failure may be due to longer photoperiod at higher latitudes during the breeding season, which may allow adults in the north to feed their chicks for longer than their southern counterparts, therefore reducing the risk of nests failing. The period of darkness, and therefore period of not feeding is also shorter in the north, meaning chicks may not lose as much mass overnight giving them an increased chance of survival.

Oak density had no detectable effect on the risk of failure during any nesting stage (Table 5.7, Table 5.8 and Table 5.9), suggesting food availability, due to oak, may not be a limiting factor of nest success, and that birds may not be reliant upon oak trees to find adequate food. This also

suggests that habitat is not likely to be driving the observed latitudinal effect. Previously, at higher latitudes than this study, an effect of habitat on fledging success was detected, with increased success when more oak was present (Shutt et al., 2018). This may be due to fledging success being defined differently to this study, and highlights brood reduction, rather than all out failure, may be more common in habitat that supports sub-optimal prey items. Despite efforts to maximise habitat variation in this study, there is not large between site variation in habitat, possibly as an artefact of nest box schemes being established to primarily monitor pied flycatchers *(Ficedula hypoleuca)*, which typically prefer oak-dominated woodlands. The limited habitat variation, in addition to the scale (site level) habitat was recorded at, should be noted when interpreting the results presented here.

There is large variation in the risk of failure across years and sites in all stages of nesting, whether considered individually or as a whole (Figure 5.5, Figure 5.6, Figure 5.7, Figure 5.8, Figure 5.9 and Figure 5.10). Treswell Wood, a site in the east midland region of the UK (Figure 5.1), for example is consistently the site with the highest risk of failure. However, the site with the lowest risk of failure differs depending on which nesting stage is considered. Site differences could arise from variables that are unable to be explored using these data, such as density dependent effects. From personal observation, Treswell Woods had a higher density of nest boxes than, for example, Warburtons and Wells Wood, which had the lowest risk of failure during egg stage, despite both sites being at similar latitude (Figure 5.1). Predation rates have been shown to be density dependent (Dunn, 1977), so differences in predation rates may explain some of the variation in failure risk between sites, that I have been unable to account for in this study.

5.5.3 Conclusions

In conclusion, the results presented here show that climate and breeding phenology exert the strongest effects on blue tit productivity. Both temperature and breeding phenology have negative effects on clutch size. However, warmer temperatures during egg and young stage lead to reductions in the risk of nest failure. This may counteract the negative effects of temperature on clutch size if fewer nests ultimately fail. In addition, populations at higher latitudes have a lower risk of failure than their southern counterparts. Despite increased clutch size at high oak densities, the risk of failure is uniform across oak densities. This suggests that with warming springs, blue tits nesting in woodlands with higher densities of oak may have larger clutch sizes, but failure risk remains constant, so may not lead to increased success. Investigation into the effects of mismatch and recruitment are needed to extend these findings into population effects.

Chapter 6: General discussion

6.1 Overview

Since the mid-20th century atmospheric warming has been occurring at an unprecedented rate, and temperatures are predicted to continue warming throughout the 21st century (IPCC, 2014), leading to alterations in many ecological processes (Parmesan, 2006; Walther et al., 2002). Changes in phenology are often regarded as an indicator of climate change (Edwards et al., 2004; Thackeray, 2016; Walther et al., 2002). Phenological change can impact trophic interactions, leading to a cascade of effects through food chains, such trophic mismatch, which can in turn alter population numbers and ecosystem functioning (Cushing, 1990; Durant et al., 2007). In temperate woodlands the tri-trophic deciduous tree-herbivorous caterpillar-insectivorous bird food chain has been used to explore the impacts of climate change (Burgess et al., 2018; Charmantier et al., 2008; Hinks et al., 2015; Nussey et al., 2005; Visser et al., 1998, 2004).

The overarching aim of my thesis was to explore the effects of both climate and habitat on the phenology and productivity of the deciduous tree-herbivorous caterpillar-insectivorous bird, woodland system. Throughout I have shown that climate influences the phenology and productivity of herbivorous caterpillars and blue tits more than the habitat with which they are residing in. I demonstrated this through analysing frass fall, from under four common UK woodland tree species at a single site in north east England, to establish the effect of temperature and host tree species on the phenology and productivity of herbivorous caterpillars. I also aimed to extend the tri-trophic food chain into a more complex food web, through utilising next-generation sequencing (NGS). However, due to difficulties extracting DNA suitable for NGS I was unable to elucidate the trophic interactions within the time frame of my PhD, and instead offer methods to overcome the difficulties I encountered. To explore blue tit phenology and productivity, I combined nest records from the British Trust for Ornithology's (BTO) Nest Record Scheme (NRS) with habitat data for 34 sites across the UK. This is the first time the effects of habitat on either phenology or productivity in blue tits have been investigated across such an extensive spatial and temporal scale.

Under two broad headings (variation in resource availability and resource usage, and predictors of blue tit phenology and productivity), I will cover the main findings of each chapter and the possible wider implications of these results. Then I will discuss how the utility of datasets collected by citizen scientists could be extended, before finally suggesting how further questions raised during this research could be addressed.

6.2 Variation in resource availability and resource usage

Phenological mismatch is believed to be one of the most important causes, and potential future cause, of species extinctions due to climate change (Cahill et al., 2013). To understand whether mismatch between trophic levels occurs, an understanding of how phenology varies across both space and time, and what drives any variation, is crucial (Thackeray, 2016).

In the oak-caterpillar-blue tit tri-trophic system, caterpillars are the least researched trophic level; studies that have considered caterpillar phenology have usually only done so at a site level (Both et al., 2009; Marciniak and Nadolski, 2007; Schöll et al., 2016; Smith et al., 2011; Visser et al., 2006) or at an even larger spatial scale (Burgess et al., 2018). To date, the phenology of frass peaks have most commonly been quantified under oak trees (Burgess et al., 2018; Smith et al., 2011), a mixture of tree species (Glądalski et al., 2017; Sisask et al., 2010; Wesołowski and Rowiński, 2014) and rarely are multiple host species investigated (except see Veen et al., 2009), despite Lepidoptera using a wide range of deciduous, and coniferous, tree species (Kennedy and Southwood, 1984). In Chapter 2, I addressed this knowledge gap by investigating frass fall under four common deciduous tree species, in the UK (beech (*Fagus sylvatica),* silver birch (*Betula pendula*), oak (*Quercus robur*) and sycamore (*Acer pseudoplatanus*)), across a single site in North East England. This allowed within year variation of frass fall to be explored, but did not have sufficient power for between year variation, due to only three years of sampling.

Lepidoptera were detected, through frass fall, under all four tree species sampled and the timing and duration of peak frass fall was consistent between species and across the site. However, not all species produced frass peaks under every tree sampled, with only oaks producing frass peaks under all trees sampled, in all three years. Frass peaks were only produced under 35% of beech trees, 36% of sycamore trees and 54% silver birch trees, across all three years. These caterpillar detection rates, between species, are similar to those from samples obtained through branch beating, described by Shutt (2017). Surprisingly, frass fall phenology was not predicted by either host bud-burst phenology or temperature, either within the site or between years, despite inter-annual variation in frass fall phenology being detected. Previously, peak frass fall has been shown to be negatively correlated with temperature both in the UK and the Netherlands (Smith et al., 2011; Visser et al., 2006). A negative relationship between frass fall and temperature would have been expected due to bud-burst, which also exhibits a negative relationship with temperature (Tansey et al., 2017), and caterpillars generally remaining synchronous (Both et al., 2009; Burgess et al., 2018). The duration of frass fall also varied inter-annually, and similarly to peak frass fall phenology, was not predicted by either yearly or within site temperature. Due to caterpillars developing faster at warmer temperatures (Buse et al., 1999; Holliday, 1985) and previous findings suggest narrower peaks occur at higher temperatures (Visser et al., 2006), my findings

were not what I hypothesised. Although frass fall was detected under all four tree species, only oak reliably produced frass peaks and accounted for approximately 70% of the total frass fall collected. When considering phenology of frass fall, there were no differences in the timing of peak frass fall, or the duration of frass fall, between tree species. This suggests that caterpillar phenology is relatively synchronous across a site and between species, so site-level estimates of frass fall phenology should be representative of a conditions experienced by consumers across a site.

The disproportionately high frass fall detected under oak reinforces the importance of oak trees as a host for Lepidopteran species (Kennedy and Southwood, 1984; Southwood et al., 2005). Due to the greater occurrence of Lepidopteran frass fall under oak, in comparison to other tree species, any management strategies that may disadvantage oak within UK woodlands could impact many insectivorous woodland birds. If Lepidoptera numbers were affected, not only tits (e.g. blue, great and coal tits (*Periparus ater*)), but also flycatchers (pied and spotted flycatchers (*Ficedula hypoleuca* and *Muscicapa striata*, respectively) and warblers (willow warbler and chiffchaff (*Phylloscopus trochilus* and *Phylloscopus collybita*, respectively) would be disadvantaged. The implications of the lack of variation in frass fall phenology between tree species would also mean that insectivorous passerine birds could not exploit caterpillars on different host tree species if they mistimed reproduction with Lepidoptera peaks on oak, and would heighten any subsequent effects on productivity of phenological mismatch. The lack of variation in frass fall phenology may have implications for insectivorous birds' productivity, and drive selection towards breeding phenology that is highly synchronous with oak, leading to a habitat specialisation. If numerous species of insectivorous woodland birds, such tits, flycatchers and warblers, were negatively affected by reduced food availability, there could also be a devastating effect on biotic interactions within woodland ecosystems, which are required to keep the ecosystem functioning.

Despite Chapter 2 increasing our understanding of Lepidopteran phenological variation, in both space and time, the diets of many insectivorous woodland birds, including blue tits, during the breeding season remains relatively unknown. Nestling diet has been shown to affect nestling quality in great tits (Wilkin et al., 2009) and blue tits (Pollock et al., 2017), with Lepidoptera, when available, making up a large proportion of nestling blue tit diets (Betts, 1955; García-Navas et al., 2013; García-Navas and Sanz, 2011; Gibb and Betts, 1963; Grzędzicka, 2018). To be able to elucidate the full impacts of climate change on blue tit productivity, and explore the full implications of potential phenological mismatch, there is a need to move from a simple food chain to a more realistic food web and to understand the phenology of multiple resources (Thackeray, 2016). In Chapter 3, I make progress towards addressing this shortfall by developing techniques to

permit the application of next generation sequencing to blue tit nestling faecal sacs to more fully understand diet. Next generation sequencing, or metabarcoding, provides a new opportunity to define diet in a non-invasive way and at a higher resolution than more traditional methods (Pompanon et al., 2012). The latter includes the use of ligatures (Grzędzicka, 2018; Johnson et al., 1980), gizzard analysis (Bourgault et al., 2006), identification of prey from video footage (García-Navas and Sanz, 2011) or field observations (Betts, 1955; Gibb and Betts, 1963) and microscopic analysis of indigestible prey items. All of these techniques have biases and limitations, some of which are overcome by metabarcoding. An essential prerequisite of metabarcoding is the provision of high quality DNA, which has been successfully amplified through polymerase chain reaction (PCR) (King et al., 2008). The extraction of high quality DNA from faecal samples has sometimes proven problematic (McInnes et al., 2017; Oehm et al., 2011; Schrader et al., 2012). In Chapter 3, I showed the issues that were faced whilst trying to extract and amplify DNA from faecal sacs of nestling blue tits. It is likely these issues arose from a combination of PCR inhibitors, which are common in faecal samples (Monteiro et al., 1997; Schrader et al., 2012), and low DNA concentration. DNA was successfully extracted using a modified version of an off-the-shelf DNA extraction kit (MO-BIO PowerSoil) followed by a secondary extraction using solid-phase reversible immobilization, with carboxylated paramagnetic beads.

In addition to problems with extractions, satisfactory DNA amplification success for sequencing could not be obtained with the universal invertebrate PCR primers tested. Unfortunately, due to these issues, sequencing was unable to be undertaken during the timeframe of my PhD, which means I was unable to provide a more complex food web and discuss the ecological implications of this. Once nestling diet has been able to be elucidated to a higher resolution, the next steps will be to relate diet composition to habitat and nestling success. It could also inform the exploration of other resources phenology in more detail, to understand how climate change may disrupt or impact the strength of current trophic interactions in the future. Information about resource usage within woodland systems could be used to inform management strategies to ensure that key resources for insectivorous birds are maximised.

6.3 Predictors of blue tit phenology and breeding success

To ensure ecological networks are resilient to climate change, a detailed understanding of responses to previous changes, and the drivers of any change, need to be fully understood. Typically, phenological studies are limited to single sites, only addressing responses to, and drivers of change, in specific geographic locations (Thackeray, 2016). In the case of the deciduous tree-herbivorous caterpillar-blue tit food chain, the majority of studies to date are limited to single sites and typically in oak dominated woodlands (Both et al., 2009; Hinks et al., 2015; Visser and Holleman, 2001; Wilkin et al., 2009). Studies have extended to multiple sites, either at a national

level (Burgess et al., 2018; Phillimore et al., 2016) or across Europe (Samplonius et al., 2018; Sanz, 2002) but the focus on oak dominated woodlands remains, despite blue tits being habitat generalists (Perrins, 1979; Robinson, 2018; Stenning, 2018). Rarely have differences in breeding phenology, and productivity, been investigated with habitat, other than broad comparisons between two habitat types (Atiénzar et al., 2010; Blondel et al., 1993; Glądalski et al., 2017; Pollock et al., 2017).

Chapters 4 and 5 used a subset of the BTO NRS' blue tit nesting records, in combination with information on tree composition, across a UK-wide network of 34 sites, to understand the relationships between blue tit phenology, productivity, climate and habitat. In Chapter 4, I showed breeding phenology, both first egg and hatching date, has advanced since 1979, and the advancement is explained by increasing spring temperatures. These finding corroborate findings in both small scale studies across Europe (Dolenec, 2007; Marrot et al., 2018; Potti, 2009) and nationwide UK studies (Burgess et al., 2018; Phillimore et al., 2016). Site level tree composition did not predict nesting phenology, contrary to expectations. Previous research has shown correlations between bud burst of oak and nesting phenology in blue tits and great tits, (Bourgault et al., 2010; Burgess et al., 2018; Hinks et al., 2015; Wilkin et al., 2007), and higher levels of synchronisation with local phenology when trees primarily used for foraging were at higher densities (Cole et al., 2015). The lack of relationship between habitat and blue tit breeding phenology may be due to the scale at which habitat was measured and that tree density, as opposed to a measure of tree phenology, was used as the habitat variable. Due to the spatial and temporal scale of this study, nest site specific tree phenology was not available. Instead the proportion, or density, of tree species at a site were used. The lack of relationship between habitat and blue tit phenology could also indicate that previous findings of correlation between tree phenology and nesting phenology do not imply direct causation, and occurs as a result of both phenological measures being temperature driven. Birds may not be using tree phenology as a cue, and therefore, once temperature is also considered, tree phenology does not predict blue tit breeding phenology (Shutt, 2017). Experimental work supports the theory that tree phenology and bird breeding phenology are correlated rather than one cueing the other, as a result of both being temperature driven, as the presentation of early leafing branches did not advance breeding in great tits (Schaper et al., 2011). The apparent lack of association between local habitat and nesting phenology, shown in Chapter 4, suggests that concerns about mismatch with continued warming (Burgess et al., 2018) are substantiated, as blue tits do not appear to use local habitat as a cue to time their breeding.

In Chapter 5, I explored the effects of climate and habitat on productivity, measured through clutch size and the risk of nest failure. Understanding demography is vital when considering the

effects of potential stressors, such as climate change, on species, but is often a neglected area of research (Selwood et al., 2015). I found that clutch size decreases as spring temperature increases, which to the best of my knowledge is the first time this has been demonstrated in this species. Previously, correlates of temperature, such as elevation, or North Atlantic Oscillation index have shown no correlation with clutch size (Sanz, 2002; Shutt et al., 2018). Oak density and clutch size demonstrated a positive relationship, with increased oak density predicting increased clutch size, but only significantly when considered as density as opposed to proportion of oak trees present at a site. The positive relationship between oak density and clutch size strengthens the importance of oak in woodlands, and also suggests that oaks do provide increased resources for nesting birds, suggested in Chapter 2 due to increased frass fall under oaks. Previously, increased oak availability has not led to increases in clutch sizes, with only increased willow availability predicting an increase in clutch size in blue tits (Shutt et al., 2018). However, in sympatric great tits increased oak density has been shown to lead to increased clutch sizes (Sanz et al., 2010). These contrasting results between sympatric species and geographic locations suggest the effects of habitat may be complex and differ geographically and between bird species. This highlights that care should be taken when extrapolating results from single geographic locations and/or single species to wider population effects, as they may not be representative. How an increase in clutch size, with increased oak density, translates into population effects, such as abundance, will depend whether the increases in productivity continue into survival and recruitment (McLean et al., 2016).

In Chapter 5, I showed that despite the positive relationship between oak density and clutch size, the positive association with oak did not continue into reducing the risk of nest failure. Oak density had no effect on the risk of nest failure, at either egg or young stage, nor when the two stages were combined. This suggests that food availability may not be limiting overall nest success, i.e. complete brood failure. However, birds nesting in sub-optimal habitats, defined by reduced food availability, may experience brood reduction (Slagsvold, 1985) as opposed to complete failure. With climate change both optimal (oak dominated) and sub-optimal (non-oak dominated) habitats may pose an equal risk of nest failure. This may be due to sub-optimal prey resources in non-oak dominated woodlands and mismatch between nestling demand and invertebrate availability in highly homogenous oak woodlands (Burgess et al., 2018). Therefore, counteracting any beneficial or detrimental effects each respective habitat may have historically provide. Despite oak woodlands being traditionally optimal habitat, recent phenological mismatch could be making these habitats sub-optimal and could offer an explanation as to why habitat did not modulate the risk of nest failure.

Climatic factors, however, decreased the risk of nest failure at both egg (FED until hatching) and young stages (hatching until fledging), shown in Chapter 5. Increased temperature sums during each respective stage decreased the risk of failure, suggesting with continued spring warming nest failure may decrease. During the egg stage of nesting, this reduced risk of failure may be due to females being in better condition during periods of warmer weather, as invertebrates are typically more active (Abram et al., 2017) and birds can forage more efficiently (Avery and Krebs, 2008). Under experimental conditions, artificially heating nests did not affect survival (Andreasson et al., 2018). However, this may be due to the effects of temperature not acting directly upon the nestlings but upon the resources which they are reliant on, which were not manipulated in Andreasson et al. (2018). Precipitation reduced the risk of nest failure during the young stage, which may be related to increased nestling mass during periods of rainfall (Mainwaring and Hartley, 2016) and heavier nestlings typically being indicative of high quality chicks (Wilkin et al., 2006). The opposite effects have been found in great tits, with reduced nestling mass with increased precipitation (Kelleri and Van Noordwijk, 1994). Future climate projections for northern temperate regions are for increased mean precipitation and likelihood of extreme precipitation (IPCC, 2014), which given the results presented here could lead to increased productivity in blue tits. The effects of precipitation are hard to quantify, as it is likely that the intensity and duration of rainfall is likely to be more important than total rainfall. However, rainfall duration and intensity is harder to quantify on a macro-scale, and is even difficult at a micro-scale, and as such could not be investigated here.

Overall, I have demonstrated that climatic factors are more important than habitat for blue tit phenology and productivity. However, the effects of climate are likely to be complex in terms of whether they are beneficial or detrimental in light of predicted future climate change. Increased temperatures will likely further advance breeding phenology, increasing the chance of mismatch, with both higher and lower trophic levels, which could lead to alterations in trophic interactions. Alterations to trophic interactions may impact population sizes of both resources and consumers, and in turn could alter ecosystem functioning. Decreased clutch sizes but reduced risk of nest failure, were demonstrated with increased temperature, which may lead to an increased number of nests producing fledglings. Increased blue tit productivity will have implications for woodland trophic cascades. For example, by providing increased food resources, in terms of increased fledgling prey, for higher trophic levels such as sparrowhawks (*Accipiter nisus*), whilst concurrently reducing herbivorous caterpillar populations. A reduction in herbivorous caterpillar numbers could possibly even lead to woodlands acting as increased carbon sinks, due to reducing herbivory damage of young leaves. Under increased atmospheric CO_2 levels, it is predicted that herbivory damage from caterpillars will increase (Couture et al., 2015), therefore insectivorous birds, such

as blue tits, could be used as a form of biological control, to control herbivorous insect populations, allowing more carbon to be fixed by woodland ecosystems.

6.4 Improving the utility of citizen science datasets and considerations when designing ecological studies

Citizen science, where volunteers collect and sometimes also analyse or process data, has revolutionised scientific data collection, and allowed data to be collected at geographical scales not previously possible by individual research teams (Bonney et al., 2014; Silvertown, 2009). Citizen science has contributed to the investigation of many of the recent big ecological questions, such as the effects of climate change, tracking of invasive species, and the effectiveness of ecological restoration, to name but a few (Silvertown, 2009). The BTO NRS was started in 1939, the oldest bird nest monitoring scheme globally, and data collected through the scheme has contributed to numerous scientific papers since its conception (Crick et al., 2003). NRS data has contributed to advancements in knowledge of basic breeding biology (e.g. Crick et al., 1993), population dynamics (e.g. Morrison et al., 2014; Siriwardena et al., 2000), highlighted species at risk due to poor breeding performance and the drivers of these trends and phenological changes (e.g. Crick et al., 1997; Crick and Sparks, 1999).

In both Chapters 4 and 5 I used citizen science data from the BTO NRS to explore the effect of climate and habitat upon blue tit phenology and productivity. Without the dedication of the numerous volunteers who collected these data I would not have been able to begin to answer these questions. However, there are a number of limitations and biases associated with the dataset that could be addressed to extend the applications of these data sets.

Despite efforts to maximise habitat variation *a priori* during site selection, habitat variation was limited between sites, which may have hindered my ability to detect the effects of specific tree species. Comparing the habitat variation between sites in Chapters 4 and 5 with a study where sites spanned a 220 km transect in Scotland (Shutt et al., 2018) showed the habitat variation between my 34 sites was less pronounced, and woodlands dominated by willow and alder were lacking. The lack of variation is likely to be as a result of the majority of long-running nest box schemes being initiated to monitor other cavity nesting species, such as pied flycatchers or redstarts (*Phoenicurus phoenicurus)*. Pied flycatchers, and redstarts, are often deemed more interesting to monitor by nest recorders due to their recent declines and red and amber conservation status, respectively, within the UK (Eaton et al., 2015). Both pied flycatchers and redstarts are cavity nesters, like the blue tit, and preferentially nest in mature deciduous woodland (Buxton, 1950; Lundberg and Alatalo, 2010), with redstarts preferring slightly more open woodland than pied flycatchers (Droz et al., 2015). Due to pied flycatchers and redstarts

being more habitat specialists than blue tits, and pied flycatchers optimal habitat being considered as mature oak woodlands (Lundberg and Alatalo, 2010), this will likely have influenced nest recorders decisions about nest box placement, and woodlands to monitor, when setting up their studies. Within the 34 sites included in this study, only a few nest box schemes were established solely with the intention of monitoring either blue tits or great tits. These inherent, potentially unintentional, biases make it difficult to address questions relating to habitat with these data sets. These biases could be addressed in a number of ways, whilst still ensuring that the guidelines are not too prescriptive on what can and cannot be included to ensure participation levels remain high (Tweddle et al., 2012). One approach could be to further highlight the importance of common species, as well as rare and declining species, as common species are responsible for many ecosystem services, and the common status, is in fact relatively rare (Gaston, 2010). In addition to highlighting what can be deduced from common species, placing emphasis upon sampling all habitats where species may occur, even though it may not be their optimal habitat, would extend the questions which these datasets could address and increase their utility when considering how to mitigate anthropogenic effects.

The spatial scale at which variables are collected at should also be considered when designing ecological studies. In Chapter 2 all variables were collected at a scale that was likely comparable to the scale at which the organism experienced it. However, when conducting macro-scale studies, such as Chapters 4 and 5, it is often not possible to obtain data spanning both the spatial and temporal extent required at a micro-scale. This can often lead to a need to decrease the resolution to answer large scale questions to depict trends at a lower resolution (Blackburn and Gaston, 2002). This leads to questions about the validity of results, due to different scales often elucidating different results, and difficulty in selecting the right scale and the right results (Blackburn and Gaston, 2002). Previous research has shown scale is important when considering blue tit phenology (Hinks et al., 2015; Wilkin et al., 2007), and often the best correlates are at a fine scale (25-75 m) (Wilkin et al., 2007). However, previous work has been limited to single site studies, where data were available at fine resolution, which is not usually the case with citizen science or macro-ecological datasets. This does not mean the patterns or processes deduced at a larger scale are not informative or should be disregarded due to the scale variables were collected at (Blackburn and Gaston, 2002), instead this should be borne in mind when interpreting the results and investigated in further studies.

6.5 Further questions and future work

I have highlighted that despite blue tits being common and a well-studied species, there are still considerable knowledge gaps. Due to their abundance, and resilience to monitoring, blue tits

offer many opportunities to answer ecological questions and expand research questions to a wider scale.

Chapter 2 provided additional information on the most understudied trophic level of the tri-trophic system, herbivorous caterpillars. However, it was still at a single site scale, only utilising one sampling method, for a single invertebrate order. Further work could be carried out with concurrent sampling for many invertebrate species, through methods such as branch beating, frass sampling and sticky traps, for example, on multiple host tree species, at multiple sites. This would give a more complete picture of the phenology and resource availability for blue tits during the breeding season, across different habitats. Invertebrate sampling could be extended into the recruitment period as well, to understand the resources that recently fledged nestlings may have access to, which may differ from what is available during the nestling period.

For invertebrate sampling to be targeted most efficiently, an obvious area of future work would be to continue the work presented in Chapter 3 by sequencing the DNA extracted from nestling faecal sacs. This approach could then be extended out to a multi-site scale, possibly through using the network of 34 sites, which was used in Chapters 4 and 5, or through appealing to BTO nest recorders and bird ringers to collect samples during routine nest monitoring and ringing activities. This would have the benefit of sampling from a wide variety of habitats, geographical locations and if samples were collected over multiple years, could allow both spatial and temporal variation in prey choices and breeding success to be explored. The addition of faecal samples from other sympatric woodland species could also inform us about niche differentiation, or overlap, that may be occurring, which could be influencing phenology or productivity through density or competitive effects not immediately apparent.

In addition to elucidating diet, DNA from nestling faecal sacs could be tested for the presence of parasitic infection, such as Coccidian infection, as parasitic load has been shown to negatively affect breeding success (Gustafsson et al., 1994; Marzal et al., 2005) and survival (Lachish et al., 2011; Sol et al., 2003). Although, a positive association between breeding success and malarial infection in blue tits has been reported, in experimentally manipulated broods, in a single Swedish population (Podmokła et al., 2014). In addition, starling (*Sturnus vulgaris*) nestlings heavily infected with Coccidian parasites have been reported to be, on average, heavier than less infected counterparts (Mazgajski and Kedra, 1998). This suggests that infection may lead to increased appetite, which may affect reproductive success if parental feeding effort cannot match nestling demand (Mazgajski and Kedra, 1998). The prevalence of parasitic infection may also be linked to climatic, or habitat, characteristics and with future climate change parasitic infections are predicted to become more prevalent (Garamszegi, 2011), which makes understanding the effects of parasites on productivity and trophic interactions important.

In Chapter 4 and 5 I showed that climate, but not habitat, was important in influencing blue tit phenology and productivity. However, how these changes translate into influencing population processes and numbers is still unknown, which makes assessing the impact of climate change particularly difficult. A major gap in our understanding is how climate and habitat affects recruitment success, as recruitment will define whether changes in phenology and nesting productivity are detrimental or not (McLean et al., 2016), and whether populations are likely to be resilient to climate change. This could be achieved by systematic monitoring post fledging, through mark-recapture/re-sighting techniques that do not influence success. These additional data could then be combined with NRS and other census data held by the BTO (such as ringing recoveries), to undertake further analyses to investigate population trends and demographic rates through Integral Projection Models (Metcalf et al., 2013), for example, which would allow the effects of continuous demographic variables e.g. first egg date, and any changes in these, to be explored in relation to their effects on population dynamics.

Although Chapter 4 and 5 used a multi-site approach, the network of 34 sites included in this thesis could also be expanded. Sites which do not submit nesting records to the BTO NRS scheme could be targeted, and may increase habitat diversity between sites. Increasing the number of sites, and therefore sample size, would increase the power to detect relationships between breeding parameters and habitat, which appear to be relatively weak. Information about optimal habitats, for blue tits, could be used to inform future woodland managements and identify areas that could benefit from nest box provisioning, for example. Optimal habitat for blue tits may not be optimal habitat for all woodland bird species. However a number of other insectivorous woodland bird species, where the data are not available to investigate such habitat effects, may also benefit.

Finally, ensuring effective science communication occurs between scientists using citizen science data and the citizen scientists collecting the data is crucial to i) maintain high levels of participation, due to tangible results being presented from their data and ii) ensure that the importance of monitoring common species, across all of their possible range, is re-iterated to maximise the potential uses of these data sets.

6.6 Conclusion

Throughout this thesis I have demonstrated that climate is more important than habitat in influencing phenology and productivity of herbivorous caterpillars and blue tits. At a single site scale, I have provided important results on how Lepidopteran resource availability varies throughout the breeding season and across four common tree species in UK deciduous woodland. These results highlight the importance of oak as a host for Lepidopteran larvae, and could be used

to inform woodland management to ensure that oak is not disadvantaged the future. If oak were to be disadvantaged, this could impact upon numerous species of insectivorous woodland birds and lead to alterations in woodland food web dynamics. Despite being unable to sequence DNA from nestling blue tit faecal sacs, to elucidate nestlings' diet within this thesis, the information gained on effective DNA extraction and amplification methods is useful in informing future studies. Expanding to a nationwide, 34 site scale, I showed that climate influenced blue tit phenology and productivity more than habitat, with increased spring temperatures leading to earlier breeding phenology, but mixed effects on productivity. These findings, combined, can be used to inform future predictions of how the deciduous tree-herbivorous caterpillar-insectivorous bird, tri-trophic food chain, and woodland ecosystems more generally, are likely to fare with continued climate change. This thesis, therefore, provides important novel insights into the effect of climate change on one of the most common bird species in the UK.

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